Screening Systems for Detecting Inhibitors of Cell Wall Transglycosylation in *Enterococcus*

Cell Wall Transglycosylation Inhibitors in *Enterococcus*

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We devised two screening systems to detect cell wall transglycosylation inhibitors. One screen utilizes a mutant of *Enterococcus faecalis* strain A256 that is dependent on vancomycin or moenomycin for growth. In the absence of transglycosylation inhibitors the strain fails to grow, while in the presence of inhibitors, cells are rescued. A second screening organism *E. faecalis* strain MDD212 utilizes a translational fusion of the *lacZ* gene to the *vanH* promoter in a derivative of *E. faecalis* that contains a vancomycin resistance determinant. Induction of β-galactosidase occurs when cells are exposed to inhibitors of transglycosylation. Our natural products drug source of fungal fermentations was tested with these screens. Several cultures that produced the same family of compounds, called the thielavins, were detected. Thielavin B inhibited the formation of peptidoglycan in an *in vitro* assay, suggesting that these screening systems can detect compounds that interfere with cell wall transglycosylation.

In vancomycin-resistant enterococci, the cell wall precursor is modified to prevent the binding of vancomycin to the terminal D-alanine residues of the muramyl pentapeptide[1~6]. This modification is mediated by the *vanH* gene product which catalyzes the formation of D-lactate[1,7] and by the *vanA* gene product, a ligase that catalyzes the formation of D-alanyl-D-lactate[1,8], the product apparently incorporated into the cell wall precursor by the host adding enzyme[1,6]. In addition VanA and VanB determinants encode functions that diminish the capacity of cells to synthesize the original precursor. The *vanX* gene encodes a D-alanyl-D-alanine peptidase which reduces the internal concentration of D-alanyl-D-alanine[9], and the *vanY* gene encodes a carboxypeptidase that hydrolyzes terminal D-alanine residues[10~13].

This elaborate mechanism for simultaneously remodeling the cell wall precursor, and diminishing the synthesis of the original precursor, is regulated by a two-component system in both VanA[14,15] and VanB[16] determinants. The *vanS* gene codes for a histidine protein kinase that regulates the state of phosphorylation of the response regulator, encoded by *vanR*. One unusual feature of this regulatory system is that not only vancomycin, but other inhibitors of cell wall transglycosylation, such as moenomycin, but not transpeptidation inhibitors such as β-lactams, have been reported to be inducers of the *van* genes[17~19]. Thus it is possible that the regulatory components of the Van determinants could be utilized to screen for compounds that inhibit cell wall transglycosylation.

In order to develop cell-based screens to detect inhibitors of transglycosylation, two approaches were investigated. One approach utilizes the rescue of a vancomycin-dependent strain. It is known that vancomycin-dependent mutants of *Enterococcus* can be isolated from the intestinal tract of patients who were treated for extended periods with high levels of vancomycin[20~22]. Recently it has been shown that the loss of the host D-alanyl-D-alanine ligase in vancomycin resistant strains

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leads to vancomycin-dependence, and that vancomycin is required to induce the VanA ligase[19,23]. Vancomycin-dependent strains can thus be utilized to screen for inducers that promote growth at low concentrations, but inhibit cell growth at high concentrations. A second approach utilizes a reporter gene fused to the *vanH* promoter, whose induction can be measured in the presence of transglycosylation inhibitors. The effectiveness of these approaches for screening drug sources is discussed.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Enterococcus faecalis strains A256[24] and *Enterococcus faecium* strain X34044[25] were provided by LOU RICE and DAVE SHLAES, Case-Western Reserve University, Cleveland, OH. *E. faecalis* strain OG1RF[26] was supplied by DON CLEWELL, University of Michigan, Ann Arbor, MI. Plasmid pDL278[27] was provided by GARY DUNNY, University of Minnesota, Minneapolis, MN. The plasmid pSK10Δ6 was obtained from RUTH SCHMIDT, Millennium Pharmaceuticals Inc., Cambridge, MA. *E. coli* strain XL1-Blue was used for all cloning experiments. Brain Heart Infusion (BHI) broth and agar plates were utilized for all experiments, supplemented with vancomycin, moenomycin, or other compounds as indicated. Growth was monitored by measuring optical density at 655 nm (Biorad Model 3550-UV plate reader).

Plasmid Constructions, Transformations, and Matings

The *vanH* promoter was cloned from strain A256 by standard PCR protocols[28], using the oligonucleotides van3 (5'CGAAGGCCTCTCTCCGGGGATGTGGTG TC3') and *vanH*rev1 (5'CGCGGATCCTCTGCCTCA TCCTGCTCACA3'), based on the published DNA sequence of the *van* promoter from *E. faecium* strain BM4147[14]. The underlined regions are *Stu*I and *Bam*HI restriction sites which are not homologous to *van* sequences. The PCR product was digested with *Bam*HI and with *Eco*RI enzymes. (The *vanH* coding region contains an *Eco*RI site.) The resulting 404 bp DNA fragment was ligated into plasmid pSK10Δ6, which had been digested at its unique *Eco*RI and *Bam*HI restriction sites, adjacent to the *lacZ* structural gene. The plasmid pMDD8, containing the translational fusion of P_{vanH}*vanH'-lacZ*, was transformed into *E. coli*.

To clone the fusion into *E. faecalis*, plasmid pMDD8 was digested with *Asp*700 and cloned into the shuttle plasmid pDL278 which was digested with *Sma*I to give plasmid pMDD76. Transformants were first selected in *E. coli*, and then recombinant plasmids were transformed into *E. faecalis* strain OG1RF (rifampicin and fusidic acid resistant, vancomycin-sensitive strain) by electroporation[27] to form strain MDD174.

Strain A256 and strain MDD174 were mated by initially co-incubating onto a BHI plate without selection. Following growth for 17 hours, cells were harvested and inoculated on BHI plates containing 64 μg of vancomycin/ml and 1000 μg of spectinomycin/ml. Exconjugants which formed single colonies after two days at 37°C were further screened for fusidic acid and rifampicin resistance. Strain MDD212 was the resulting strain, carrying the VanA determinant as well as the *van-lacZ* fusion.

Susceptibility Testing

Strain *E. faecium* X34044 was tested for susceptibility in microtiter trays. Dilutions of compounds or fermentation extracts were dried in microtiter wells. Cells were grown to mid-log phase and the optical density of 150 μl at 655 nm was measured (Biorad Model 3550-UV plate reader). Cells were diluted to an OD of 0.01. Plates were incubated without shaking at 37°C for 6 hours. Growth inhibition is indicated when the optical density is less than 0.1, compared to an approximate OD of 0.3 for untreated cultures.

β-Galactosidase Assays

The β-Galactosidase assay for whole cells was conducted as described[29]. Ten μl of culture samples was added to 65 μl of Z-buffer containing 0.2 mg/ml of sodium desoxycholate and 0.1 mg/ml of CTAB (hexadecyltrimethylammonium bromide) containing 666 μg/ml ONPG (*o*-nitrophenyl β-D-galactopyranoside). After 2 hours incubation at 30°C, 30 μl of stop solution (1 M Na_2CO_3) was added, and the optical density at 405 nm was measured (Biorad Model 3550-UV plate reader). A zero time reading of 10 μl of cells in Z-buffer and stop solution was subtracted to determine the absolute enzyme activity of the culture.

LC/MS Analysis

Two ml extracts of active fermentation broths were dried and resuspended in 500 μl of methanol/water (1 : 1 v/v). The solution was filtered (Nalgene 5 micron syringe filter) and 250 μl was injected into the HPLC (Hewlett-Packard Model 1090 HPLC) using a C-18 microsorb short column. The flow rate was 1 ml/minute, using conditions described previously[30]. Fractions were tested for activity in the rescue screen, and for antibacterial

activity. The active fractions were pooled, reinjected onto the column using the same conditions, with 10% of the effluent being directed to the mass-spectrometer. The material was analyzed using the negative ion mode.

Assay for Peptidoglycan Synthesis

The particulate enzyme preparation from *E. coli* cells and the reaction mix for detecting peptidoglycan formation was as described[31]. UDP-muramyl pentapeptide was provided by Dr. PETER E. REYNOLDS, Cambridge University, and UDP-[^{14}C]*N*-acetylglucosamine was from Amersham. Samples were subjected to thin layer chromatography (Whatman Silica Gel K6 250 μm 60A) instead of paper chromatography. Autoradiograms were developed after 18 hours exposure, using the Kodak transcreen LE system. The signal intensity of the peptidoglycan spot (at the origin) was determined for reactions exposed to thielavin B or to moenomycin, compared to untreated controls.

Results

Isolation and Characterization of a Vancomycin-Dependent Derivative of *E. faecalis* Strain A256

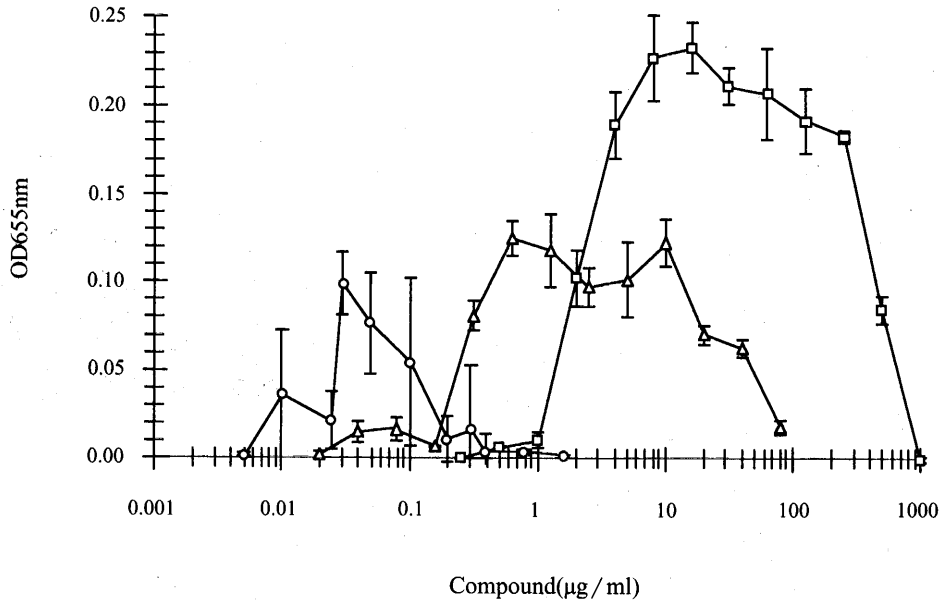
The prevalence of vancomycin-dependent strains from vancomycin-rich environments[20,21] suggests that mutations leading to vancomycin-dependence confer a selective advantage in the presence of vancomycin. In order to isolate a vancomycin dependent derivative, the vancomycin resistant strain A256 was grown overnight in BHI containing 64 μg of vancomycin/ml and inoculated onto a BHI agar plate containing 64 μg of vancomycin/ml. A very high concentration of vancomycin (10 mg/ml) was then spotted in the middle of the plate and the plates were incubated at 37°C overnight. The next day super-resistant colonies that required vancomycin for growth were found growing at the edge of the zone of inhibition. One such strain A256-5 was used for the rescue screen.

The Rescue Screen

To test for rescue of A256-5 cells in liquid medium, extracts of active fermentation broths were dried in wells of 96-well microtiter plates. Cells were grown to mid-log phase in BHI containing 64 μg/ml vancomycin and washed 3 times in BHI without vancomycin. After resuspension, the optical density of the cell suspension was measured in the plate reader (75 μl of cell suspension, OD 655 nm, Biorad Model 3550-UV plate reader). The cell suspension was inoculated with samples after dilution

Fig. 1. Rescue screen.

□, Vancomycin; △, thielavin B; ○, moenomycin.



Cells of strain A256-5 were grown to mid-log phase in BHI with vancomycin, diluted to 0.00001 OD per well of a microtiter plate, as described in the text, and incubated with the indicated compounds. After 17 hours at 37°C, the optical density was determined by subtracting the zero time reading. An active sample showed ≥0.04 OD at 655 nm. Values are the average of at least three experiments. Error bars indicate 1 standard deviation from the mean.

to an OD of 0.00001 (approximately 2,500 cells per well). Cells were grown overnight at 37°C. After 17 hours, growth to greater than OD 0.04 was observed in wells containing either moenomycin or vancomycin (Figure 1). When this low inoculum was used, no growth was observed in untreated wells. If the inoculum was increased to a starting OD of 0.001, then untreated cells occasionally exhibited an OD of greater than 0.04, due to the overgrowth within the well of a mutant derivative that no longer requires inducer. The rescue screen was very sensitive to vancomycin, and rescue occurred at a broad concentration range (2 to 500 μg/ml). The screen was even more sensitive to moenomycin, but only at concentrations below the MIC. Moenomycin was sometimes detected when cells were exposed to a concentration of 10 ng/ml, and was detected routinely at 30 ng/ml (Figure 1), although the amplitude of the growth signal was reduced when compared with cells exposed to vancomycin.

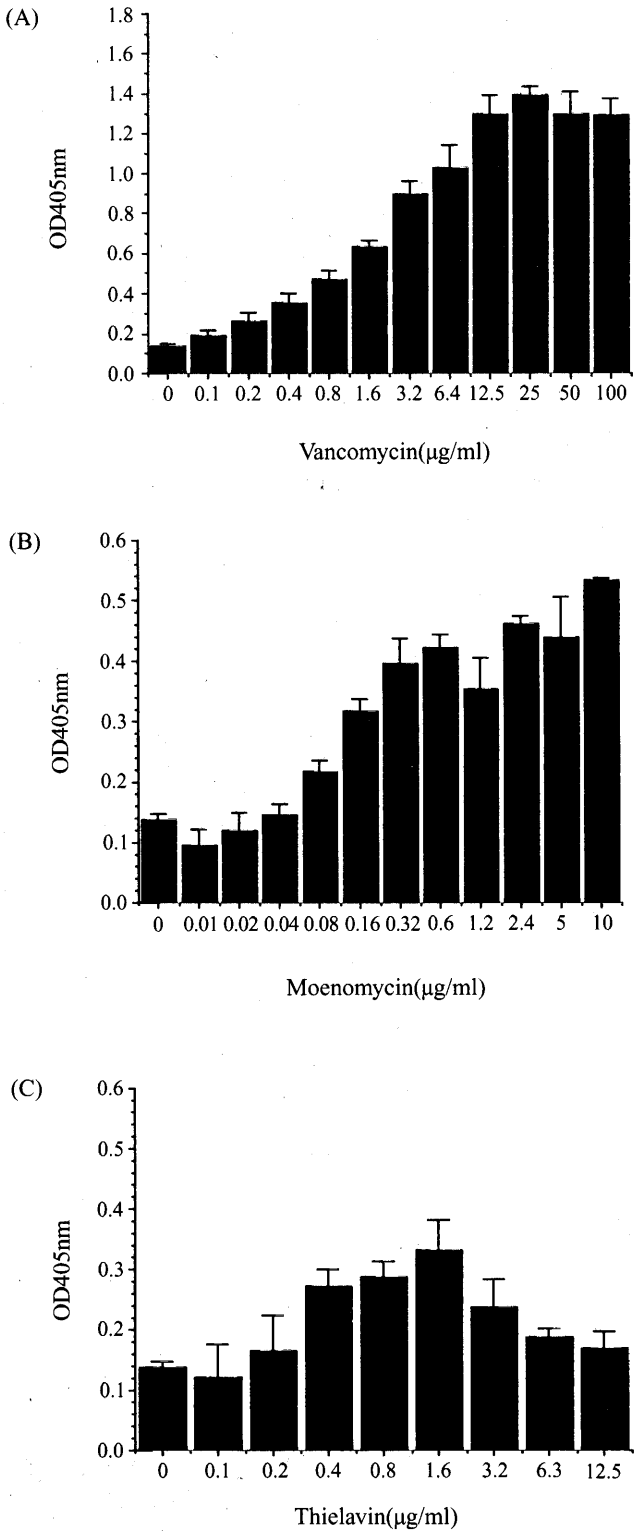
Although it is possible to run a rescue screen using an agar plate format (Data not shown), the microtiter plate format was more sensitive to our control compound, moenomycin. In addition samples can be assessed by objective criteria in the microtiter plate format.

Development of the Reporter Screen

One disadvantage of the rescue screen is that rescue cannot occur in the presence of an abundant level of a sufficiently potent transglycosylation inhibitor. For example, if moenomycin were present at 1 μg/ml, it would be impossible to detect it in the rescue screen, because it exceeds the MIC. This drawback can be overcome in part by screening samples at multiple concentrations as described below. Another approach that might be more concentration-independent is the use of a reporter gene system to measure the induction caused by exposure to samples within a shorter time-frame.

A translational fusion was created between the *vanH* transcriptional and translational signals and the *lacZ* gene and introduced into *E. faecalis* as detailed in Materials and Methods. It was found that the transformant produced a low, constitutive level of β-galactosidase (Data not shown). However, when the vancomycin resistance determinant from strain A256 was mated into the transformant, the resulting strain, MDD212, was found to produce β-galactosidase in response to a two hour exposure to moenomycin or vancomycin (Figure 2). Although both vancomycin and moenomycin induced, it is interesting that β-galactosidase levels were three times higher when vancomycin was

Fig. 2. Reporter screen.



Strain MDD212 was grown to mid-log phase, and cells were diluted to 0.01 OD 655nm per well of a microtiter plate, and exposed to the indicated compounds for 2 hours. Ten μl samples of cells were assayed for β-galactosidase as described in Materials and Methods. Activity is OD 405 nm, subtracting a zero time reading. Values are the average of at least three experiments. Error bars indicate 1 standard deviation from the mean.

Table 1. Compounds that do not test positive in the rescue screen. Samples were tested in 2-fold dilutions.

Compound	A256-5	
	MIC[a] (μg/ml)	High concentration[b] (μg/ml)
Ampicillin[c]	0.4	2500
Bacitracin	16	500
Fosfomycin	2	500
D-Cycloserine	63	1000
Spectinomycin	125	1000
Ciprofloxacin	0.2	100
Chloramphenicol	2	1000
Erythromycin[d]	>500	500
Kanamycin	250	1000
Tetracycline	16	1000
Fusidic Acid	2	1000
Rifampicin	1	1000
Triton X 100	34	135
Sodium desoxycholate	67	135
1-*o*-octyl-β-D-Glucopyranoside	67	135
SDS	17	135
CTAB	0.4	100

[a] MIC was determined by measuring growth in BHI containing 64 μg/ml of vancomycin to satisfy the vancomycin-dependent phenotype.
[b] Eighteen 2-fold dilutions were made starting with the high concentration indicated, for the rescue screen.
[c] In the case of ampicillin twentyfour 2-fold dilutions were made starting with the high concentration indicated.
[d] The strain A256-5 is also erythromycin resistant.

the inducer. As with the rescue screen, the threshhold concentration of moenomycin required for induction (80 ng/ml) was less than that of vancomycin. In comparing the two screening methods, it is clear that the rescue screen was more sensitive at lower concentrations of the transglycosylase inhibitor, moenomycin, whereas the reporter screen was more effective at higher concentrations. Because we were particularly interested in new activities that were not related to vancomycin, moenomycin was selected as the control compound in formatting a screen.

Specificity of the Rescue and Reporter Screens

The rescue screen and the reporter screen were tested against the agents listed in Table 1. A broad range of concentrations were tested, both below and above the MIC of strain A256-5. Only vancomycin and moenomycin, the transglycosylation inhibitors, were active in the rescue and reporter screens. These screening systems are very discriminating in that inhibitors of protein, DNA, or RNA synthesis failed to induce. Other cell wall-active agents, such as bacitracin and fosfomycin, also failed to induce. Furthermore, detergents failed to induce, suggesting that non-specific cell surface disruptive agents will not exhibit activity in these screening systems.

Screening of Fermentation Samples

The rescue screen was tested with samples from the Millennium natural products drug source, derived from fungal fermentations. In parallel, growth inhibition of the multiply-resistant *E. faecium* strain X34044 (a vancomycin-resistant, but not dependent strain) was also assessed. Eight thousand samples were tested as dried films in microtiter wells. In order to maximize the chances of observing rescue, two concentrations, differing by a factor of 10, were tested. An activity that was growth-inhibitory at the high concentration, for instance, could exhibit rescue at the ten-fold lower (sub-inhibitory) concentration.

Seventy-nine samples of the 8000 tested were active in the Rescue Screen. Eight of these seventy-nine active cultures also showed antibacterial activity, and were subjected to our dereplication system.

Identification of Rescue Screen Activity

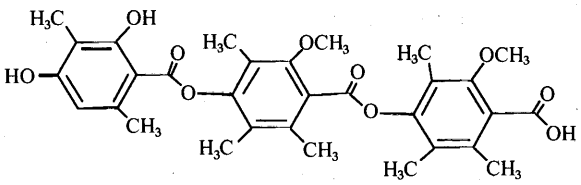
Fermentation samples from culture AA15352 from the Millennium natural products drug source contained antibacterial activity and rescue activity that co-purified during fractionation. To identify the active component(s), strain AA15352 was grown in 1 liter of potato dextrose medium (Difco) for 5 days at 28°C. The supernatant was separated from mycelium and was extracted twice with an equal volume of ethyl acetate. The ethyl acetate layer was evaporated to give an oil (120 mg) which was separated using a reverse phase C-18 HPLC (8 ml/minute flow rate, water/methanol gradient: 95% H_2O/MeOH for 8 minutes, 95% H_2O to 100% MeOH from 8 to 40 minutes and 100% MeOH from 40 to 56 minutes; Dynamax C-18 (5 mm) column, 21.6 × 250 mm in size.). Thielavin B (10 mg), a crystalline material, eluted at around 38 minutes. The physical and chemical data of this isolated thielavin B matched well with that reported in the literature. ^{1}H NMR in CD_3OD: δ 2.06 (3H), 2.14 (3H), 2.17 (3H), 2.25 (3H), 2.27 (3H), 2.29 (3H), 2.42 (3H), 2.65 (3H), 3.82 (3H), 3.83 (3H), 6.38 (1H). The structure of thielavin B is illustrated in Figure 3.

Dereplication of Samples with Rescue Activity and Antibacterial Activity

It was important to eliminate extracts from further consideration if they were likely to contain the same active component as AA15352. Therefore extracts were subjected to reverse-phase HPLC as described in Materials and Methods. Fractions were tested for activity in the rescue screen and for antibacterial activity against *E. faecium* strain X34044. Peak fractions were analyzed by mass spectroscopy in the negative ion mode. As part of the dereplication process, the producing cultures were identified (Table 2).

Dereplication analysis was based on three independent parameters that indicated that the active component in all cases was the family of thielavin compounds: (1) Activity eluted at either 18.0 or 18.8 minutes in each case. (2) The peak fractions contained a compound of exactly the *m/z* of thielavin A and/or B. (3) One of the producing organisms was identified as the ascomycete *Thielavia terricola*, a common soil fungus with ellipsoidal to fusiform ascospores and a terminal germ pore known to produce thielavins[32]. The others were of the genus *Thielavia*, but were not identified to species. It was concluded that the active component(s) in all cases were very likely to be the thielavins.

Fig. 3. Structure of thielavin B.



Activities of Thielavin B

Purified thielavin B was active from 0.5 to 10 μg/ml in both the rescue screen (Figure 1), and in the reporter screen (Figure 2). The fact that thielavin B induced the *van* genes in both screens at a broad concentration range suggested that it was a strong inducer. To obtain additional evidence that thielavin B induced the *van* genes to a physiological meaningful level, cells of *E. faecalis* strain A256 were challenged with 256 μg/ml of vancomycin after overnight growth in BHI that contained no vancomycin, or contained 10 μg/ml vancomycin or 1 μg/ml of thielavin B, which prepared cells for the challenge. Cells that were not pre-induced with vancomycin or thielavin B grew slowly after shifting to the high vancomycin medium. Cells that were grown overnight in the presence of 10 μg/ml of vancomycin, or 1 μg/ml of thielavin B, grew comparably better than uninduced cells (Figure 4). Therefore thielavin B induced to a physiologically meaningful extent.

Activity of Thielavin B *In Vitro*

To confirm that thielavin B inhibited cell wall biosynthesis *in vitro*, we prepared a particulate enzyme preparation capable of carrying out the late steps of cell wall biosynthesis as described in Materials and Methods. Figure 5 shows that thielavin B inhibits the formation of peptidoglycan with an IC_{50} of 5 μg/ml.

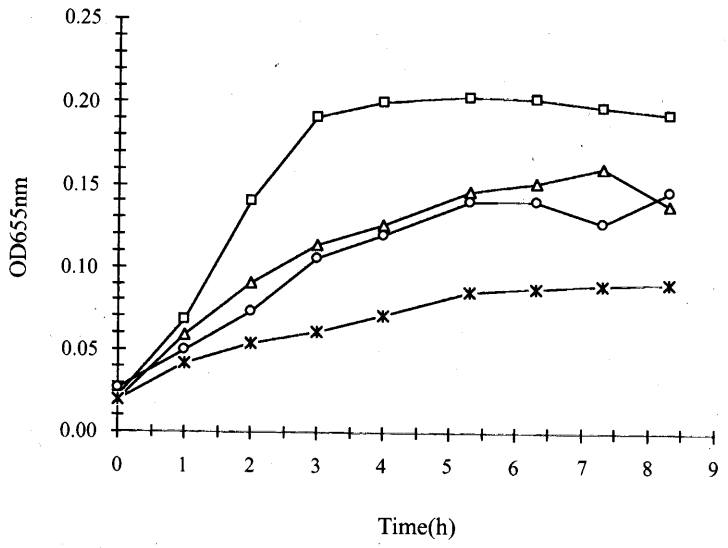
Table 2. Dereplication of activities by taxonomic identification and by LC/MS.

Phylogeny		LC-mass spectroscopy analysis		
Culture[a]	Species identification	UV retention time (minutes)	Molecular ion/MS	Compound
AA15352	*Theilavia terricola*	18.0	565	Thielavin B
AA15222-2	*Theilavia* sp.	18.7	537	Thielavin A
AA15222-3	*Theilavia* sp.	18.0	565	Thielavin B
AA16615	*Theilavia* sp.	18.0	565	Thielavin B
		18.8	537	Thielavin A
AA16598	*Theilavia* sp.	18.2	565	Thielavin B
		18.8	537	Thielavin A
AA15222-1	*Theilavia* sp.	18.0	565	Thielavin B
		18.8	537	Thielavin A

[a] The culture designation in the Millennium strain collection is designated by AA and the 5-digit accession number. The number following the dash indicates different growth conditions.

Fig. 4. Vancomycin challenge.

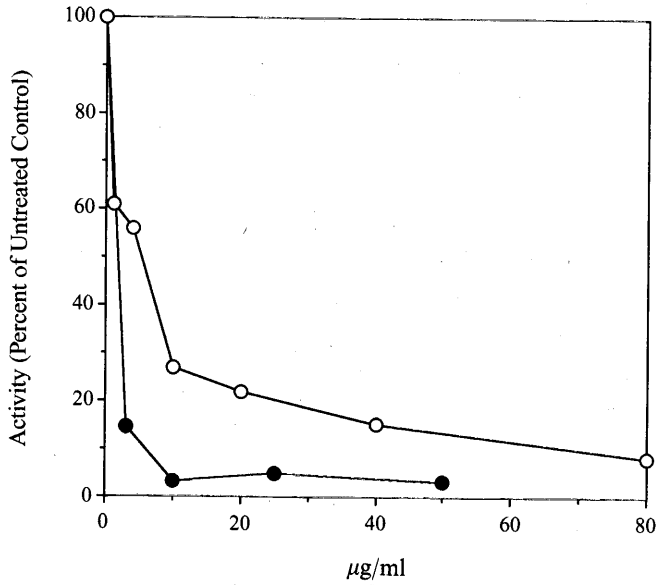
□, Van0-Van0 μg/ml; △, Van10-Van256 μg/ml; ○, ThB1-Van256 μg/ml; ∗, Van0-Van256 μg/ml.



Strain A256 was grown overnight in BHI broth with or without 10 μg/ml vancomycin or 1 μg/ml thielavin B. Cells were diluted the next day in BHI without vancomycin or with 256 μg/ml vancomycin and grown in microtiter wells at 37°C. Cell density was measured at 655 nm.

Fig. 5. *In vitro* inhibition of peptidoglycan synthesis.

○, Thielavin B; ●, moenomycin.



Samples were tested for inhibition of peptidoglycan biosynthesis as described in Materials and Methods. The activity of samples in the presence of thielavin B or moenomycin is shown as percent product relative to the untreated control.

Discussion

Two screens to detect compounds that interact with the VanR/VanS regulatory system have been described. The rescue screen, in which growth of a vancomycin-dependent strain is the signal, was sensitive to vancomycin, and especially to moenomycin. The reporter screen, in which induction of β-galactosidase is the signal, was less sensitive to moenomycin, a compound distinct

in structure and binding properties from vancomycin. However, the reporter screen has the advantage that induction was detected even at concentrations approaching and surpassing the MIC.

An effective screening system was described previously which utilized a fusion of the *cat* gene, cloned to the *van* regulatory region. Growth was observed on agar medium in the presence of chloramphenicol[18]. The rescue screen that we describe has the advantage that it is run in a microtiter plate format, with an objective outcome, in a high-throughput mode. The rescue screen is ideal in terms of sensitivity and simplicity, provided that one has the luxury of testing at two different sample concentrations, differing by a factor of 10. The reporter screen, or the *cat* screen[18] might be more appropriate if only one concentration is to be tested, or alternatively, the rescue screen could be conducted using an agar plate format (Data not shown). It should also be noted that a system for monitoring *van* expression was described in *B. subtilis*, which might also be applied to screening[33].

Because both moenomycin and vancomycin, which have dissimilar structures, are inducers, the cell responds to a physiological signal relating to the state of cell wall biosynthesis, as suggested by others[17~19,34]. The detection of moenomycin at a lower concentration, relative to vancomycin, observed here and in other studies[17~19,34] may be related to the greater susceptibility of *E. faecalis* to moenomycin. If the regulatory components respond to the inhibition of cell wall biosynthesis, then moenomycin will have such effects at a lower concentration than vancomycin for these vancomycin-resistant strains. However, it is interesting that the amplitude of the signal was greater when cells were exposed to vancomycin, compared to moenomycin, in both the rescue and reporter screens, as well as in other studies[17~19,34], suggesting that inhibition of transglycosylation is not the only factor determining the level of expression of the *van* genes.

Both the rescue and reporter screens were induced by inhibitors of transglycosylation, and not by other classes of compounds, including inhibitors of other steps in cell wall biosynthesis, such as bacitracin and fosfomycin (Table 1). However, it should be noted that others have suggested that bacitracin induced the expression of *van* genes from the *vanH* promoter in *Enterococcus*[18,34] and in *B. subtilis*[33]. It is possible that strain differences account for these apparent discrepancies.

Thielavin A and B have been reported previously as potent inhibitors of prostaglandin biosynthesis[35,36]. However we are not aware of any reports of its antibacterial activity. It is encouraging that thielavins were consistently found to be active in the rescue screen (Figure 1), when 8000 samples were screened from our natural products drug source. Thielavins were found to be active in the reporter screen as well (Figure 2), and thielavin B was active in the vancomycin challenge experiment (Figure 4). Therefore thielavin B induces the *van* genes. Furthermore, thielavin B was found to inhibit the formation of peptidoglycan *in vitro*, at a concentration that is close to the MIC for *Enterococcus* (Figure 5). The rescue and reporter screening systems appear to be effective approaches to identify compounds that interfere with cell wall transglycosylation.

Acknowledgments

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