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Induction Signals for Vancomycin Resistance Encoded by the *vanA* Gene Cluster in *Enterococcus faecium*

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The induction of vancomycin resistance in enterococci containing the *vanA* gene cluster is thought to be controlled by a two-component sensor-response regulator system encoded by *vanR* and *vanS*. Eight inducing compounds were identified by screening a panel of more than 6,800 antibiotics and synthetic compounds including the three tested glycopeptides (vancomycin, avoparcin, and ristocetin), two other cell wall biosynthesis inhibitors (moenomycin and bacitracin), two cyclic peptide antibiotics (antibiotic AO341β and polymyxin B), and a macrocyclic lactone antibiotic (moxidectin). Induction activity by structurally unrelated antibiotics suggests that the induction signal is not a structural feature of vancomycin.

The *vanA* gene cluster encodes inducible resistance to the glycopeptide antibiotics vancomycin and teicoplanin. The genetics and resistance mechanisms of this determinant have been studied in detail elsewhere (2, 16). Glycopeptide resistance results from the enzymatic replacement of the D-alanine-D-alanine target of these antibiotics by D-alanine-D-lactate (3, 5, 6, 8, 10). Control of the expression of *vanA* gene cluster expression depends on a putative two-component regulatory system encoded by the *vanS* and *vanR* genes (4). On the basis of strong sequence homology with bacterial response regulator systems, the *vanS* gene is believed to encode a membrane-associated sensor containing a histidine protein kinase motif within its cytoplasmic domain. By the same criteria, the *vanR* gene is believed to encode a response regulator protein which contains phosphorylation and DNA-binding motifs. In systems of this type, specific interactions with the extracellular domain of the sensor result in protein autophosphorylation and the subsequent transfer of a phosphate group to a cognate response regulator protein. Phosphorylation of the response regulator modulates its activity to produce transcriptional activation or repression (2, 4).

In strains with the *vanA* gene cluster, the action of a signal molecule on the extracellular domain of the VanS protein is believed to lead to the activation of the VanR response regulator protein, increasing its activity as a transcriptional activator of structural genes encoding enzymes for peptidoglycan precursor synthesis (2, 4). However, the exact identity of the signal molecule acting on the VanS membrane sensor has not been determined. In this paper, we further characterize the nature of the induction signal by assaying more than 6,800 compounds for induction and characterizing 8 active compounds that were identified from this compound file.

MATERIALS AND METHODS

Strains, media, and chemicals. *Enterococcus faecium* strains BM4138::pAT89 (pAT87), JH2-2(pAT80), and JH2-2(pAT78) (4) were provided by Patrice Courvalin. The strains were grown on appropriately supplemented brain heart infusion (BHI) media (Difco Laboratories, Detroit, Mich.). Esculin was purchased from Baker Chemical Co., Phillipsburg, N.J. Ferric citrate and cell wall pentapeptide were purchased from Sigma Chemical Co., St. Louis, Mo. Antibodies were either from Sigma Chemical Co. or from the Cyanamid chemical library. Sensi-Discs were purchased from BBL, Cockeysville, Md. Protein assay solution was from Bio-Rad Laboratories, Richmond, Calif.

CAT assay. *E. faecium* BM4138::pAT89(pAT87) was grown overnight to stationary phase at 37°C in BHI broth supplemented with 60 μg of spectinomycin per ml. Overnight cultures were diluted 1:4 in the same medium and were grown for 3.5 h at 37°C until late log phase. Chemicals at two concentrations that were less than the MIC (approximately 1/10 and 1/5 of the MIC) were added to the cultures, and the mixtures were incubated for an additional 2 h. The cells were centrifuged, resuspended in 50 mM Tris-HCl (pH 7.5) containing 145 mM NaCl, and disrupted by a W-225 Sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) until the cells reached >50% breakage. The supernatant was used for enzyme assays. Protein concentrations were determined by the Bio-Rad protein assay method according to the manufacturer's instructions. Chloramphenicol acetyltransferase (CAT) assays were carried out according to the method described by Shaw (14).

Petri plate induction assays. The development of a petri plate assay, in which CAT activity is measured by bacterial growth in the presence of chloramphenicol, was necessary to test large numbers of compounds. In this assay, one part of an overnight culture of strain BM4138::pAT89(pAT87) was mixed with 100 parts of BHI agar supplemented with 60 μg of spectinomycin, 60 μg of chloramphenicol, 1 mg of esculin, and 500 μg of ferric citrate per ml. Aliquots (20 μg) of chemicals to be tested were applied onto 6-mm paper discs (Schleicher & Schuell, Inc., Keene, N.H.), and these were placed on the surface of solidified media.The plates were incubated at 37°C for 2 days. Growing cells hydrolyze the glycoside esculin to esculetin and glucose. Esculetin then reacts with the iron salt to form a dark-brown-black complex (9) (Fig. 1). By scoring of color change, vancomycin-stimulated growth could be easily observed. In a serial dilution experiment, as little as 78 ng of vancomycin per disc could be detected on the basis of growth and the color reaction.

Disc diffusion test for *vanA*-dependent activity. A secondary assay was developed to determine whether the activity of compounds detected in the petri plate induction assay required the presence of the *vanA* gene cluster. One part of an overnight culture of cells either carrying [strain BM4138::pAT89(pAT87)] or lacking [strain JH2-2(pAT78)] the *vanA* gene cluster was mixed with 100 parts of BHI agar supplemented with 60 μg of spectinomycin per ml. Paper discs carrying 20 μg of test compounds were placed on the solidified agar surface near chloramphenicol C30 Sensi-Discs. The interactions between chemicals tested and chloramphenicol were recorded after incubation of the plates at 37°C for 2 days. In this assay, asymmetric zones of chloramphenicol inhibition would be expected near compounds which decrease the susceptibility of the cells to chloramphenicol. Such compounds should diffuse into the chloramphenicol zone and rescue the growth of the test strain. Production of such zones with the control strain would indicate that the action of the compound does not require the presence of the *vanA* gene cluster.

RESULTS

Arthur and coworkers (4) constructed an *E. faecium* strain [BM4138::pAT89(pAT87)] in which plasmid pAT89, containing *vanR* and *vanS*, is integrated into the chromosome of strain BM4138 and in which plasmid pAT87 places a promoterless *cat* gene into an operon containing *vanH*, *vanA*, and *vanX*. The inserts in these plasmids and their relationship to the vanco-

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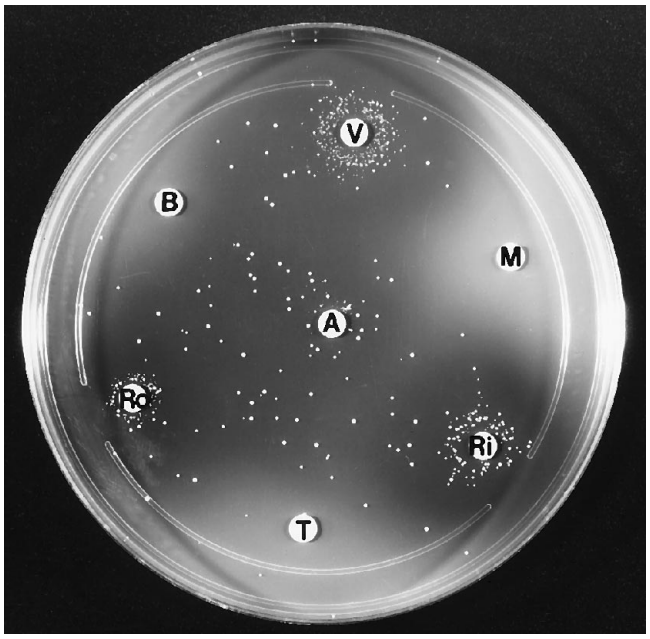

FIG. 1. Petri plate assay for vancomycin resistance induction. The responses to several representative compounds are shown. Test samples applied to paper discs were applied to the surface of the agar as described in Materials and Methods. V, vancomycin; B, bacitracin; Ro, robenidine; T, tetracycline; Ri, ristocetin; M, moenomycin; A, avoparcin.

mycin resistance transposon Tn*1546* are shown diagrammatically in Fig. 2. Using this construct, Arthur et al. were able to investigate the regulation of vancomycin resistance through the use of CAT activity as a reporter. The use of this CAT reporter system should also facilitate the identification and characterization of antibiotics which produce induction of this operon.

Eight antibiotic compounds were selected for initial testing. Vancomycin was employed as a positive control, and two other structurally related glycopeptides, ristocetin and avoparcin, were selected as additional representatives of this chemical class. Three other cell wall-acting antibiotics (moenomycin, bacitracin, and methicillin) were chosen to test the activities of antibiotics acting on other cell wall biosynthesis targets. Tetracycline, a protein synthesis inhibitor, and novobiocin, a DNA gyrase inhibitor, were selected to represent antibiotics with other mechanisms of action. CAT activities were measured after the antibiotic treatment as described in Materials and Methods.

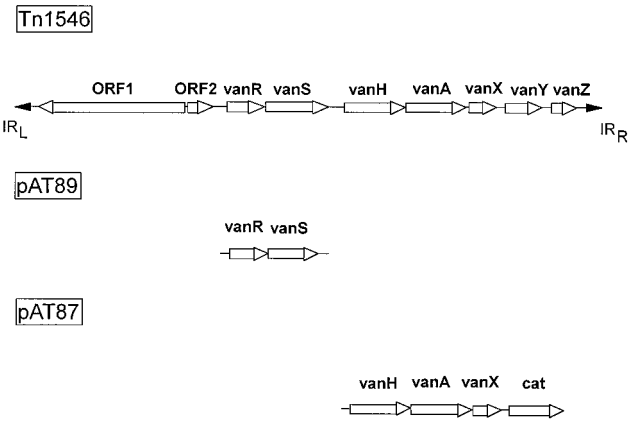

FIG. 2. Diagrammatic presentation of the inserts in plasmids pAT87 and pAT89 and their relationship to the vancomycin resistance transposon Tn*1546*. IR_L and IR_R, inverted left and right repeats, respectively.

TABLE 1. CAT induction activities of selected antibiotics and active compounds from the petri plate assay

Compound	MIC (μg/ml)	Concn (μg/ml)	CAT activity[a]	Induction
None	NA[b]	NA	536; 739	NA
Vancomycin[c]	>100	50	3,820	6.1
		100	3,760	5.9
Avoparcin[c]	>100	50	11,700	18
		100	11,900	19
Ristocetin[c]	>100	50	5,409	8.6
		100	5,310	8.3
Moenomycin[c]	2.5	0.2	2,080	3.3
		0.5	3,190	5.0
Bacitracin[c]	50	5	2,110	3.3
		10	1,920	3.0
Methicillin	12.5	2	680	1.1
		5	702	1.1
Tetracycline	100	10	557	0.87
		20	511	0.80
Novobiocin	25	2	607	0.95
		5	880	1.38
Robenidine	2.5	0.2	742	1.2
		0.5	1,000	1.6
Polymyxin B[c]	>100	50	1,160	1.8
		100	2,170	3.4
AO341β[c]	25	2	2,140	3.4
		5	3,800	6.0
AF283α (Biphenomycin A)	>100	50	660	1.0
		100	694	1.1
Moxidectin[c]	25	2	2,480	3.6
		5	2,650	4.2

[a] Nanomoles per milliliter per minute per milligram of protein.
[b] NA, not applicable.
[c] Data analysis by analysis of variance with Dunnett's *t* tests indicated that CAT activity values obtained after treatment with these antibiotics differed from untreated control values at or greater than at the 95% confidence level.

Vancomycin treatment produced an approximately 6-fold increase in CAT activity (Table 1). Avoparcin and ristocetin behaved similarly to vancomycin, producing approximately 18- and 8-fold increases in CAT activities, respectively. Of the three other cell wall antibiotics that were tested, significant increases in CAT activity were observed for moenomycin (approximately 4-fold) and bacitracin (approximately 3-fold), while methicillin produced no significant change in CAT activity. Tetracycline and novobiocin were also largely inactive, although novobiocin produced a small increase in CAT activity when it was tested at the higher concentration.

To identify additional compounds with potential induction activities, three panels of compounds were tested in the petri plate induction screening assay. The first panel of compounds, including a variety of β-lactam antibiotics and all of the previously tested compounds, was composed of 36 antibacterial antibiotics chosen to represent a wide variety of mechanisms of action. A second panel was composed of 116 antibiotics, including a variety of structurally diverse antibiotics which are commonly recognized as antibacterial agents, as well as compounds with other biological activities (antifungal agents, antitumor agents, and endectosides). The third panel, which consisted of 6,688 compounds from the Cyanamid chemical library, was selected on the basis of chemical diversity.

Four antibiotics from the first panel were active in the screen. In addition to vancomycin, the two other glycopeptide

antibiotics, avoparcin and ristocetin, were active. Surprisingly, none of the other cell wall-acting agents, including moenomycin and bacitracin, was active. Potentially, this was due to the antibacterial activities of these compounds. A growth response may be inhibited if induction produced by these compounds occurs only at concentrations that are inhibitory or partially inhibitory to bacterial growth. The fourth active compound from the first panel was polymyxin B. However, colistin, an antibiotic which is structurally and mechanistically related to polymyxin B, was inactive. Activity was seen with the following three antibiotics in the second group: antibiotic AF283α (biphenomycin A), antibiotic AO341β, and moxidectin (1, 7, 13, 15). All of the active antibiotics from this panel are fairly high-molecular-weight compounds with complex structures.

The third panel tested was composed of chemically synthesized compounds including many structurally simple chemical types. Nine active compounds were identified in this group. These compounds largely resembled one another, and one representative compound, robenidine, was chosen for further characterization. Robenidine is a 344-Da amino guanidine anticoccidial agent used in veterinary medicine to treat protozoan disease in poultry and has not been reported to have commercially useful antibacterial activity (12).

There are a number of mechanisms by which compounds could produce activity in the assay described above. One potential mechanism is through the direct antagonism of the action of chloramphenicol, for example by binding to or reacting with chloramphenicol or by displacing chloramphenicol from its binding site on the bacterial ribosome. Therefore, a disc diffusion test was performed to score the reversal of chloramphenicol inhibition by the production of asymmetric chloramphenicol inhibition zones.

Six of the eight active compounds identified in the prior assay (avoparcin, ristocetin, vancomycin, polymyxin B, robenidine, and, more weakly, moxidectin) produced asymmetric, cut zones on plates inoculated with the strain carrying the *vanA* gene cluster. However, no such effects were observed on plates inoculated with the vancomycin-susceptible control strain. This result demonstrates that the induction of chloramphenicol resistance by these six compounds requires the presence of a *vanA* gene cluster-regulated *cat* gene and excludes *vanA* gene cluster regulation and/or *cat*-independent mechanisms for the action of these compounds. The *vanA* induction effects of AF283α (biphenomycin A) and AO341β were not confirmed in this experiment. These compounds produced no alteration in the shape of the chloramphenicol zone of inhibition. However, activity in this assay requires precise placement of the discs carrying the test compounds. Therefore, all compounds were further tested for the induction of CAT enzyme activity.

The CAT enzyme assay was employed to characterize the petri plate assay-active compounds for *vanA* induction activity (Table 1). As before, each compound was tested at two concentrations that were less than the MIC. Polymyxin B, moxidectin, and peptide antibiotic AO341β were all active in this test. Polymyxin B produced induction ratios of approximately 2.5-fold, while moxidectin and antibiotic AO341β produced induction ratios of approximately 4- to 4.5-fold. These compounds were not as effective inducers as the glycopeptides but were similar or superior to moenomycin and bacitracin. Antibiotic AF283α (biphenomycin A) was inactive, and robenidine was at best weakly active (approximately 1.4-fold induction). This is not totally surprising, since the activity of AF283α (biphenomycin A) was not confirmed in the disc assay. These data do not suggest a ready explanation for the incomplete correlation between the petri plate and cell-free assays. Perhaps some compounds may enhance the activity of CAT in the petri plate assay in a manner that does not affect the cell-free assay. However, despite the incomplete correlation, the petri plate assay was useful in identifying a number of additional novel *vanA*-inducing compounds.

Lastly, the wide variety of active compounds differing in structure and antibacterial mechanism indicates that the inducing signal must be generated by the cell in response to antibiotic treatment. In a crude attempt to identify this signal, CAT activity was measured after treatment of the cells with a peptidoglycan precursor pentapeptide, Ala-D-γ-Glu-Lys-D-Ala-D-Ala. Treatments were performed at 50 and 100 μg/ml to ensure that the test was performed with adequate levels of inducing compound. No induction was observed by the pentapeptide in this experiment, indicating that this cell wall metabolite is probably not the inducing signal (data not shown).

DISCUSSION

In this study, we have used an operon fusion to characterize the induction signal for the *vanA* resistance determinant. Active compounds can be placed into three groups. The three tested glycopeptides were clearly the best inducers, and all produced induction ratios of 6-fold or greater. While we measured an approximately 6-fold induction with vancomycin, Arthur and coworkers reported a 38-fold induction of activity using a similar level of vancomycin (4). This difference is largely due to the fact that we have consistently observed higher basal levels of CAT activity than those that were reported in the previous study, potentially because we consistently assayed CAT activity at a later point in the growth curve.

A second group of compounds consisting of moenomycin, bacitracin, polymyxin B, antibiotic AO341β and moxidectin all produced induction, but in the range of an approximately 3- to 4-fold increase relative to untreated control. A third group of compounds were active in the plate assay. However, these compounds (robenidine and AF283α [biphenomycin A]) showed little effect, at best about 1.5-fold induction, in the enzyme induction assay. Moenomycin and bacitracin were active in the enzyme induction assay but negative in the plate assay, potentially because activity in the plate assay requires cell growth and the concentrations of these two compounds that are required for induction produce cell damage that limits subsequent growth. In a prior study, Handwerger and Kolokathis (11) tested a panel of antibiotics for the induction of vancomycin resistance by pregrowing a vancomycin-resistant *E. faecium* strains with test compounds and then measuring the subsequent growth rate in the presence of a subinhibitory level of vancomycin. Of the compounds tested, only pretreatments with vancomycin and moenomycin led to increased subsequent growth rates in the presence of vancomycin, leading these authors to conclude that moenomycin acts as an inducer of vancomycin resistance.

Induction by structurally diverse compounds suggests that the induction signal for *vanA* operon-encoded vancomycin resistance is not a structural feature of vancomycin and may be a cell wall degradation product or other cell metabolite produced in the presence of certain antibacterial agents. Two of the non-glycopeptide inducers inhibit cell wall biosynthesis. Moenomycin is a phosphoglycolipid antibiotic which inhibits glycan transglycosylation from the undecaprenyl-pyrophosphate-disaccharide-pentapeptide substrate (10). Bacitracin is a cyclic peptide antibiotic that inhibits cell wall biosynthesis through an action on the C_{55}-isoprenyl pyrophosphate carrier molecule that is required for the transport of peptidoglycan precursor disaccharide-pentapeptides across the plasma membrane (10).

Inducing compounds that do not have demonstrated cell wall activity include the cyclic peptide antibiotics polymyxin B, AO341β, and moxidectin. Polymyxin B has been shown to act on the plasma membrane and does not directly affect cell wall biosynthesis (10). AO341β is an incompletely characterized cyclic peptide antibiotic with an unknown mechanism of action (15). Moxidectin is a macrocyclic lactone endectocide antibiotic with no significant antibacterial activity. This compound acts on a ligand-gated chloride channel present in insects and helminths (1, 13).

All of the active compounds were examined for the presence of common structural features. All tested glycopeptide antibiotics and three of the other active compounds (bacitracin, polymyxin B, and antibiotic AO341β) are similar in that they contain or are composed of cyclic peptides and all contain leucine or leucine analogs. Aromatic amino acids are also present in all of the glycopeptides and in polymyxin B and bacitracin. However, there are no aromatic amino acids in antibiotic AO341β. Threonine is present in both polymyxin B and AO341β. Beyond these similarities, no amino acid is common among all of the induction-active peptides. Structural similarities among the glycopeptides, peptide antibiotics, moxidectin, and moenomycin are even less clear. One property shared by all of the active compounds is that they either are all hydrophobic molecules or have hydrophobic domains. This suggests a common potential for action on the plasma membrane. Future studies will address this possibility.

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