

# A Vancomycin-Inducible LacZ Reporter System in *Bacillus subtilis*: Induction by Antibiotics That Inhibit Cell Wall Synthesis and by Lysozyme

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Received 6 June 1996/Accepted 26 August 1996

**We have constructed a *Bacillus subtilis* strain in which expression of a *vanH::lacZ* gene fusion is regulated by VanR and VanS of *Enterococcus faecium*. This construct allows a nonpathogenic bacterial strain to be used as a model system for studying regulation of vancomycin resistance. Antibiotics and enzymes that affect cell wall biosynthesis and stability were tested for the ability to induce *lacZ* expression. As a result, fosfomicin and D-cycloserine were added to the group of peptidoglycan synthesis inhibitors shown to induce expression from the *vanH* promoter. Induction by cell wall hydrolytic enzymes, as well as by antibiotics whose actions may lead to the accumulation of chemically different peptidoglycan precursors, raises the possibility that models that postulate induction by peptidoglycan precursors are wrong.**

Inducible resistance to vancomycin and related glycopeptide antibiotics in *Enterococcus* spp. (9, 11, 13, 24, 28) has emerged in recent years as a major health problem in the treatment of infectious diseases (22, 29, 31). The inducible form of resistance can also serve as a transitional step to constitutive resistance, with important consequences for the clinical effectiveness of antibiotics that belong to this group (12, 15, 25, 26).

The VanA type of resistance is one of the major forms of inducible resistance to glycopeptide antibiotics. It is specified by the VanA complex operon, which consists of two coordinately expressed operons, *vanRS* and *vanHAX*. The *vanRS* operon specifies the two-component system histidine kinase receptor VanS and its transcriptional response regulator VanR, while *vanHAX* specifies enzymes that are responsible for the synthesis and biochemical modification of cell wall peptidoglycan precursors which make bacteria resistant to glycopeptide antibiotics (2, 3). The activated VanS receptor phosphorylates the transcriptional regulator, VanR, which in turn activates transcription of the *vanHAX* operon (for details, see references 17 and 33). The molecular mechanisms of VanS receptor activation and its ligands are not known, and a study of the virulent bacteria or opportunistic pathogens expressing glycopeptide resistance requires special attention to the containment of these strains.

To address these problems, we have developed a new cloning tool and used it to construct a model vancomycin-inducible system in the nonpathogenic strain *Bacillus subtilis* 168. In this strain, the *Enterococcus faecium vanRS* operon regulates expression of LacZ from the *E. faecium vanH* promoter. We have tested the system to measure the inducing activities of antibiotics that inhibit peptidoglycan synthesis and cell wall hydrolytic enzymes.

## MATERIALS AND METHODS

**Plasmid pAU101.** The general methods used in this work to construct recombinant plasmids and to introduce them into *Escherichia coli* were as described in

reference 26. PCR was performed by using the general methods described by Mullis and Faloona (21). A 257-bp *vanH* cassette bounded by *Bam*HI-compatible cohesive ends, 5'GATC, and containing the *vanH* promoter, together with the *vanH* Shine-Dalgarno sequence and the first three amino acid determinants of VanH, was obtained by performing two parallel PCRs with primer sets 3714-3715 and 3716-3717, respectively (Table 1). DNA from *E. faecium* A634 was used as the template, and proofreading *Pfu* polymerase was used for amplification. The two flush PCR products were mixed in equal amounts, melted, and reannealed; the resultant cassette preparation was ligated with plasmid pMD428 (8) at its *Bam*HI site. The mix-melt-reanneal step generates four duplex products: the two original flush DNA duplexes, and two heteroduplexes, one with 5' overhangs and the other with 3' overhangs. The linearized vector, prepared by digestion with restriction endonuclease *Bam*HI, will ligate only with the reannealed product which contains the complementary 5'GATC overhanging ends. Thus, the method described can generate a cloning cassette with potentially any combination of cohesive and flush ends by a procedure that does not require restriction endonucleases. The ligation mixture was used to transform *E. coli* JM109 (20), and transformants were selected for resistance to ampicillin. Transformants were checked for incorporation of the *vanH* cassette in the desired orientation by PCR with primers 3716 and 3957, and one plasmid clone, pAU101, was selected for use in the next step.

***B. subtilis* BAU-101.** The general methods used to transform *B. subtilis* were as described by Henkin and Chambliss (16). Plasmid pAU101 DNA prepared in *E. coli* was used to transform *B. subtilis* 168, after which cultures were selected for chloramphenicol resistance. Since pMD428 lacks a *B. subtilis*-compatible origin of replication, transformants selected in this way contain the *vanH::lacZ* cassette integrated into the *amyE* locus by homologous recombination with the *amyE* partial sequences that flank the multiple cloning site of pMD428. One colony containing the *vanH* cassette, *B. subtilis* BAU-101, was selected for the next step.

**Plasmids pAU102 (*vanRS*), pAU103 (*vanR*), and pAU104 (*vanS*).** A cassette containing *vanRS* bounded by an upstream flush end and downstream *Bam*HI cohesive end was obtained by performing two concurrent PCRs with primer sets 4912-4913 and 4912-4914, respectively. *E. faecium* A634 DNA was used as the template. The mixture of two PCR products was melted and reannealed; the resultant cassette preparation was ligated with the *E. coli*-*B. subtilis* shuttle vector plasmid pHB201 (6) at its *Pme*I and *Bam*HI restriction sites. In pAU-102, *vanRS* is inserted 81 nucleotides downstream of the *cat-86* promoter that is present in pHB201. The ligation mixture was used to transform *E. coli* JM109, and transformants, selected on the basis of resistance to ampicillin, were checked for incorporation of the *vanRS* cassette in the desired orientation. Similarly, cassettes containing *vanR* alone or *vanS* alone were prepared by using primer pairs 4912-5335 and 4912-5336 for *vanR* (plasmid pAU103) and 5334-4913 and 5334-4914 for *vanS* (plasmid pAU104).

***B. subtilis* BAU-102, BAU-103, and BAU-104.** DNA preparations of plasmids pAU102, pAU103, and pAU104, were used to transform *B. subtilis* BAU-101, and then cultures were subjected to selection for erythromycin resistance. The resultant transformants were designated BAU-102, BAU-103, and BAU-104, respectively.

**Demonstration of induced  $\beta$ -galactosidase activity on solid medium.** Solid bottom agar medium for plate assays contained 1 $\times$  Spizizen minimal medium

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TABLE 1. PCR primers<sup>a</sup>

Primer	5'-3' DNA sequence	Use	Coordinates
3714	<u>GAT CCC</u> AGA CTT GGT TGA TAA AAG GAG G	VanH promoter forward 1	2230-2253
3715	GTT ATT CAT AAT TAA GAC CAA CCC	VanH promoter reverse 1	2458-2481
3716	CCA GAC TTG GTT GAT AAA AGG AGG	VanH promoter forward 2	2230-2253
3717	<u>GAT CGT</u> TAT TCA TAA TTA AGA CCA ACC C	VanH promoter reverse 2	2458-2481
3957	GGG GAC GAC GAC AGT ATC GGC CTC	LacZ sequence in pMD428	
4912	GAT AAA ATA CTT ATT GTG GAT GAT GAA CAT	VanRS forward	438-467
4913	TTA GGA CCT CCT TTT ATC ACC AA	VanRS reverse 1	2235-2258
4914	<u>GAT CTT</u> AGG ACC TCC TTT TAT CAA CCA A	VanRS reverse 2	2235-2258
5334	ATA AAT TGA AAA ATA AAA AAA ACG ACT AT	VanS forward	1110-1139
5335	TTA TTT TTC AAT TTT ATA ACC AAC CCC	VanR reverse 1	1110-1139
5336	<u>GAT CTT</u> ATT TTT CAA TTT TAT AAC CAA CCC C	VanR reverse 2	1100-1126

<sup>a</sup> Primers and their associated sequence coordinates were based on the *vanRS* and partial *vanH* DNA sequence reported by Arthur et al. (3). Nucleotide sequences which are underlined do not occur as such in the published sequence and represent modifications that were made to generate the cohesive ends used for cloning.

(SMM) (14), agar (1.5%), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal; 40  $\mu$ g/ml), and erythromycin (10  $\mu$ g/ml). Soft overlay agar was prepared as for bottom agar except that 0.75% agar was used. Pour plates were prepared by mixing 4 ml of top agar with 300  $\mu$ l of BAU-102 overnight-grown cells and pouring the resultant cell suspension onto solidified bottom agar. Antibiotic disks were placed on the solidified top agar, and plates were incubated overnight at 37°C and for an additional day at 4°C for complete color development.

**Measurement of induced  $\beta$ -galactosidase activity in solution.** Cell were tested in the exponential phase of growth. An inoculum of *B. subtilis* containing one of the constructs was grown overnight in a 50-ml Erlenmeyer flask at 37°C in LB medium containing erythromycin (10  $\mu$ g/ml) and chloramphenicol (25  $\mu$ g/ml); 250  $\mu$ l of the overnight culture was added to 10 ml of SMM (14) containing 10% (wt/vol) sucrose and 10  $\mu$ g of erythromycin per ml in a 50-ml Erlenmeyer flask. Sucrose was used to prevent lysis of the bacteria in the presence of inhibitors of cell wall synthesis or lytic enzymes. After 1.5 to 2 h of incubation with shaking at 30°C, cells reached an optical density of 0.25 to 0.30 at 660 nm and were tested for induction by transfer of 0.5-ml samples of culture to polypropylene tubes (17 by 100 mm; Falcon no. 2059). Test antibiotics or enzymes were added, and samples were incubated for an additional 4 h.  $\beta$ -Galactosidase activity was measured by using the fluorogenic substrate 4-methyl-umbelliferyl- $\beta$ -D-galactopyranoside (MUG) as described by Youngman (35). To measure  $\beta$ -galactosidase activity, 50  $\mu$ l of cell culture was incubated with 10  $\mu$ l of MUG stock solution (MUG stock solution [400  $\mu$ g/ml] in dimethyl sulfoxide) for 1 h at 25°C. Reactions were stopped by adding 50  $\mu$ l of the incubation mixture to 2.5 ml of assay buffer containing 60 mM  $K_2HPO_4$ , 40 mM  $KH_2PO_4$ , and 100 mM NaCl. Fluorescence was determined in an SLM-Aminco fluorimeter with excitation at 365 nm and emission at 450 nm. Cell growth was determined by measuring total protein concentration with the Bio-Rad (Bradford) protein assay as specified by the supplier. An arbitrary unit of  $\beta$ -galactosidase activity was defined as the fluorimeter reading normalized to total protein concentration in micrograms per milliliter.

**Chemicals.** Except as indicated, enzymes and fine chemicals were obtained from Sigma. The glycopeptide LY-191145 was a gift of Eli Lilly and Co. The glycopeptide teicoplanin was a gift of Marion-Merrill Dow Inc.

## RESULTS

**Construction of a *vanH::lacZ* fusion which is inducible by vancomycin.** When induction of resistance to an antibiotic is

linked to its inhibitory action on bacterial growth, the usual growth-based assays may fail to respond to a prospective inducer if the inducer eventually kills the test culture during the assay. A reporter gene linked to expression of an enzyme which is synthesized during induction would be helpful in avoiding false-negative observations. We therefore constructed a LacZ reporter system to determine the range of antibiotics that induce vancomycin resistance and to measure the dose response to test inducers. The composition of our reporter strain is summarized in Fig. 1.

**Demonstration of inducing activity on solid medium.** Inducibility of *B. subtilis* was tested on X-Gal indicator plates. Results are shown in Fig. 2. The blue halo, indicating induction, was consistently seen at the margin of the inhibition zone, which implies that the inducing concentration of the antibiotic is lower than but close to its killing concentration. Kanamycin, which inhibits ribosome function, was included as a negative control. We note strong induction by all of the peptidoglycan synthesis inhibitors tested. For reviews of the peptidoglycan synthesis pathway and its inhibitors, see references 7 and 30.

**Measurement of induction as a function of antibiotic concentration.** To obtain a quantitative measure of induction, we measured LacZ activity in cell cultures growing in the presence of antibiotics. Results of induction as a function of antibiotic concentration are shown in Fig. 3. All antibiotics which inhibit cell wall synthesis strongly induced  $\beta$ -galactosidase in the reporter strain BAU-102. The concentration of inducing antibiotic that was required ranged from less than 100 ng/ml (the glycopeptide LY-191145) to more than 100  $\mu$ g/ml for fosfomycin, D-cycloserine, and bacitracin. Especially interesting is the finding that the early cell wall synthesis inhibitors D-cycloserine and fosfomycin can induce our reporter system. If the hypoth-

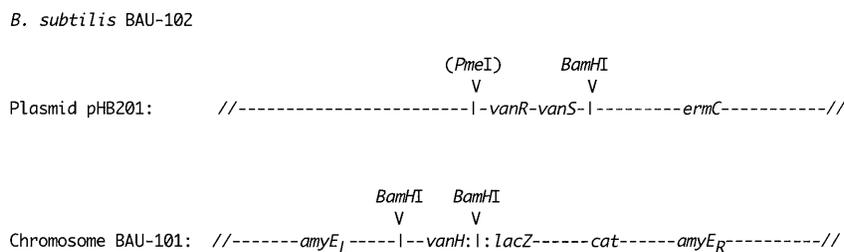


FIG. 1. Construction of the inducible *vanH::lacZ* *B. subtilis* reporter strain BAU-102. A *vanH* cassette with *Bam*HI cohesive ends, containing the first three codons of the VanH open reading frame, was ligated into the *Bam*HI multiple cloning site of plasmid pMD428 in phase with *lacZ*. The resultant construct, containing *cat-86* for selection and bounded by *amyE* gene fragments, was introduced into *B. subtilis* by transformation, and integrants into the chromosome by site-specific recombination were selected with chloramphenicol. The resultant *B. subtilis* strain was designated BAU-101. Plasmid pHB201, containing the *vanRS* operon cassette, was obtained by PCR with a flush upstream end and a 5'GATC overhang downstream. The resultant cassette was ligated with pHB201 digested with *Pme*I and *Bam*HI and introduced into *B. subtilis* BAU-101 by transformation and selection for erythromycin resistance. The resultant *B. subtilis* strain was designated BAU-102.

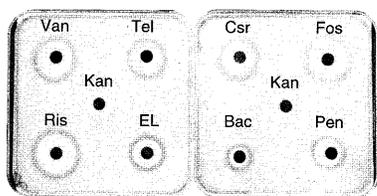


FIG. 2. Survey of antibiotics for inducing activity, using the reporter strain and the disk diffusion method. Filter paper disks containing test antibiotics were placed on a lawn of *B. subtilis* BAU-2 growing on SMM containing X-Gal (40  $\mu\text{g/ml}$ ). Antibiotics and their respective amounts (micrograms per disk) were as follows: vancomycin (Van), 25; teicoplanin (Tei), 25; ristocetin (Ris), 25; LY-191145 (EL), 25; D-cycloserine (Csr), 250; fosfomycin (Fos), 2,000; bacitracin (Bac), 250; penicillin G (Pen), 50; and kanamycin (Kan) 25. Plates were incubated overnight at 37°C for cell growth and stored at 4°C for an additional 24 h for complete color development.

esis of VanS induction by cell wall precursors (1, 13) is correct, these results indicate that early cell wall intermediates may also function as the activators of VanS.

**Comparative induction by two groups of antibiotics.** We were unable to obtain a full dose-response curve in our concentration dependence experiments because the higher concentrations of antibiotics killed the cells. For this reason, we compared the inducing activities of antibiotics at the concentration that half inhibited cell growth on the basis of comparing total cell protein with that of untreated control cells. As shown in Fig. 4, antibiotics that inhibit cell wall synthesis induced  $\beta$ -galactosidase much more strongly than did the antibiotics kanamycin, trimethoprim, thiostrepton, rifampin, and novobiocin, which do not.

**Activation of *LacZ* by either late or early inhibitors cannot be attributed to cross talk.** Cross talk, i.e., phosphorylation of bacterial response regulators by heterologous histidine kinase receptors, is a well-known phenomenon. To estimate the relative contribution of cross talk to our observations, we compared *LacZ* induction in *B. subtilis* BAU-102 (which expresses both VanR and VanS) and BAU-103 (which expresses only VanR). We used two late-stage cell wall synthesis inhibitors, vancomycin and teicoplanin, and two early-stage inhibitors, D-cycloserine and fosfomycin. As shown in Fig. 5, all four

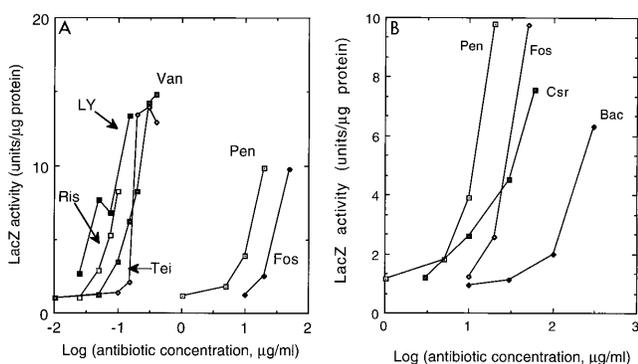


FIG. 3. Induction of *vanH:lacZ* as a function of concentration of antibiotics that inhibit cell wall synthesis. All antibiotics that were tested strongly induced. Growing liquid cultures of *B. subtilis* BAU-102 were supplemented with test antibiotics for 4 h at the concentrations shown. Cells were collected, and *LacZ* activity was quantified as described by Youngman et al. (35) with the fluorogenic substrate MUG and normalized for activity of control untreated cells and total protein. Abbreviations for antibiotics used: Bac, bacitracin; Csr, D-cycloserine; LY, LY-191145; Fos, fosfomycin; Pen, penicillin G; Ris, ristocetin; Tei, teicoplanin; Van, vancomycin.

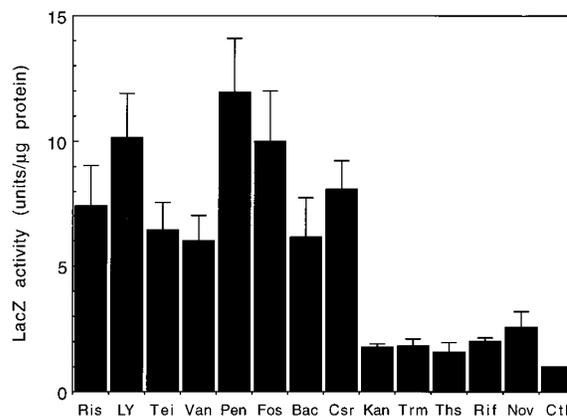


FIG. 4. Induction of *vanH:lacZ* in the BAU-102 reporter strain at the antibiotic concentration that inhibited cell growth by 50%, measured on a protein basis (average and standard deviation,  $n = 3$ ). All data were normalized for activity of control untreated cells and total protein. The inhibitors of peptidoglycan synthesis and the respective concentrations (in micrograms per milliliter) were as follows: ristocetin (Ris), 0.1; LY-191145 (LY), 0.075; teicoplanin (Tei), 0.3; vancomycin (Van), 0.2; penicillin G (Pen), 30; fosfomycin (Fos), 300; bacitracin (Bac), 300; and D-cycloserine (Csr), 100. The negative controls were as follows: kanamycin (Kan), 16; trimethoprim (Trm), 4; thiostrepton (Ths), 0.2; rifampin (Rif), 0.025; and novobiocin (Nov), 2. Fosfomycin, bacitracin, and D-cycloserine strongly induced  $\beta$ -galactosidase activity but weakly inhibited cell growth and at the above concentrations produced less than 50% inhibition.

antibiotics demonstrated *lacZ* induction in BAU-103 but to a markedly lower degree than in BAU-102. No significant  $\beta$ -galactosidase activity was observed in strains BAU-101 and BAU-104 lacking VanR (data not shown). This result indicates that (i) most of *LacZ* activation in the reporter strain is dependent on VanS receptors and (ii) cross talk, i.e., phosphorylation of VanR by other kinases (possibly including a *B. subtilis* homolog of VanS), may influence transcription from the *vanH* promoter.

All antibiotics that strongly induced our reporter strain also inhibit cell wall synthesis, and incubation of bacteria with these antibiotics damages the cell wall. To test the possibility that cell

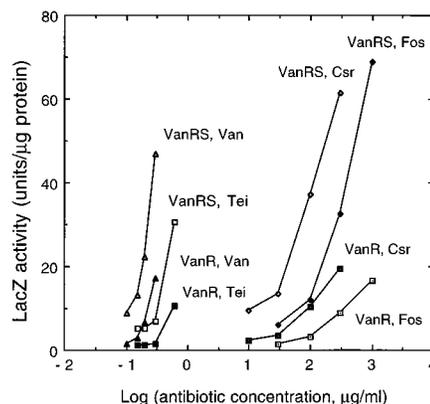


FIG. 5. Induction of *vanH:lacZ* in the presence and absence of VanS. Induction of *vanH:lacZ* was tested in the reporter strains BAU-102 (*vanRS*) and BAU-103 (*vanR*) by antibiotics that inhibit the later stage (vancomycin [Van] and teicoplanin [Tei]) or earlier stages (fosfomycin [Fos] and D-cycloserine [Csr]) of cell wall synthesis. A low level of induction was seen even if the response regulator VanR alone was present and can be attributed to other histidine kinases that are resident in *B. subtilis* and are capable of cross talking with VanR. Activity is expressed in relative units (fluorimeter reading/total protein). Values for control untreated cells were 4.8 (BAU-102) and 0.87 (BAU-103).

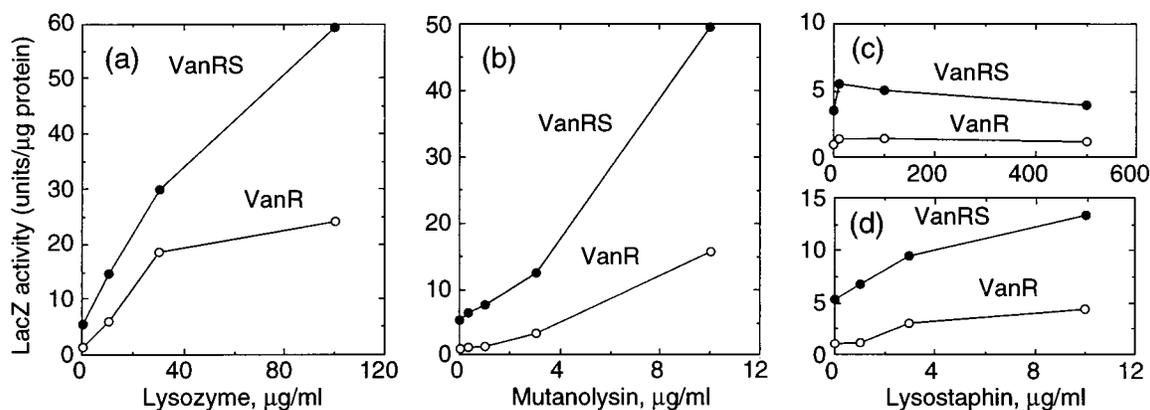


FIG. 6. Induction of *vanH:lacZ* in BAU-102 (*vanRS*) and BAU-103 (*vanR*) by cell wall lytic enzymes lysozyme (a), mutanolysin (b), and lysostaphin (high [c] and low [d] concentration ranges).

wall damage per se activates our reporter system, we tested three hydrolytic enzymes, lysozyme, mutanolysin, and lysostaphin. Lysozyme and mutanolysin were reported by Banerjee et al. (4) to cleave the  $\beta$ 1 $\rightarrow$ 4 glycosidic bond between GlcNAc and MurNAc, whereas lysostaphin (27, 32) cleaves the glycyglycine linkage (18, 23) and has been reported to lack activity against *B. subtilis* cell walls (27).

Figure 6 shows dose-response curves for induction of BAU-102 and BAU-103 by hydrolytic enzymes. Lysozyme and mutanolysin strongly induced  $\beta$ -galactosidase activity in the reporter strains in a dose-dependent manner. Results with lysostaphin varied and were weakly positive (Fig. 6c and d), suggesting the presence of a low level of glycyglycine bridge residues.

## DISCUSSION

We have developed a LacZ reporter system that demonstrates induction of *vanH* independently of cell survival in a nonpathogenic strain, *B. subtilis* 168. Previous attempts (5) to transfer vancomycin resistance to *B. subtilis* were unsuccessful. We show here that it is possible to reconstitute, in *B. subtilis*, at least the regulatory mechanism leading to induction of *vanRS* and transcriptional activation of *vanH*.

Previous studies of induction of vancomycin resistance in *E. faecium* have used assays based on (i) growth in challenging concentrations of vancomycin after preincubation with test inducer (1, 5, 13, 28), (ii) growth of a vancomycin-dependent strain in the presence of test inducer (11), (iii) increased density of a 39-kDa protein band by polyacrylamide gel electrophoresis (9, 13, 28), or (iv) measurement of chloramphenicol acetyltransferase activity in cells carrying *van::cat* fusion constructs (3). Growth-based assays cannot be used reliably to identify antibiotics which induce the resistance-related enzymes if the induced cells fail to survive induction. It is useful, therefore, to study induction of such mechanisms in assays that do not depend on cell survival.

Two biologically useful observations come out of this initial report of the construction and characterization of our *B. subtilis* vancomycin-inducible reporter strain: (i) the vancomycin resistance system is induced by cell wall hydrolytic enzymes and early-phase inhibitors of cell wall synthesis, D-cycloserine and fosfomycin, and (ii) the induction by cell wall hydrolytic enzymes and structural dissimilarities between the antibiotics which induce suggest that their interaction with VanS is indirect and may be a complex function of other factors (see also references 1, 11, and 19).

The molecular mechanism of VanS induction is unknown. Recent observations of induction of VanA resistance by non-glycopeptide antibiotics indicate the possibility that vancomycin and its analogs are not the natural ligands for the VanS receptor. Handwerger and Kolokathis (13) observed induction of resistance by vancomycin and moenomycin, but not by D-cycloserine, bacitracin, or penicillin, all of which induced our construct. Allen and Hobbs (1) were able to demonstrate induction of resistance with late-stage inhibitors of cell wall biosynthesis (penicillin G, bacitracin, and vancomycin) but not with early-stage inhibitors (D-cycloserine and fosfomycin). On the basis of induction by late-stage but not early-stage inhibitors of peptidoglycan synthesis, they concluded that pentapeptide precursors serve as the inducers (ligands) of the VanS receptor. Our results with late-stage inhibitors confirm their experimental findings as well as those of Handwerger and Kolokathis (13) and extend these observations to include early-stage cell wall synthesis inhibitors as well. For induction, we were able to use concentrations of fosfomycin and D-cycloserine higher than those used by Allen and Hobbs (1) because the LacZ reporter system does not depend ultimately on cell survival. Species differences between *B. subtilis* and *E. faecium* might also have contributed to observed differences between the two sets of observations.

If the hypothesis of activation by cell wall peptidoglycan precursors is correct, then induction by fosfomycin and D-cycloserine indicates that VanS can be activated by direct interaction with all peptidoglycan precursors, beginning with UDP-GlcNAc. Since the cell wall precursors UDP-GlcNAc through UDP-N-acetylmuramylpentapeptide have UDP-GlcNAc as a common structural element, it is possible that this element would be the one recognized by VanS; however, the finding that all antibiotics which inhibit peptidoglycan synthesis together with cell wall hydrolytic enzymes induce makes the hypothesis of induction by peptidoglycan precursors tenuous and suggests the possibility of another mechanism.

Cross talk in the VanRS system has been described by Fisher et al. (10). They reported the ability of a VanS kinase fragment to phosphorylate the PhoB transcriptional activator in vitro, while Arthur et al. (3) previously reported the complementary observation, namely, that insertional inactivation of VanS failed to abolish induced resistance in *Enterococcus* spp. They attributed this residual resistance to cross talk between expressed VanR and some other histidine kinase receptors. LacZ expression in our system was demonstrable in a strain transformed only with *vanR*; in the absence of *vanS*, *lacZ* was

induced by teicoplanin, vancomycin, fosfomycin, and D-cycloserine, but to a lesser degree than in the presence of VanS (Fig. 5).

We were surprised to observe induction of the VanS reporter system by lysostaphin, which has been reported to lack activity toward *B. subtilis* cell walls (27). The observed induction was much lower than that which was obtained with either lysozyme or mutanolysin and was observed reproducibly. Lysozyme is a proteolytic enzyme that cleaves glycyglycine linkages in the pentaglycine bridge moiety of staphylococcal peptidoglycan, (18, 32). Studies of Young (34) indicated the presence of equimolar amounts of serine and glycine in fragments of *B. subtilis* cell wall which they analyzed, while Robinson et al. (23) noted the association between peptide bridge serine content and insusceptibility to digestion with lysostaphin. The low-level induction seen in our system may indicate the presence of a limited number of glycyglycine target sites in *B. subtilis* peptidoglycan. The presence of traces of a contaminating lytic enzyme is unlikely since we did not see a stronger inductive effect even with very high concentrations of the enzyme (100 and 500  $\mu\text{g/ml}$ ). The same level of induction seen at 10, 100, and 500  $\mu\text{g}$  of lysostaphin per ml favors the hypothesis of a limited number of fully susceptible sites in the *B. subtilis* cell wall.

In conclusion, strong induction of our reporter system by all late cell wall inhibitors, early inhibitors, and, finally, cell wall hydrolytic enzymes brings into question a cell wall precursor mechanism as the basis for VanS activation. These results suggest an alternative hypothesis for VanS activation whereby VanS senses cell wall destabilization by an as yet unknown mechanism.

#### ACKNOWLEDGMENTS

This work was supported by research grant AI-18283 from the National Institutes of Health.

We thank D. Maki and R. C. Moellering, Jr., for *Enterococcus* strains and M. Dahl and H. Bron for plasmids. Teicoplanin was a gift of Marion-Merrill Dow through E. Böhme. LY-191145 was a gift of Eli Lilly and Co. through T. Nicas.

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