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In vivo characterization of the type A and B vancomycin-resistant enterococci (VRE) VanRS two-component systems in *Escherichia coli*: A nonpathogenic model for studying the VRE signal transduction pathways

(crossregulation/cross talk/PhoB/PhoR/sensor histidine kinase)

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ABSTRACT ***Escherichia coli* reporter strains modeling the high-level type A and B vancomycin resistances of *Enterococcus faecium* BM4147 and *Ent. faecalis* have been developed to study the respective VanR-VanS two-component regulatory systems. P_{vanH}-, P_{vanRa}-, P_{vanY}-, and P_{vanRb}-*lacZ* fusions report on expression from the vancomycin-resistant enterococci promoters of the type A *vanRSHAXYZ* and type B *vanRSYWHBX* gene clusters. These strains also express from single-copy chromosomal genes $vanR_a$, $vanR_b$, or $vanRS_b$ behind their respective promoter (P_{vanRa} or P_{vanRb}) or $vanS_a$ or $vanS_b$ behind the rhamnose-inducible P_{rhaB}. Results show that activation (phosphorylation) of the response regulator $VanR_a$ by its sensor kinase $VanS_a$ leads to transcriptional activation of both P_{vanH} and P_{vanRa}. Additionally, $VanR_b$ activates its cognate promoters P_{vanY} and P_{vanRb}, although this occurs only in the absence of $VanS_b$ and presumably is caused by $VanR_b$ phosphorylation by an unidentified endogenous *E. coli* kinase. Thus, $VanS_b$ interferes with activation of $VanR_b$, probably by acting as a phospho-$VanR_b$ phosphatase. Although both $VanR_a$ and $VanR_b$ activate their cognate promoters, neither activates the heterologous P_{vanR}, P_{vanH}, or P_{vanY}, arguing against the interchangeability of type A and B two-component regulatory switches in vancomycin-resistant enterococci. $VanR_a$ also is activated by the nonpartner kinase PhoR. Because this occurs in the absence of its inducing signal (P_i limitation), PhoR autophosphorylation apparently is regulated *in vivo*. Furthermore, the activation of $VanR_a$ caused by cross talk from PhoR in the absence of a signal allows distinction of cross talk from crossregulation as the latter, but not the former, responds to environmental cues.**

Vancomycin-resistant enterococci (VRE) have become clinically problematic human pathogens with high mortality and incidence increasing alarmingly over 35-fold from 0.4% to 14% in hospital intensive-care units from 1989 to 1993 (1). Clinical isolates of VRE have been divided into three types based on the level of resistance (type A and B, high-level resistance; VanC, low- or moderate-level resistance), inducible versus constitutive resistance (type A and B, inducible; VanC, constitutive), and inducibility by both vancomycin and teicoplanin (type A) or inducibility by vancomycin only (type B; refs. 2–4). At the molecular level, types A and B resistance result from the same substitution of an ester linkage (D-Ala-D-lactate) for an amide linkage (D-Ala-D-Ala) at the termini of peptidoglycan chains involved in cell wall synthesis; VanC resistance instead involves a switch to D-Ala-D-Ser termini (5, 6).

Type A and B strains have six genes in common, ones encoding a two-component sensor kinase-response regulator system ($VanS_a$, $VanR_a$ and $VanS_b$, $VanR_b$), three enzymes (VanH, VanA/VanB, and VanX) that bring about the switch to D-Ala-D-lactate peptidoglycan chains with a 1,000-fold lower affinity for vancomycin, and the D,D-carboxypeptidase (VanY). In type B VRE, *vanY* is upstream of *vanH,* whereas in type A the corresponding *vanY* comes after *vanX*. The consequence of this reordering is that while the promoters controlling the two-component regulatory system genes are P_{vanRa} and P_{vanRb}, respectively, the promoters for the structural genes are instead P_{vanH} and P_{vanY} (Fig. 1). Most previous genetic and enzymatic studies trying to define the molecular logic of how these proteins function in VRE have focused on the type A system. Courvalin and colleagues (7) also have demonstrated that mutations of type B $VanS_b$ can result in teicoplanin-inducible resistance.

To characterize the two-component VRE signal transducing proteins, we previously have studied $VanS_a$ and $VanR_a$ *in vitro* and *in vivo* by using *Escherichia coli* (8, 9). We now report studies on the *in vivo* function and selectivity of $VanS_a$ and $VanR_a$ along with similar ones of $VanS_b$ and $VanR_b$ on expression from the type A and B promoters (Fig. 1). We examined $VanR_a$ and $VanR_b$ for transcriptional activation of its cognate and noncognate promoters. We tested the requirements of $VanS_a$ and $VanS_b$ for activation (phosphorylation) of $VanR_a$ and $VanR_b$. Additionally, we tested for cross talk between these heterologous VRE signaling proteins, comparing interactions between them to those with the response regulator PhoB and the sensor kinase PhoR of the *E. coli* Pho regulon (10) as a basis for distinguishing specific and nonspecific (cross talk) interactions. Our results indicate that, unlike the VanH, VanA/VanB and VanX enzymes required for vancomycin resistance, the type A and type B two-component regulatory systems evolved independently.

MATERIALS AND METHODS

Media, Chemicals, and Other Reagents. Luria–Bertani, 3-(*N*-morpholino)propanesulfonic acid (Mops), and tryptone-yeast extract media are described elsewhere (11). Ampicillin was added at 100 μg per ml; kanamycin, at 12.5 or 50 μg per ml, gentamicin, at 5 or 15 μg per ml, and chloramphenicol, at 5 and 20 μg per ml to select antibiotic-resistant integrants or maintain plasmids, respectively. L-rhamnose was used at 1.1 mM for induction. After

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Abbreviations: BG, β-galactosidase; MBP, maltose binding protein; VRE, vancomycin-resistant enterococci.

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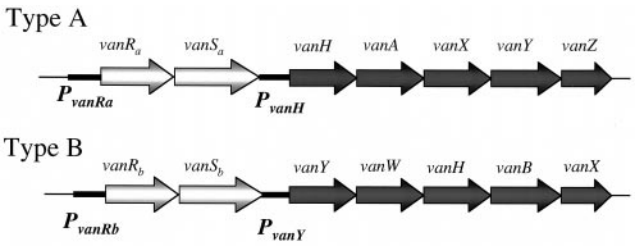

FIG. 1. Organization of type A and type B VRE gene clusters.

integration, the integrants were grown without an antibiotic. *Pfu* DNA polymerase (Stratagene) was used to generate DNAs for cloning. All PCR-amplified fragments were cloned and sequenced as double-stranded plasmid DNAs in a core facility.

Bacteria. All bacteria are derivatives of *E. coli* K-12 BW13711 (11). Conditional replication ($oriR_{R6K\gamma}$) plasmids were propagated at low or high copy number in the pir^+ host BW23473, its isogenic *pir-116* host BW23474, or similar hosts (12, 13). BW24381 (*lacI*^{q} $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ Δ*phoBR580* Δ*creABCD154* *rpoS* (Am) $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$ Δ(*ackA pta*)*160*) is a derivative of BW23660 (9) into which the $\Delta rhaBAD_{LD78}$ (14) and Δ(*ackA pta*)*160* (9) mutations were introduced by P1*kc* transduction essentially as described elsewhere (15). Its derivative BW25124 was made by recombining the P_{vanH}-*lacZ* fusion in pJS110 onto the chromosome via allele replacement (13) and then introducing the Δ*phoR574* mutation (9) by P1*kc* transduction. pJS110 was made by cloning the *Pst*I–*Bsi*WI, P_{vanH}-*lacZ* DNA fragment from pJS79 into the allele-replacement *lacZ* transcriptional fusion vector pWJ18 (W. Jiang, A.H., and B.L.W., unpublished results).

Construction of *lacZ* Transcriptional Fusions. The P_{vanH} and P_{vanRa} promoters were PCR-amplified by using pAT87 (16) as template and the primer JS-P27 (GCAGTCGAC-CGGAAAG-CAATGATAACTATACGACG) with JS-P29 (GCAGGATC-C-TCTGAAGAACGAAAACGGCTCGTTC) and JS-P31 (GCAGTCGAC-ATGTATCTAGGGCTTCATTATACAGG) with JS-P32 (GCAGGATCC-CTTAATAATTTATCAGATT-ATAGGGCCG), respectively (extensions containing a restriction site precede hyphens). The 240-bp P_{vanH} and 255-bp P_{vanRa} PCR products were cloned as *Bam*HI–*Sal*I fragments into pAH-125 to make pJS79 and pJS81, respectively. The P_{vanY} and P_{vanRb} regions were amplified by using genomic DNA of *Ent. faecalis* V583 (from Daniel Sahm, Jewish Hospital, St Louis, MO; ref. 17) as template and the primer JS-P21 (GCAGTCGAC-TCACAG-ATATTCCAGCCGGACAAATTGTCC) with JS-P26 (GCA-GGATCC-TTTGCAATAAAACTACGATTTGTGGC) and JS-P15 (GCAGTCGAC-TTAAACGGTATATTTCGGAAG-AAC) with JS-P19 (GCAGGATCC-ATTTAAGAAGATAA-CATAACAGTCTG), respectively. The 554-bp P_{vanY} and 182-bp P_{vanRb} PCR products were similarly cloned into pAH125 to make pJS52 and pJS51, respectively. To integrate these plasmids at *att*P21, a fragment encoding chloramphenicol resistance of pCAH54 (18) and $attP_{P21}$ of pAH95 (A.H. and B.L.W., unpublished results) was introduced by exchanging the *Nhe*I–*Sph*I fragments with the corresponding one in pIADL46 (from Ivan Lessard, Harvard Medical School, Boston, MA) to make pJS94, pJS96, pJS97, and pJS98, respectively. pAH125 is a derivative of pSK49 (12) for making *lacZ* transcriptional fusions (S.-K. Kim, A.H., and B.L.W., unpublished results).

Plasmids. pAH85, pAH66, and pAH67 (12) are derivatives of pSK49 that synthesize $PhoB^{wt}$, $PhoB_{M17V,\ E87D}$, and $PhoB_{T97A}$, respectively, under P_{phoB} control. pAH151 synthesizes PhoR under P_{rhaB} control (14) in a pSK49 derivative with $attP_{HK022}$ and encoding gentamicin resistance of pAH70 and pAH143 (A.H. and B.L.W., unpublished results). pMP1 is a derivative of pSK49 carrying the same P_{rhaB}-$phoR^+$ fusion. pJS87 synthesizes full-length $VanS_a$ with a C-terminal His_6 tag ($vanS_{H6}$) under P_{rhaB} control. pMP3 is a derivative of pSK49 carrying the same P_{rhaB}-$vanS_{H6}$ fusion. The $vanS_{H6}$ fragment was constructed by PCR using pSLF40 (9) and the primers JS-P3 (GAATTC-TG-TACGGAGTCAAGCCATATGTTG) and JS-P7 (A ATAGT-CGAC-TTATTAgtggtggtggtggtggtgGGACCTCCTTTTATC-AACCAAGTC) according to conditions above (the His_6 coding region is in lowercase). The 1,216-bp $vanS_{H6}$ product was cloned into pUC19 as an *Eco*RI–*Sal*I fragment to make pJS60 for sequencing and then into pAH151 as an *Nde*I–*Sal*I fragment to make pJS87. pJS126 is a derivative of pSLF55 with $attP_{P21}$ and encoding chloramphenicol resistance pSLF55 synthesizes $VanR_a$ from its native promoter (9). pMP2 is a derivative of pSK49 with the P_{rhaB}-$vanS_b$ fusion. pJS107 and pJS109 are similar plasmids that express $vanR_b$ and $vanRS_b$, respectively, behind P_{vanRb}. The $vanR_b$ and $vanS_b$ fragments were generated by using *Ent. faecalis* V583 genomic DNA and the primer JS-P15 (GCAGTCGAC-T-TAAACGGTATATTTCGGAAGAAC) with JS-P16 (CGCT-CTAGA-ATAAGACACAAATTGCTGTGC) and JS-P17 (GCACTGCAG-CATATGACCATGGCGGGTGTAGGTTA-CCGATTGG) with JS-P18 (TTCTCTAGA-TTGTTTCATAT-GCCGTTTGTG), respectively. The 964-bp $vanR_b$ and 1,641-bp $vanS_b$ PCR products were cloned with *Sal*I and *Xba*I or *Pst*I and *Xba*I into pAH143, to make pJS45 and pJS46, respectively. The P_{vanRb}-$vanR_b$ fragment was subcloned as an *Sph*I–*Bam*HI fragment from pJS45 into pAH85 to make pJS107. The *Sac*II–*Xba*I $vanS_b$ fragment was subcloned from pJS46 into pJS45 to make the P_{vanRb}-$vanRS_b$ plasmid pJS47. The P_{vanRb}-$vanRS_b$ fragment then was subcloned as an *Sph*I–*Bam*HI fragment from pJS47 into pAH85 to make pJS109.

***attP* Plasmid Integration and PCR Testing of Copy Number.** Plasmids were integrated into the chromosome of cells expressing the respective integrase and verified by PCR to have a single integrated plasmid as described elsewhere (9, 12, 18).

Cell Growth and Enzyme Assay. Cells were grown and assayed for bacterial alkaline phosphatase and β-galactosidase (BG) as described (11). Units are nanomoles of product made per min per cell culture at OD_{420} at 37°C and 28°C, respectively.

Purification of $VanS_b$ and Protein Phosphorylation Experiments. A maltose binding protein (MBP) fusion to the cytosolic domain of $VanS_b$ ($'VanS_b$, residues V146M to L447) was constructed by using pJS46 as template and the primer MKP5 (GCAGGTGTGGGATTGCTTCATATG-GGGCTGACAA-TTCGG) with MKP6 (GTACCCGGGGATCC-TCTAGATT-GTTTCATATGC). The 1,224-bp $'vanS_b$ fragment was cloned with *Nde*I into a derivative of pMAL-c (New England Biolabs) called pIADL16 (from I. Lessard) to make pJS119. Overproduction, purification, and autophosphorylation of MBP-$'VanS_b$ was accomplished as described for MBP-$'VanS_a$ (19).

RESULTS

An Improved *E. coli* Reporter System for Studying VRE Two-Component Systems. *lacZ* transcriptional fusions to P_{vanH}

Table 1. Activation of P_{vanH} and P_{vanRa} by $VanR_a$

Regulator, kinase	*lacZ* fusion*	β-Galactosidase Sp Act −Rha	β-Galactosidase Sp Act +Rha
None	P_{vanH}	0.7 ± 0.1	N.D.
$VanR_a$	P_{vanH}	0.9 ± 0.3	N.D.
$VanR_a$, P_{rhaB}-$vanS_a$	P_{vanH}	0.8 ± 0.1	31.2 ± 4.1
None	P_{vanRa}	12.8 ± 0.9	N.D.
$VanR_a$	P_{vanRa}	14.7 ± 1.7	N.D.
$VanR_a$, P_{rhaB}-$vanS_a$	P_{vanRa}	10.4 ± 1.7	46.7 ± 4.2

Cells were assayed after 16–20 hr growth in 0.10% glycerol-Mops-2 mM P_i medium without (−Rha) or with (+Rha) rhamnose. Specific activity (Sp Act) units are nanomoles of product formed per cell optical density at 420 nm. Means of three or more determinations with SD are given. N.D., not determined.

*Strains are integrants of BW24381 with single copies of the plasmids in parenthesis: JCS182 (pJS94), JCS184 (pJS96), JCS196 (pJS94, pSLF55), JCS207 (pJS96, pSLF55), JCS230 (pJS94, pSLF55, pJS87), and JCS345 (pJS96, pSLF55, pJS87).

Table 2. Activation of P_{vanRb} and P_{vanY} by $VanR_b$

Regulator, kinase	*lacZ* fusion*	Carbon source	β-Galactosidase Sp Act −Rha	β-Galactosidase Sp Act +Rha
None	P_{vanY}	Fru	7.6 ± 1.2	N.D.
$VanR_b$	P_{vanY}	Fru	42.5 ± 3.8	N.D.
$VanRS_b$	P_{vanY}	Fru	2.6 ± 0.5	N.D.
None	P_{vanRb}	Fru	8.5 ± 0.9	N.D.
$VanR_b$	P_{vanRb}	Fru	40.8 ± 0.5	N.D.
$VanRS_b$	P_{vanRb}	Fru	2.3 ± 0.4	N.D.
$VanR_b$, P_{rhaB}-*vanS*$_b$	P_{vanY}	Gly	59.2 ± 3.0	6.7 ± 0.8
$VanR_b$, P_{rhaB}-*vanS*$_b$	P_{vanY}	Fru	44.7 ± 2.6	12.5 ± 1.3
$VanR_b$, P_{rhaB}-*vanS*$_b$	P_{vanY}	Glu	47.9 ± 0.4	40.5 ± 2.2
$VanR_a$	P_{vanY}	Fru	8.7 ± 1.3	N.D.
$VanR_a$, P_{rhaB}-*vanS*$_a$	P_{vanY}	Fru	7.4 ± 0.8	7.6 ± 1.0
$VanR_a$	P_{vanRb}	Fru	11.0 ± 1.4	N.D.
$VanR_a$, P_{rhaB}-*vanS*$_a$	P_{vanRb}	Fru	8.4 ± 0.9	8.7 ± 0.7
$VanR_b$	P_{vanH}	Fru	0.9 ± 0.5	N.D.
$VanR_b$	P_{vanRa}	Fru	13.6 ± 3.5	N.D.

Cells were assayed after growth in 0.06% glucose (Glu)-, 0.10% glycerol (Gly)-, or 0.06% fructose (Fru)-Mops-2 mM P_i medium as in Table 1. N.D., not determined.

*Strains are integrants of BW24381: JCS186 (pJS97), JCS188 (pJS98), JCS197 (pJS97, pSLF55), JCS198 (pJS98, pSLF55), JCS199 (pJS94, pJS107), JCS200 (pJS96, pJS107), JCS201 (pJS97, pJS107), JCS202 (pJS98, pJS107), JCS237 (pJS97, pJS107, pJS102), JCS240 (pJS97, pJS109), JCS241 (pJS98, pJS109), JCS347 (pJS97, pSLF55, pJS87), and JCS349 (pJS98, pSLF55, pJS87).

and P_{vanRa} were made to improve on the signal-to-noise ratio of previously engineered strains. The basal levels of earlier P_{vanH}- and P_{vanRa}-*lacZ* fusions ranged from 28.5 ± 9.8 to 69.3 ± 6.4 BG units (BGU; ref. 9). These elevated levels interfered with their use in experiments directed toward probing structural determinants for VanRS interactions. Their high background probably resulted from an RNase III processing site upstream of *lacZ* in pWJ19 that stabilizes the mRNA and is derived from pTL61 (20). The new fusions lack this site as the *lacZ* junction in pAH125 is from pRS415 (21). Basal levels of the new type A fusions in the absence of $VanR_a$ were 0.7 ± 0.1 BGU for P_{vanH} and 12.8 ± 0.9 BGU for P_{vanRa} (Table 1). The basal levels of the type B P_{vanY}-*lacZ* and P_{vanRb}-*lacZ* fusions were 7.6 ± 1.2 and 8.5 ± 0.9 BGU, respectively (Table 2).

It is generally more meaningful to study regulatory effects when the relevant genes are in single copy. This is especially true in studies of the VRE and Pho two-component systems. Abnormal regulatory effects have been repeatedly observed when using multicopy *phoB, phoR*, or *vanS* plasmids, which presumably result from effects of plasmid copy number on synthesis of their gene products (15, 22, 23). It therefore was critical to construct reporter strains in which both the promoter fusions and regulatory genes are in single copy. The use of strains with the *vanR* and *vanS* genes stably recombined on the chromosome also afforded tighter control of these regulatory genes and circumvented problems that can arise from inconsistent gene dosages.

All reporter strains express *vanR* alone or together with *vanS* from its native promoter (P_{vanRa} or P_{vanRb}). Many express *vanR* from its promoter and *vanS* or *phoR* under tight control from the rhamnose-inducible promoter P_{rhaB} (14). These genes and fusions were recombined onto the chromosome by use of conditional replication, integration, and modular plasmids that have different phage attachment (*attP*) sites and antibiotic resistances (refs. 9, 12, 14, and 18; *Materials and Methods*; A.H. and B.L.W., unpublished results). The promoter-*lacZ* fusions were recombined onto the chromosome either by use of an analogous *lacZ* vector or one that provides homologous sequences for allele replacement at the *lac* locus.

Activation of P_{vanH} and P_{vanRa} by $VanR_a$ and $VanS_a$. Previous studies had shown that $VanR_a$ and $VanS_a$ both were required for *ca.* 6- to 45-fold activation of a P_{vanH}-*lacZ* fusion in *E. coli* (9). Although no activation of a P_{vanRa}-*lacZ* fusion was apparent in that study, the basal level was high. As shown in Table 1, the new P_{vanH}-*lacZ* fusion was activated *ca.* 40-fold only in the presence of both $VanR_a$ and $VanS_a$ and the new P_{vanRa}-*lacZ* fusion was similarly activated *ca.* 5-fold. Apparently, the high basal activities of earlier fusion strains interfered with detecting activation of P_{vanRa} by $VanR_a$. Activation of P_{vanRa} is consistent with phospho-$VanR_a$ (P-$VanR_a$) binding P_{vanRa} with higher affinity than free $VanR_a$ (19).

Activation of P_{vanY} and P_{vanRb} by $VanR_b$. Both the P_{vanY}- and P_{vanRb}-*lacZ* fusions show *ca.* 5-fold activation by $VanR_b$. Unexpectedly, this activation occurs in the absence of $VanS_b$ (Table 2). Many response regulators are phosphorylated by nonpartner histidine kinases (24). It therefore is likely that $VanR_b$ activation results from its being phosphorylated by an unidentified *E. coli* kinase. Although $VanR_b$ also might have been activated by acetyl phosphate (25), this was ruled out here because our reporter strains are genetically blocked in acetyl phosphate synthesis.

Evidence of an *in Vivo* $VanS_b$ Phosphatase. Many two-component sensor proteins act both as a kinase and as phosphatase toward their partner response regulators. Using analogous reporter strains, we previously showed that $VanS_a$ acts as a P-$VanR_a$ phosphatase by demonstrating that $VanS_a$ blocked activation of $VanR_a$ by PhoR or acetyl phosphate (9). Accordingly, $VanS_b$ was tested for interference with the activation of $VanR_b$. As shown in Table 2, no activation of the P_{vanY}- or P_{vanRb}-*lacZ* fusion was apparent when *vanR*$_b$ and *vanS*$_b$ were expressed together from P_{vanRb}. Similar *ca.* 17-fold decreases were seen for both type B VRE promoters. Curiously, the expression levels of both fusions in presence of P_{vanRb}-*vanRS*$_b$ were reduced *ca.* 3-fold below the basal levels in the absence of $VanR_b$, suggesting that free $VanR_b$ might act as a repressor. Further evidence that $VanS_b$ is responsible for inhibiting activation comes from experiments in which *vanR*$_b$ and *vanS*$_b$ were expressed independently. The P_{rhaB}-*vanS*$_b$ strains also showed reduced activation of P_{vanY} with the levels varying inversely with catabolite repression conditions. Glycerol results in the lowest, fructose results in an intermediate, and glucose results in the highest catabolite repression of P_{rhaB} (14). In agreement, greatest inhibition occurred upon induction in glycerol medium and the lowest, in glucose medium (Table 2). Together, these data suggest that $VanS_a$ and $VanS_b$ are poised at different kinase-phosphatase set points; $VanS_a$ acts as a kinase under conditions where $VanS_b$ is a phosphatase. Finally, $VanS_a$ and PhoR were tested for phosphatase function toward P-$VanR_b$. As expected, no interference by them was seen.

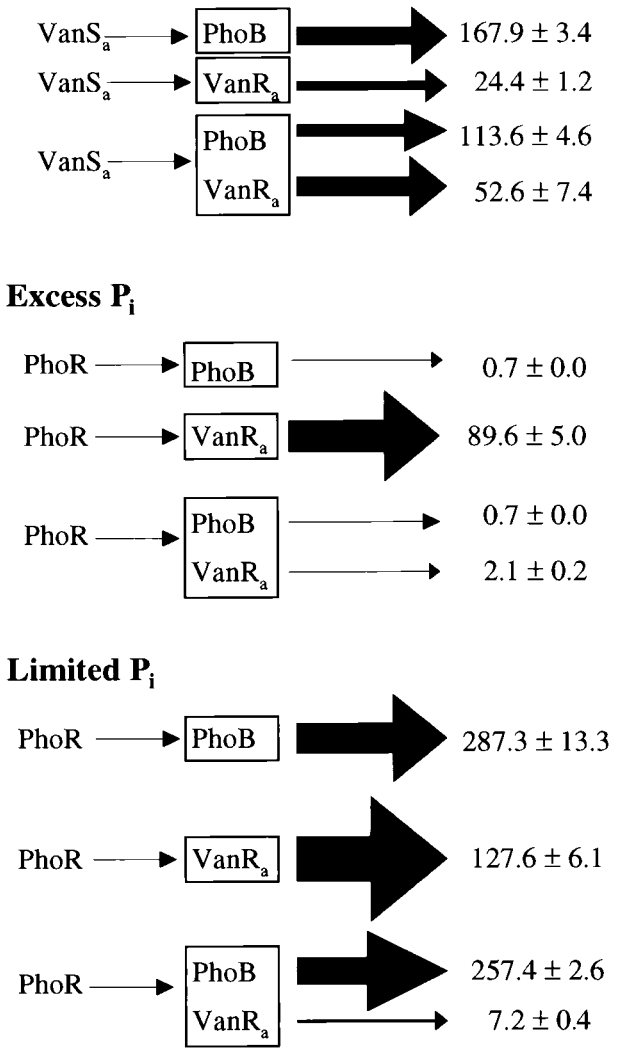

FIG. 2. Competition between VanR and PhoB. Expression of *phoA* (encoding bacterial alkaline phosphatase, Bap) and a P_{vanH}-*lacZ* fusion report on PhoB and VanR$_a$ activation, respectively. VanS$_a$ or PhoR were synthesized under P_{rhaB} control in cells expressing *phoB*, *vanR*$_a$, or both behind its native promoter. Bap and BG were assayed as in Table 1 in medium with rhamnose and 2 mM (Excess) or 0.1 mM (Limited) P$_i$. Arrows on the right are in proportion to Sp Act values (nmol product made per min per OD_{420}). With no rhamnose, Bap and BG Sp Act values of all strains were *ca.* 0.1 ± 0.1 and 1.0 ± 0.2, respectively. Strains are integrants of BW25053 (Δ*phoBR*) or BW25124 (Δ*phoR*): JCS289 (pJS126, pMP1), JCS297 (pMP3), JCS304 (pJS126, pMP3), JCS338 (pMP1), and JCS342 (pMP3).

Promoter Specificity of the VRE Response Regulators. Both type A and type B VRE promoters were tested for activation by their cognate and noncognate response regulators. Conditions leading to activation of P_{vanH} and P_{vanRa} by VanR$_a$ (Table 1) were without effect on expression of P_{vanY} or P_{vanRb} (Table 2). Likewise, conditions leading to activation of P_{vanY} and P_{vanRb} by VanR$_b$ were without effect on P_{vanH} and P_{vanRa} (Table 2).

***In Vivo* Competition Between VanR$_a$ and PhoB.** Previous kinetic studies of MBP-′VanS$_a$ with VanR$_a$ and PhoB provided a quantitative estimate of the overall efficiency and specificity of phosphotransfer from P-VanS$_a$ (8). By acting as a slow heterologous substrate for P-MBP-′VanS$_a$ with a k_{xfer} of 0.2 min^{-1} and K_M of 95 ± 30 μM, PhoB was a competitive inhibitor of P-VanR formation. In those studies, VanR$_a$ had a k_{xfer} of *ca.* 60 min^{-1} and K_M of 3 μM for phosphotransfer from P-MBP-′VanS$_a$. Experiments therefore were done to see whether competition occurs between VanR$_a$ and PhoB *in vivo*. As shown in Fig. 2, VanS$_a$ activated *phoA* and P_{vanH}-*lacZ* expression *ca.* 1,120- and 18-fold, respectively, when only PhoB or VanR$_a$ was present. When both were present, VanS$_a$ activated *phoA* and P_{vanH}-*lacZ* expression *ca.* 760- and 36-fold, respectively. Although a modest reduction of PhoB activation by VanS$_a$ occurred in the presence of VanR$_a$, it is unclear why VanR$_a$ was stimulated, as these experiments were carried out in the absence of deliberate stimulation of VanS$_a$.

Results from similar experiments with PhoR provided convincing evidence of *in vivo* competition. With excess P$_i$, PhoR activated *phoA* and P_{vanH}-*lacZ* expression *ca.* 7- and 90-fold, respectively, when only PhoB or VanR$_a$ was present (Fig. 2). Activation of *phoA* under these conditions was an apparent consequence of PhoR overproduction from P_{rhaB} (data not shown). When both PhoB and VanR$_a$ were present, activation of VanR$_a$ by PhoR was <2-fold, whereas activation of PhoB was unaffected. The *ca.* 40-fold reduced activation of P_{vanH} caused by PhoB indicates that a PhoB-PhoR interaction interferes with PhoR autophosphorylation when P$_i$ is in excess.

Under limited P$_i$ conditions, PhoR activated *phoA* and P_{vanH}-*lacZ* expression *ca.* 2,900- and 90-fold, respectively, when only PhoB or VanR$_a$ was present (Fig. 2). High activation of PhoB under these conditions was anticipated. These results also showed that activation of VanR$_a$ by PhoR is unresponsive to P$_i$ limitation. In agreement, competition between PhoB and VanR$_a$ occurred with limited P$_i$ as well. An *ca.* 2,600-fold activation of *phoA* expression was accompanied with only a *ca.* 7-fold activation of P_{vanH}-*lacZ* expression when both response regulators were present. That is, *ca.* 13-fold reduction of P_{vanH}-*lacZ* expression resulted because of the presence of PhoB.

Absence of Cross Talk from VanS$_b$ *in Vivo*. VanS$_b$ was tested for activation of both VanR$_a$ and PhoB. No activation of VanR$_a$ by VanS$_b$ occurred under conditions leading to activation of VanR$_a$ by VanS$_a$ or PhoR (Table 3). VanS$_b$ also was tested for activation of PhoB^{wt}, PhoB$_{M17V, E87D}$, and PhoB$_{T97A}$. The latter are altered recognition (AR) mutants showing 200- to 400-fold enhanced activation by ′VanS$_a$ (12). Yet, no activation by VanS$_b$ occurred (data not shown).

***In Vitro* Analysis of VanS$_b$.** Unable to demonstrate VanS$_b$ kinase *in vivo*, an MBP-′VanS$_b$ fusion protein was purified by using the same strategies used to study both VanS$_a$ (26) and PhoR (M.K.P., J.C.S., B.L.W., and C.T.W., unpublished results). Consistent with the *in vivo* results, MBP-′VanS$_b$ showed no autophosphorylation in the absence or presence of detergents intended to facilitate proper folding (0.002 to 1.0% SDS, Brij 58, or Nonidet P-40). To investigate whether the truncation or overproduction of MBP-′VanS$_b$ was deleterious to its folding, full-length VanS$_b$ also was overproduced as an N-terminal, His-tagged, or MBP-VanS$_b$ fusion protein. In no case would VanS$_b$ undergo autophosphorylation, even in the membrane fraction. Although it is possible that VanS$_b$ kinase is particularly labile, it is also reasonable to suppose that under these conditions VanS$_b$ is heavily biased as a P-VanR$_b$ phosphatase.

DISCUSSION

Although we previously have reported *in vitro* studies on purified native VanR$_a$ and an MBP-′VanS$_a$ fusion protein to the VanS$_a$ cytoplasmic domain (8, 19, 26) and *in vivo* studies on VanR$_a$ and VanS$_a$ in *E. coli* (9, 15), little information has been available describing *in vitro* and *in vivo* features of the type B regulatory proteins (17). When P_{vanRa}- and P_{vanH}-*lacZ* fusions were tested for activation by VanR$_a$ and VanS$_a$ in previous *E. coli in vivo* studies (9), activation of P$_{vanH}$, but not P_{vanRa}, was observed. Using reporter strains that provide improved signal-to-noise ratios, a 5-fold increase in P_{vanRa} expression was seen when VanS$_a$ synthesis was driven from the tightly regulated P_{rhaB} (14). This P_{vanRa} response is about one-eighth as strong as the 40-fold activation of P_{vanH}. These *E. coli* strains now provide a more faithful readout of the type A VRE promoters.

Initial results on VanR$_b$ in *E. coli* showed *ca.* 5-fold activation of both type B VRE promoters, P_{vanRb} and P_{vanY}, in the absence

Table 3. Activation of $VanR_a$ and $VanR_b$ by $VanS_a$, $VanS_b$, and PhoR

Regulator, kinase	*lacZ* fusion*	Carbon source	β-Galactosidase Sp Act −Rha	β-Galactosidase Sp Act +Rha
None	P_{vanH}	Fru	0.7 ± 0.1	N.D.
$VanR_a$	P_{vanH}	Fru	0.9 ± 0.3	N.D.
$VanR_a$, P_{rhaB}-$vanS_a$	P_{vanH}	Fru	0.7 ± 0.0	15.2 ± 1.9
$VanR_a$, P_{rhaB}-$vanS_b$	P_{vanH}	Fru	0.8 ± 0.1	0.8 ± 0.1
$VanR_a$, P_{rhaB}-$vanS_b$	P_{vanH}	Gly	0.7 ± 0.1	0.7 ± 0.1
$VanR_a$, P_{rhaB}-*phoR*	P_{vanH}	Fru	0.7 ± 0.2	21.2 ± 1.3
$VanR_a$, P_{rhaB}-*phoR*	P_{vanH}	Gly	0.8 ± 0.2	47.6 ± 8.4
None	P_{vanY}	Fru	7.6 ± 1.2	N.D.
$VanR_b$	P_{vanY}	Fru	42.5 ± 3.8	N.D.
$VanR_b$, P_{rhaB}-$vanS_a$	P_{vanY}	Fru	38.7 ± 2.4	43.1 ± 1.8
$VanR_b$, P_{rhaB}-$vanS_a$	P_{vanY}	Gly	22.7 ± 2.7	37.4 ± 3.3
$VanR_b$, P_{rhaB}-*phoR*	P_{vanY}	Fru	32.2 ± 2.3	34.2 ± 1.1
$VanR_b$, P_{rhaB}-*phoR*	P_{vanY}	Gly	26.0 ± 5.2	37.0 ± 2.6

Cells were assayed as in Table 2. N.D., not determined.
*Strains are integrants of BW24381; JCS182 (pJS94), JCS186 (pJS97), JCS196 (pJS94, pSLF55), JCS201 (pJS97, pJS107), JCS229 (pJS94, pSLF55, pAH151), JCS230 (pJS94, pSLF55, pJS87), JCS231 (pJS94, pSLF55, pJS102), JCS238 (pJS97, pJS107, pJS87), and JCS239 (pJS97, pJS107, pAH151).

of $VanS_b$, consistent with the phosphorylation of $VanR_b$ by an unidentified endogenous *E. coli* kinase. This cross talk to $VanR_b$ is in contrast to the behavior of $VanR_a$ in *E. coli* where no significant activation of P_{vanRa} or P_{vanH} has been observed in the absence of $VanS_a$. That $VanR_a$ and $VanR_b$ are recognized by different *E. coli* kinases was unexpected; it is explicable by the fact that $VanR_a$ and $VanR_b$ share only 34% sequence similarity whereas the VanH, VanA/VanB and VanX resistance enzymes share more than 67% sequence similarity (17).

A second distinction between the type A and B VRE systems in *E. coli* was the behavior of the sensor kinases. In these studies, $VanS_b$ exhibited only a phosphatase and no discernable kinase activity toward its cognate response regulator. In contrast, $VanS_a$ exhibited both kinase and phosphatase activity under similar conditions (9). The issue of balance between phosphoaspartyl-response regulator-phosphatase and phosphohistidinyl-autophosphorylation-kinase has been noted elsewhere, but only for $VanS_a$ rather than $VanS_b$ (Fig. 3). While studying control of $VanR_a$ by $VanS_a$ in *Ent. faecium* BM4147, Arthur *et al.* (27) showed that $VanR_a$ was constitutively active in the absence of $VanS_a$, presumably because of cross talk from an unidentified enterococcal kinase. In the presence of $VanS_a$ and absence of vancomycin or teicoplanin, $VanS_a$ acted predominantly as a phosphatase to reduce P-$VanR_a$-dependent transcription. Only in the presence of a glycopeptide inducer did $VanS_a$ shift from a phosphatase to kinase.

In this study, $VanS_b$ apparently was locked as a phosphatase. To what degree this reflects its true phosphatase/kinase balance in VRE remains to be determined. Along with the unrelieved $VanS_b$ phosphatase, there was no indication that $VanS_b$ phosphorylates $VanR_a$ or PhoB. In contrast, $VanS_a$ phosphorylates both. The inability of $VanS_b$ to activate PhoB was especially surprising. Under similar conditions, five other nonpartner kinases activate $PhoB^{wt}$ and $PhoB^{AR}$, making $VanS_b$ the sole exception (A.H., S.-K. Kim, and B.L.W., unpublished results). Because of amplification of PhoB synthesis resulting from its autogenous positive control, PhoB activation should provide a very sensitive test for cross talk (10), providing further evidence that $VanS_b$ is in a kinase inactive state under these conditions. Although the absence of kinase activity for $VanS_b$ is an anomaly, more rigorous experiments are required to prove that $VanS_b$ is not a kinase. Nevertheless, these results support the hypothesis that the $VanS_b$-$VanR_b$ system is regulated largely or exclusively through a phosphatase on/off balance rather than phosphatase/kinase balance. Accordingly, $VanS_b$ might act solely as a negative regulator of the type B system, whereby its phosphatase activity would be inhibited in the presence of vancomycin, thus permitting phosphorylation of $VanR_b$ by a different kinase in VRE and allowing P-$VanR_b$ to act as a positive regulator.

$VanR_a$ and $VanR_b$ are much less similar than the type A and B VanH, VanA/VanB and VanX enzyme pairs. Likewise, VanS similarities are low, with only 23% similarity between them (17). The disparity between the high similarities for the triad of peptidoglycan-modifying enzymes VanHAX and the low similarities for the regulatory proteins suggests that there was a single origin for VanHAX in VRE, e.g., from one of the vancomycin-producer organisms (28), but that distinct signaling systems were recruited independently for inducible resistance. This teleological

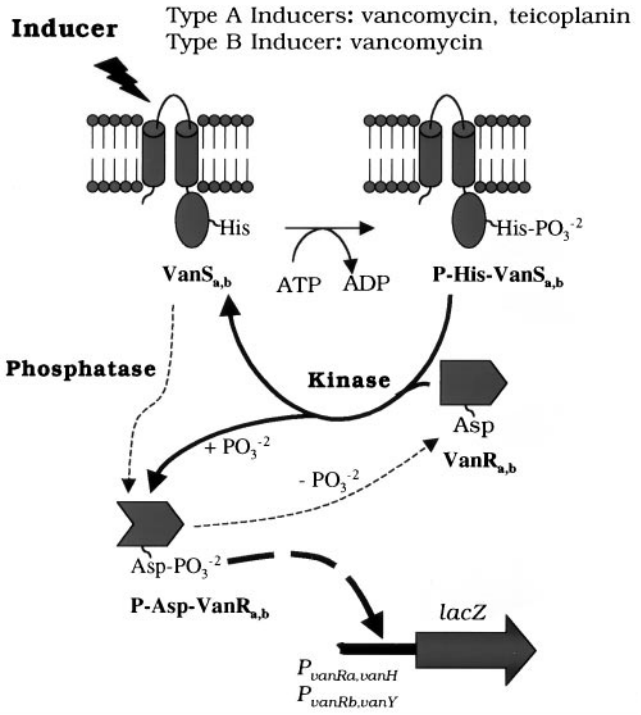

FIG. 3. Signaling in the VanS-VanR phosphorelays and activation of the type A and B VRE promoters. Sensor kinase reaction scheme involves its autophosphorylation and phosphotransfer to the response regulator (thick solid arrows) leading to activation of the respective promoters (thick dashed arrow). Phosphatase reaction of the sensor kinase dephosphorylates the cognate response leading to down-regulation of the promoter in the absence of external stimulus (thin dashed arrows). While $VanS_a$ has both kinase and phosphatase activities, whether $VanS_b$ has a kinase activity has not been established (see text).

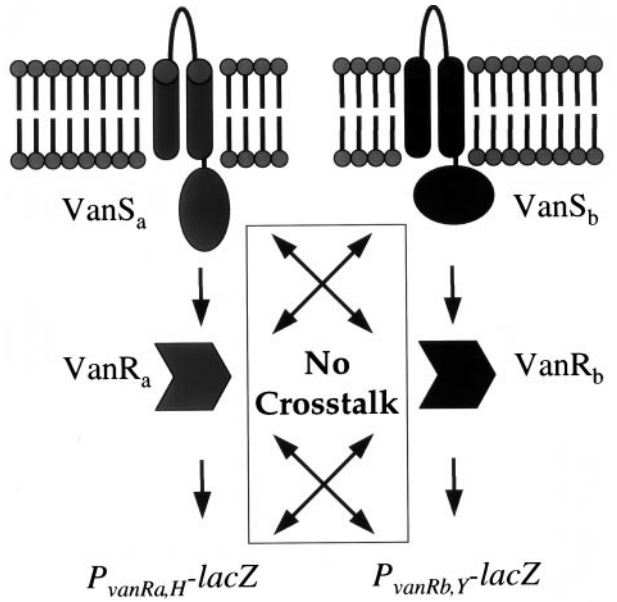

FIG. 4. Type A and B VRE two-component regulatory circuits. A signal transduction event from one sensor (VanS$_a$ or VanS$_b$) to its cognate response regulator (VanR$_a$ or VanR$_b$) leads to activation of the type A (P_{vanH} or P_{vanRa}) and type B (P_{vanY} or P_{vanRb}) promoters without apparent cross talk between the two systems.

explanation for the differences between type A and type B VRE is now supported by several complementary findings. Fig. 3 illustrates different inducers. The failure of type B VRE to acquire teicoplanin resistance results from an inability of teicoplanin to cause induction (7), presumably reflecting different ligands binding to VanS$_a$ and VanS$_b$. Additionally, no apparent cross talk occurs (Fig. 4), so that mismatches of VanS$_a$-VanR$_b$ or VanS$_b$-VanR$_a$ would not produce peptidoglycan alterations. Finally, even though both pairs of VRE promoters function in *E. coli*, there was no cross activation.

It remains uncertain whether VanS$_a$ or PhoR activates VanR$_b$ by cross talk. Because VanR$_b$ is activated by an unknown kinase in *E. coli*, only modest effects because of VanS$_a$ (1.6-fold) or PhoR (1.4-fold) were seen (Table 3). Greater effects might not have been evident if P_{vanY} expression were already near maximally activated under these conditions.

Activation of VanR$_a$ by PhoR and PhoB by VanS$_a$ are clear examples of cross talk (Table 3, Fig. 2). It is noteworthy that high activation of VanR$_a$ by PhoR occurred only in the absence of PhoB. In an earlier study, PhoB was activated by the kinase EnvZ only in the absence of its cognate response regulator OmpR (23). Although activation of PhoB by EnvZ failed to respond to an environmental signal(s), the significance of that finding was unclear as the ligand(s) that stimulates EnvZ is unknown (29). It therefore is intriguing that activation of VanR$_a$ by PhoR was strongly inhibited by PhoB under both excess and limited P$_i$ conditions. These results also show that PhoR autophosphorylation (or P-PhoR phosphotransfer) is somehow down-regulated under P$_i$ excess conditions in a PhoB-dependent manner. Such an effect supports the model that PhoB and PhoR exist in an "inhibition complex" under these conditions (30). The absence of normal (*ca.* 2,000-fold) control by P$_i$ over activation of VanR$_a$ by PhoR suggests that interactions between nonpartner proteins because of cross talk are unregulated. Yet, interactions between some nonpartner proteins are clearly physiologically regulated. Activation of PhoB by the kinase CreC responds to unknown catabolites (31). Likewise, nonpartner interactions between the NarX-NarL and NarQ-NarP two-component proteins are regulated (32). Hence, nonpartner interactions indicative of cross talk would appear to be uncontrolled whereas those indicative of "cross-regulation" would be physiologically regulated (25, 33).

There is substantial interest in developing inhibitors to one or more of the five necessary and sufficient proteins in the type A and type B VRE pathways to obviate vancomycin resistance (34). The sensor kinase-response regulator transcription factors are reasonable sites to target for interdiction of the signal that leads to glycopeptide antibiotic resistance. This work to elucidate the apparent differences between the VanRS$_a$ and VanRS$_b$ systems ultimately may help to define a compound that will inhibit both type A and B VRE. Thus, inhibitors of the *Bacillus subtilis* KinA-SpoOF two-component regulatory system recently have been shown to be antibacterial against *Ent. faecalis* ATCC 29212 and *Ent. faecalis* OC3041. It will be interesting to see whether compounds such as RWJ-49815 inhibit the VanRS$_a$ and VanRS$_b$ pairs equivalently, studies that will be enabled by using *E. coli* reporter systems similar to ones described here in *in vivo* assays.

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