JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5903–5913 Vol. 179, No. 18
0021-9193/97/$04.00+0

Transcriptional Regulation of the *Enterococcus faecium* BM4147 Vancomycin Resistance Gene Cluster by the VanS-VanR Two-Component Regulatory System in *Escherichia coli* K-12

ANDREAS HALDIMANN,[1] STEWART L. FISHER,[2]† LARRY L. DANIELS,[1]‡
CHRISTOPHER T. WALSH,[2] AND BARRY L. WANNER[1]*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 29 April 1997/Accepted 14 July 1997

An *Escherichia coli* K-12 model system was developed for studying the VanS-VanR two-component regulatory system required for high-level inducible vancomycin resistance in *Enterococcus faecium* BM4147. Our model system is based on the use of reporter strains with *lacZ* transcriptional and translational fusions to the P_{vanR} or P_{vanH} promoter of the *vanRSHAX* gene cluster. These strains also express *vanR* and *vanS* behind the native P_{vanR} promoter, the arabinose-inducible P_{araB} promoter, or the rhamnose-inducible P_{rhaB} promoter. Our reporter strains have the respective fusions stably recombined onto the chromosome in single copy, thereby avoiding aberrant regulatory effects that may occur with plasmid-bearing strains. They were constructed by using allele replacement methods or a conditionally replicative *attP* plasmid. Using these reporter strains, we demonstrated that (i) the response regulator VanR activates P_{vanH}, but not P_{vanR}, expression upon activation (phosphorylation) by the partner kinase VanS, the noncognate kinase PhoR, or acetyl phosphate, indicating that phospho-VanR (P-VanR) is a transcriptional activator; (ii) VanS interferes with activation of VanR by PhoR or acetyl phosphate, indicating that VanS also acts as a P-VanR phosphatase; and (iii) the conserved, phosphate-accepting histidine (H164) of VanS is required for activation (phosphorylation) of VanR but not for deactivation (dephosphorylation) of P-VanR. Similar reporter strains may be useful in new studies on these and other interactions of the VanS-VanR system (and other systems), screening for inhibitors of these interactions, and deciphering the molecular logic of the signal(s) responsible for activation of the VanS-VanR system in vivo. Advantages of using an *E. coli* model system for in vivo studies on VanS and VanR are also discussed.

Vancomycin is a glycopeptide antibiotic that is currently used for the treatment of gram-positive bacterial infections, especially ones caused by methicillin-resistant *Staphylococcus aureus* species (49). Vancomycin acts by binding to the terminal D-Ala-D-Ala moieties of bacterial cell wall precursors and effectively inhibits the transpeptidation and transglycosylation steps of the cell wall assembly process, thereby rendering the cell susceptible to osmotic shock. Over the past 10 years, a number of *Enterococcus* strains with high-level inducible resistance to vancomycin have been identified (11), and the relative incidence of these strains has increased sharply in the last 3 years (39). High-level resistance to the antibiotic has been found to require five plasmid-borne genes: *vanR*, *vanS*, *vanH*, *vanA*, and *vanX*.

VanR and VanS comprise a two-component regulatory system (2, 51) that regulates transcription of the genes responsible for conferring resistance: *vanH*, *vanA*, and *vanX* (52). Two-component regulatory systems are signal transduction pathways commonly used by prokaryotes to sense and adapt to stimuli in the environment; as many as 50 different ones may exist in a single bacterium such as *Escherichia coli* (29). Analogous signal transduction pathways have recently been identified in both eucaryotes (38) and archaea (32). These systems are characterized by a sensor histidine kinase (often a transmembrane signaling kinase such as VanS) that undergoes autophosphorylation on a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue on the response regulator protein (in this case, VanR) that usually acts as a transcriptional activator. Like many signaling kinases, VanS has an N-terminal domain with two transmembrane segments flanking an extracellular domain that is believed to act as the signal sensing domain and a C-terminal cytoplasmic transmitter domain with autophosphorylation and phosphotransfer activities. Biochemical studies on the cytoplasmic domain of VanS (M95 to S384) have shown that it is readily autophosphorylated on a histidine residue (H164) in the presence of ATP and that phospho-VanS (P-VanS) is capable of efficient phosphotransfer to an aspartate residue on VanR (D53) (51). Genetic studies have implicated VanR as a transcriptional activator of the vancomycin resistance genes (2). To date, two VanR-responsive promoters have been identified: the promoter P_{vanR}, for expression of *vanRS*, and the promoter P_{vanH}, for expression of *vanHAX*. The in vivo transcriptional start site of P_{vanH} (but not of P_{vanR}) has also been mapped. Studies using purified VanS and VanR have validated these predictions. Gel mobility shift analyses have shown that P-VanR has a greater binding affinity than the nonphosphorylated protein for both promoter regions of the vancomycin resistance operon, with the strongest binding near P_{vanH} (50% effective concentration of 40 nM [15]). DNase I footprinting analyses have suggested that VanR forms an oligomeric struc-

* Corresponding author. Phone: (765) 494-8034. Fax: (765) 494-0876. E-mail: BLW@bilbo.bio.purdue.edu.

† Present address: Department of Metabolic Diseases, Hoffmann-LaRoche, Inc., Nutley, NJ 07110.

‡ Present address: Biotechnology Centre, Faculty of Pure and Applied Sciences, University of the West Indies, Mona, Kingston 7, Jamaica.

ture upon phosphorylation, as the observed footprint was larger than that expected for DNA binding by a monomeric protein (ca. 80 bp for P_{vanH}; ca. 40 bp for P_{vanR}). Furthermore, the footprinting studies indicated that the VanR promoters contain a 12-bp, AT-rich consensus sequence. Finally, the protected regions identified in the footprinting analyses suggested that upon phosphorylation, VanR served dual roles in vivo, as a transcriptional activator at P_{vanH} and as a repressor of transcription at P_{vanR}. A repressor role for P-VanR was based solely on the location of the protected region, as the P_{vanR} start site has not been verified experimentally.

In addition to activation of VanR, the cytoplasmic domain of VanS is also capable of in vivo activation and in vitro phosphorylation of the *E. coli* response regulator PhoB (8). In wild-type cells, PhoB, together with its cognate sensor kinase PhoR, controls the expression of the Pho regulon in response to inorganic phosphate (P_i) limitation (44). This cross-reactivity between VanS and PhoB has provided a convenient in vivo assay for VanS activation and has allowed for genetic analyses on the factors that regulate the VanS-VanR two-component signal transduction system. For example, we have identified peptide fragments of VanS capable of inhibiting activation of PhoB by VanS (8). These fragments may correspond to an interaction domain. We have also exploited this system to identify critical residues of PhoB that are involved in the phosphotransfer pathway and recognition of VanS (12). These studies relied upon PhoB being a poor substrate for phosphotransfer from P-VanS; biochemical studies have shown that the kinetic efficiency (k_{cat}/K_m) is 10,000-fold higher for VanR than that measured for PhoB (9). This large kinetic difference between VanR and PhoB as substrates for P-VanS prompted us to develop an in vivo *E. coli* reporter system to study VanR phosphorylation and transcriptional activation. It was felt that this system would not only give an important reference point for our studies on VanS and PhoB but would also provide a genetic system to analyze VanR structure-function relationships in vivo, as well as an additional, potentially more sensitive in vivo assay of VanS activation.

Here we describe the regulation of the P_{vanR} and P_{vanH} promoters of the *vanRSHAX* gene cluster by VanR and VanS in an *E. coli* model system. By use of the appropriate strains, we examined the in vivo activation of VanR by VanS, mutant forms of VanS, the nonpartner sensor kinase PhoR, and acetyl phosphate. We also tested whether VanS, like many two-component histidine kinases, has a dual role and is required for both the activation of VanR and deactivation of P-VanR. Our results are discussed.

MATERIALS AND METHODS

Media, chemicals, and other reagents. Most materials have been described elsewhere (43). Luria-Bertani, tryptone-yeast extract (TYE), and glucose M63 agar media were routinely used as complex and minimal agar. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg per ml for detection of β-galactosidase. All regulation studies were carried out with broth or agar media buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS) and containing 2 mM P_i unless indicated otherwise. MOPS agar media contained 0.2% D-glucose, D-fructose, D-gluconate, glycerol, or 1% pyruvate as the sole carbon source without or with 1.3 mM L-arabinose or 1.1 mM L-rhamnose. Most antibiotics and chemicals were from Sigma (St. Louis, Mo.) and were used at the following concentrations: ampicillin at 100 μg per ml to maintain plasmids; kanamycin at 10 or 50 μg per ml to select kanamycin-resistant (Kan^r) integrants or maintain plasmids, respectively; and tetracycline at 12.5 μg per ml to select tetracycline-resistant (Tet^r) transductants. Except for plasmid-bearing strains, cells were routinely grown without an antibiotic. *Taq* DNA polymerase (Promega, Madison, Wis.) was used for testing the copy number of an integrated plasmid. Vent DNA polymerase (New England Biolabs, Beverly, Mass.) was used to generate DNAs for cloning. Oligonucleotides were obtained from IDT, Inc. (Coralville, Iowa).

Bacteria. Unless noted otherwise, bacteria are derivatives of *E. coli* K-12 strain BW13711 (43) and were constructed by P1*kc* transduction (43). Strain constructions usually involved two steps. In the first step, a linked auxotrophic (or carbon utilization negative) marker was introduced by selecting Tet^r transducants. These transductants were then made prototrophic (or carbon utilization positive) with P1*kc* grown on an appropriate donor, and the resultant transductants were scored for loss of antibiotic resistance and other relevant phenotypes. All strains assayed are described in Table 1. Strains used as donors included BW7261 (*leu-63*::Tn*10* [8]), BW21391 (*leu-63*::Tn*10* [this study]), BW12070 [Δ(*creABCD thr*)::Tn*5*-132 (8)], BW13635 (*proC*::Tn*5*-132 [8]), BW18101 (*nuoG*::Tn*10 purF* [unpublished data]), BW20891 (Δ*creBCD153* [20]), BW21254 (Δ*creABCD154* [19]), BW22826 (Δ$araBAD_{AH33}$ [13]), and JW383 (*zii-510*::Tn*10 metF159* [obtained from M. Berlyn]); only relevant markers are indicated. The constructions of BW21437 (Δ*ackA158*), BW21530 (Δ*phoR574*), BW21770 (Δ*ackA159*), BW22623 (Δ*pta-163*), BW22624 [Δ(*ackA pta*)*160*], and BW23231 (Δ*phoBR580*) are described below. Plasmids requiring the Π protein (the *pir* gene product) for replication were maintained in BW23473 (*pir*$^+$ *hsdR514 endA recA1* [12]), BW23474 (*pir-116 hsdR514 endA recA1* [12]), or other appropriate hosts (26).

Construction of Δ*ackA*, Δ*pta*, and Δ(*ackA pta*) mutants. Four deletions of the *ackA-pta* region were constructed. The Δ*ackA158* and Δ*ackA159* mutations remove a 0.4-kbp *Kpn*I and a 1.1-kbp *Sca*I-to-*Kpn*I fragment internal to *ackA*, respectively; the Δ(*ackA pta*)*160* mutation removes a 2.5-kbp *Xmn*I fragment internal to the adjacent *ackA* and *pta* genes; the Δ*pta-163* mutation removes a 1.7-kbp *Psh*AI-to-*Bam*HI fragment internal to *pta* (Fig. 1A). An allele replacement plasmid carrying Δ*ackA158* was made in three steps. First, the 5.2-kb *Mlu*I fragment of pMK800 (obtained from A. Nakata [23]) was deleted to make pLD56; second, the *Kpn*I fragment was deleted to make pLD57; then, the 7.4-kbp *Bam*HI fragment containing the Δ*ackA158* mutation was cloned into the vector pLD53 (26). The resultant plasmid pLD62 was used to recombine the Δ*ackA158* mutation onto the chromosome of BW13711. Tet^r transformants were selected following electroporation; after being purified once in the absence of tetracycline, tetracycline-sensitive recombinants were selected as described previously (27). Ones showing an AckA$^-$ phenotype (poor growth with acetate as a carbon source and fluoroacetate resistance) were verified as described elsewhere (26). A plasmid carrying the Δ*ackA159* mutation was made in two additional steps. First, pLD57 was digested with *Sca*I, ligated to a *Kpn*I linker (phospho-GGGTACCC), digested with *Kpn*I, and religated to make pLD58; then the 6.7-kb *Bam*HI fragment containing the Δ*ackA159* mutation was cloned into pLD53; the resultant plasmid pLD63 was used to recombine the Δ*ackA159* mutation onto the chromosome of BW21254 as described above.

To construct the Δ(*ackA pta*)*160* and Δ*pta-163* mutations, the *ackA-pta* chromosomal region was first cloned using the mini-Mu vector pMW11 (50) by complementation of the *phoR creC* $\Delta(ackA\ pta)_{TA3516}$ mutant BW21073 (6). Overproduction of AckA and Pta from a multicopy plasmid presumably leads to increased acetyl phosphate levels, resulting in activation of PhoB in the absence of PhoR and CreC and consequent expression of *phoA*. Transductants expressing *phoA* at a high level were screened for ones that, based on the restriction pattern, carried plasmids for the *ackA-pta* region. The 4.3-kbp *Mlu*I-to-*Sma*I fragment of one such plasmid was subcloned into *Eco*RV- and *Mlu*I-digested pLITMUS38 (New England Biolabs); the resultant plasmid was named pAH24. The *Xmn*I fragment was deleted from pAH24 to create pAH25 with the Δ(*ackA pta*)*160* mutation. pAH24 was also digested with *Bam*HI, filled in with T4 DNA polymerase, digested with *Psh*AI, and religated to create pAH27 with the Δ*pta-163* mutation. The 1.7- and 2.8-kbp *Sal*I-to-*Spe*I fragments of pAH25 and pAH27 were subcloned into pWM45 (26) to make pAH28 and pAH29, respectively. The latter were used to recombine the Δ(*ackA pta*)*160* and Δ*pta-163* mutations onto the chromosome of BW21591 (Δ*phoR574* Δ*creBCD153* [this study]) as described above.

Construction of Δ*phoR* and Δ*phoBR* mutants. Two deletions of the *phoBR* operon (Fig. 1B) were made. The Δ*phoR574* mutation removes 1.3 kbp including the entire *phoR* coding region; the Δ*phoBR580* mutation removes 2.0 kbp of the entire *phoBR* operon. pBC6Δ*Pst*I (46) contains the *phoBR* operon within a 4.7-kbp *Hin*dIII-to-*Eco*RI fragment. pSK2 (9) carries the same region in which *Nde*I and *Bam*HI sites were introduced by site-specific mutagenesis at the *phoB* start codon and 13 bp beyond the *phoB* stop codon, respectively. The *phoB* stop codon precedes the *phoR* start codon by 60 bp. A native *Ase*I site lies at the *phoR* stop codon. The Δ*phoR574* mutation was made by joining the 0.5-kbp *Sma*I-to-*Bam*HI fragment for the 3′ end of *phoB* to the 1.3-kbp *Ase*I-to-*Eco*RI fragment immediately downstream of the *phoR* coding region. To do this, pBC6Δ*Pst*I was digested with *Ase*I, filled in with T4 DNA polymerase, and digested with *Eco*RI. The released 1.3-kbp fragment was ligated to *Sma*I- and *Eco*RI-cut pBlueScriptIISK− (Stratagene, La Jolla, Calif.) to make pAH7. The same region was then subcloned from pAH7 as a *Bam*HI-to-*Sal*I fragment into pWM45 to make pAH12. pSK2 was cut with *Sma*I, ligated to a *Not*I linker (phospho-TTGCGGCCGCAA), and then digested with *Not*I and *Bam*HI, after which the released 0.5-kbp fragment was ligated to similarly cut pAH12 to make pAH14. The latter was used to recombine the Δ*phoR574* mutation onto the chromosome of BW13711; recombinants were verified as described elsewhere (26).

A complete *phoBR* deletion was made by using pAH7 and pSK47 (19). The latter has the 4.7-kbp *Pst*I-to-*Eco*RI *phoBR* operon fragment of pSK2 (Fig. 1B) cloned into the backbone of pSK50Δ*uidA2* (12, 19). The 2.6-kbp fragment of pSK47 containing *phoR* and *brnQ*′ was replaced with the 1.3-kbp fragment of

TABLE 1. Bacterial strains used

Strain[a]	Genotype[b]	Pedigree[c]	Source[d]
BW23441	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*phoR*(M1-D431)$_{AH35}$	BD792 via BW23400	Pro$^+$ PhoB$^-$ with P1 on BW23231
BW23477	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*phoR*(M1-D431)$_{AH35}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23441	Amp with pINT-ts, then Kan^r Amp^s with pSLF55
BW23664	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-′*vanS*(M95-S384[H164Q])$_{SLF46}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1 on BW22963
BW23665	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-NFLAG-′*phoR*(M63-D431)$_{AH41}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1 on BW22964
BW23666	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384)$_{AH44}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1 on BW22967
BW23667	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-′*vanS*(M95-S384)$_{AH49}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1 on BW23025
BW23668	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384[H164Q])$_{AH52}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1 on BW23095
BW23670	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*phoR*(M1-D431)$_{AH35}$ Δ(*rhaBAD*)$_{LD78}$::P_{vanR}-*vanR*$_{SLF60}$	BD792 via BW23650	Met$^+$ Tet^s Rha$^-$ with P1 on BW23589
BW23671	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*phoR*(M1-D431)$_{AH35}$ Δ(*rhaBAD*)$_{LD78}$::P_{vanR}-*vanRS*$_{SLF63}$	BD792 via BW23650	Met$^+$ Tet^s Rha$^-$ with P1 on BW23591
BW23715	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) Δ(*araBAD*)$_{AH33}$ *rpoS*(Am) Δ(*rhaBAD*)$_{LD78}$::P_{rhaB}, P_{vanR}-*vanRS*$_{SLF63}$	BD792 via BW23701	Leu$^+$ Ara$^-$ with P1 on BW22826
BW23734	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) Δ(*araBAD*)$_{AH33}$ *rpoS*(Am) Δ(*rhaBAD*)$_{LD78}$::P_{vanR}-*vanR*$_{SLF60}$	BD792 via BW23700	Leu$^+$ Ara$^-$ with P1 on BW22826
BW23928	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23649	Leu$^+$ Ara$^-$ with P1*kc* on BW23660
BW24019	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(pr)1$_{SLF71}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-′*vanS*(M95-S384)$_{AH49}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23929	Pro$^+$ with P1 on BW23976
BW24025	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(pr)1$_{SLF71}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384)$_{AH44}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23930	Pro$^+$ with P1 on BW23976
BW24065	*lacI*^{q} *rrnB*$_{T14}$ P_{vanR}-*lacZ*(pr)$_{SLF70}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-′*vanS*(M95-S384)$_{AH49}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23929	Pro$^+$ with P1 on BW23926
BW24066	*lacI*^{q} *rrnB*$_{T14}$ P_{vanR}-*lacZ*(pr)$_{SLF70}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384)$_{AH44}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23930	Pro$^+$ with P1 on BW23926
BW24086	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(pr)2$_{SLF74}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384)$_{AH44}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23930	Pro$^+$ with P1 on BW24026
BW24088	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(pr)2$_{SLF74}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(EcoB) Δ(*araBAD*)$_{AH33}$::P_{araB}-′*vanS*(M95-S384)$_{AH49}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$)	BD792 via BW23929	Pro$^+$ with P1 on BW24026
BW24267	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoR574* Δ*creABCD154* *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*(M1-S384)$_{AH44}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$) Δ*ackA159*	BD792 via BW24252	Pur$^+$ Tet^s with P1 on BW21770
BW24270	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoR574* Δ*creABCD154* *rpoS*(Am) Δ(*araBAD*)$_{AH33}$::P_{araB}-*vanS*[M1-S384 [H164Q])$_{AH52}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$) Δ*ackA159*	BD792 via BW24253	Pur$^+$ Tet^s with P1 on BW21770
BW24273	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoR574* Δ*creABCD154* *rpoS*(Am) Δ(*araBAD*)$_{AH33}$ *att*λ::pSLF55(P_{vanR}-*vanR*$^+$) Δ*ackA159*	BD792 via BW24254	Pur$^+$ Tet^s with P1 on BW21770
BW24386	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoR574* Δ*creABCD154* *rpoS*(Am) Δ(*araBAD*)$_{AH33}$ Δ*ackA159* Δ(*rhaBAD*)$_{LD78}$::P_{vanR}-*vanR*$_{SLF60}$	BD792 via BW24366	Met$^+$ Tet^s with P1 on BW23589
BW24387	*lacI*^{q} *rrnB*$_{T14}$ P_{vanH}-*lacZ*(op)$_{SLF48}$ Δ*phoR574* Δ*creABCD154* *rpoS*(Am) Δ(*araBAD*)$_{AH33}$ Δ*ackA159* Δ(*rhaBAD*)$_{LD78}$::P_{vanR}-*vanRS*$_{SLF63}$	BD792 via BW24366	Met$^+$ Tet^s with P1 on BW23591

[a] All bacteria are derivatives of *E. coli* K-12, except that the *phn*(EcoB) locus is from *E. coli* B (45).

[b] All known mutations are given. The prime (′) signifies that the gene is deleted on the side of the prime. The subscript T14 refers to four tandem copies of the *rrnB* terminator. The subscript TA3516 refers to the name of the original strain in which this deletion arose (48). Other subscripts correspond to plasmids carrying the original deletion or fusion that was recombined onto the chromosome by allele replacement. In another study, we recently discovered that the *E. coli* K-12 strain BD792 and its descendants contain an *rpoS*(Am) mutation present also in many other *E. coli* K-12 lines (17).

[c] The parental strain from another laboratory and its most immediate ancestor in this laboratory.

[d] See Materials and Methods.

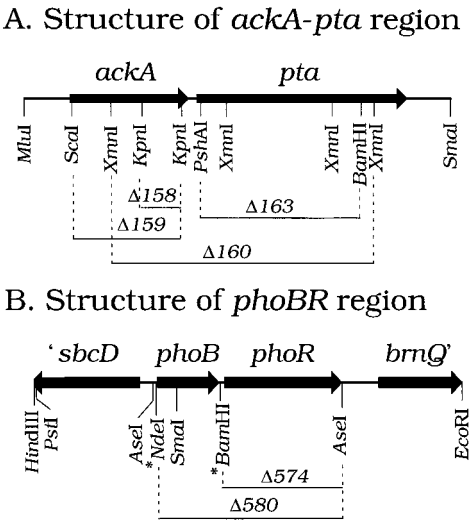

FIG. 1. Construction of *ackA-pta* and *phoBR* deletions. Arrows show gene orientations. (A) Structure of the *ackA-pta* chromosomal region. The segment in pAH24 is shown. (B) Structure of the *phoBR* chromosomal region. The segment in common in pBC6Δ*Pst*I (46), pSK2 (9), and pSK47 (19) is shown. Sites with an asterisk are present only in pSK2 and pSK47; these were introduced by site-directed mutagenesis. The truncated arrowhead indicates that the 3′ end of *sbcD* is absent in these plasmids.

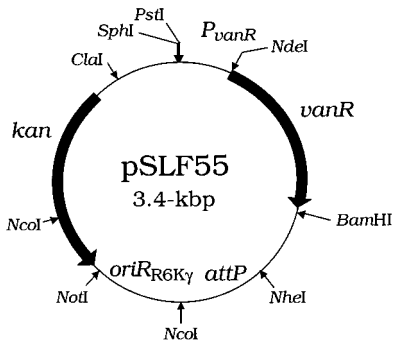

FIG. 2. Structure of the $oriR_{R6K\gamma}$ *attP* P_{vanR}-*vanR* plasmid. Arrows show the orientations of *vanR* and *kan*.

pAH7 containing only *brnQ′*. To do this, pSK47 was digested with *Bam*HI, filled in, and then digested with *Eco*RI; pAH7 was digested with *Sac*I, filled in, and then digested with *Eco*RI. The appropriate fragments were joined to create pLD86. To delete *phoB*, pLD86 was digested with *Nde*I and *Sac*II, filled in, and religated. The resultant plasmid pLD88 was used to recombine the Δ*phoBR580* mutation onto the chromosome of BW22878 (*pstS21* [this study]).

Construction of *lacZ* transcriptional and translational fusions. pSLF48 has a *lacZ* transcriptional, operon [*lacZ*(op)] fusion to P_{vanH}; pSLF70 has a *lacZ* translational, protein [*lacZ*(pr)] fusion to the start codon of *vanR*; pSLF71 and pSLF74 have *lacZ*(pr) fusions to the start codon and codon 100 of *vanH*, respectively. pSLF48 was made by cloning the 747-bp *Eco*RI-to-*Sal*I fragment for the P_{vanH} promoter region of pAT89 (2) into pWJ13 (17). pSLF70, pSLF71, and pSLF74 were constructed by cloning PCR-generated fragments using the respective P_{vanH} and P_{vanR} primers (Table 2). These were cloned as blunt or *Pst*I-to-*Bam*HI fragments into pSPORT1 (Gibco-BRL, Bethesda, Md.) for DNA sequencing to create pSLF69, pSLF68, and pSLF75, respectively, and then subcloned as *Pst*I-to-*Bam*HI fragments into pWJ20 (17). pWJ13 and pWJ20 are derivatives of pLD53 (26) that allow for construction of *lacZ*(op) and *lacZ*(pr) fusions, respectively, that can be recombined onto the *E. coli* chromosome at the *lac* locus by allele replacement, effectively replacing *lacP* with the respective foreign promoter. pWJ13 contains the polylinker region including the RNase III processing site preceding *lacZ* in pTL61L (obtained from T. Linn [24]). pWJ20 has the same polylinker region joined to the *Bam*HI site at codon 8 of *lacZ* in pRS414 (obtained from R. Simons [35]).

Construction of *phoR*, *vanR*, and *vanS* plasmids. pAH35 encodes full-length PhoR (residues M1 to D431); pAH41 encodes an N-terminal truncated PhoR (residues M63 to D431; denoted ′PhoR) with an N-terminal FLAG epitope, denoted NFLAG; pAH44 encodes full-length VanS (residues M1 to S384); pAH49 encodes an N-terminal truncated VanS (residues M95 to S384; denoted ′VanS); pAH52 encodes full-length VanS (residues M1 to S384) in which its autophosphorylation (H164) site has been changed to glutamine; and pSLF46 encodes a similar N-terminally truncated VanS (residues M95 to S384 with the H164Q change). These plasmids are derivatives of pAH33, a vector for constructing fusions to P_{araB} that can be recombined onto the chromosome at the *araCBAD* locus by allele replacement (13). The resulting recombinants express the corresponding *phoR* or *vanS* coding region behind the arabinose-regulated P_{araB} promoter. The required fragments were generated by PCR, cloned into pSPORT1, pSPORT1::*merR*, or pSPORT1::NFLAG-*merR* (obtained from B. Ballard), and then subcloned into pAH33 by using compatible sites. pSPORT::*merR* and pSPORT1::NFLAG-*merR* contain a synthetic *merR* (5) without or with an NFLAG sequence.

pSLF55 (Fig. 2) expresses *vanR* under control of P_{vanR} in a Kan^r *attP* suicide vector with the R6Kγ plasmid replication origin ($oriR_{R6K\gamma}$). pSLF55 was con-

TABLE 2. Oligonucleotides used

Primer[a]	Sequence[b]
P1-*att*λ, P135	GAAGGACGTTGATCGGGCGGGG
P2-*attP*, P143	CAGTGACACAGGAACACTTAACG
P3-*attP*, P144	CACGATAATATCCGGGTAGG
P4-*att*λ, P136	GGCGCAATGCCATCTGGTATCAC
′phoR 3′, P96	CGGGATCCCGACTAGTCGCTGTTTTTGG
′phoR 5′, P95	GGAATTCGTCATATGACCCCGCCACC
phoR 3′, P109	GGAATTCGTCATATGCTGGAACGGC
phoR 5′, P110	CGGGATCCGCTGAGCTCAGTCGCTGTTTTTGGC
P_{vanH1} 3′	CTAGGATCCGTCATATGTAAGACCAACCCTTTCTGTG
P_{vanH1} 5′	GCACTGCAGCTGTCCGGAGGAGGTCCTAAGAGATGTATATAA
P_{vanH2} 3′	TTTGGATCCGTGTCGACAGTGATGCCCATTC
P_{vanH2} 5′	AACTGCAGGTACCGGTCCGGAATTCAA
P_{vanR} 3′	CTAGGATCCGTCATATGTATCACCCTTTCACATAAGTTTGCCC
P_{vanR} 5′	GCACTGCAGCTGTCCGGAATGTATCTAGGGCTTCATTATACAGG
vanR 3′	CAGTTGCGCCGATACAAAAAATTCTGCTGAGCTCGGATCCAGATC
vanR 5′	ACTGAAGCTTCATATGAGCGATAAAATACTTATTGTG
vanS1 3′	GAGGGATCCGAGCTCAGGACCTCCTTTTATCAAC
vanS1 5′	GGTCTAGACATATGTTGGTTATAAAATTGAAAAATAAAA
vanS2 3′	CCCTCGAGGGATCCTTAGGACCTCCTTTTATCAAC
vanS2 5′	TTGGTACCATATGCTTTCAAAATTCGCAAAATAC

[a] The locations of P1-*att*λ, P2-*att*P, P3-*att*P, and P4-*att*λ are shown in Fig. 3.
[b] Underlined bases correspond to noncomplementary ones.

structed in two steps. First, the *Nde*I-to-*Bam*HI fragment encoding PhoB in pSK49 (12) was replaced with a similar fragment encoding VanR. The resultant plasmid was named pSLF49. Then the upstream region was replaced with the *Pst*I-to-*Nde*I fragment of pSLF70 containing P_{vanR}. pSLF60 and pSLF63 are derivatives of pLD78, a vector for cloning fragments adjacent to P_{rhaB} that can be recombined onto the chromosome at the *rhaRSBAD* locus by allele replacement (6). These plasmids contain also P_{vanR}. Hence, pSLF60 and pSLF63 express *vanR* and *vanRS*, respectively, under control of P_{vanR}. In pSLF63, but not in pSLF60, the respective insert is in line behind P_{rhaB}, thus allowing also for rhamnose-activated transcription of *vanRS* expression.

Molecular biology. The fragments encoding full-length and truncated PhoR were generated by using primers P109 and P110 and primers P95 and P96 (Table 2), respectively. The fragments encoding full-length VanS and $VanS_{H164Q}$ were generated by using the *vanS1* primers; the fragments encoding truncated ′VanS and ′$VanS_{H164Q}$ were generated by using the *vanS2* primers. The fragment encoding VanR was generated by using the *vanR* primers. The 261-bp P_{vanR} fragment in pSLF70 was generated by using the 5′ and 3′ P_{vanR} primers (Table 2); the 222-bp P_{vanH} fragment in pSLF71 was generated by using the P_{vanH1} primers; the 747-bp P_{vanH} fragment in pSLF74 was generated by using the P_{vanH2} primers. The *phoR* fragments were generated by using pBC6Δ*Pst*I (46) as the template. All *van* sequences were amplified by using pAT89 or a subclone carrying wild-type or appropriate mutant DNA as the template. Particular 5′ extensions were in some cases complementary to adjacent vector sequences. All PCR-amplified DNAs were sequenced on both strands, using dye terminators and an Applied Biosystems model 373A automated sequencer at the Dana Farber Cancer Institute Molecular Biology Core Facility (Harvard Medical School, Boston, Mass.).

***attP* plasmid integration.** Integration of $oriR_{R6K\gamma}$ *attP* plasmids was done by using pINT-ts essentially as described elsewhere (12). pINT-ts is an ampicillin-resistant, low-copy-number plasmid with a temperature-sensitive replicon which synthesizes λ integrase (Int) under the control of λ*cI*857 (14). It is therefore temperature sensitive for plasmid replication; it also expresses *int* upon shift to elevated temperatures (above 37°C). An ampicillin-resistant transformant carrying pINT-ts was grown in S.O.B. medium (33a) with ampicillin (100 μg per ml) at 30°C, transformed with pSLF55 by electroporation as described previously (26), incubated at 37°C for 1 h and 43°C for 30 min, spread onto TYE agar containing kanamycin (10 μg per ml), and incubated at 37°C. Several Kan^r colonies were purified on TYE agar (without kanamycin), screened for ampicillin-sensitive ones (to verify loss of pINT-ts), and then tested for copy number. Once constructed, integrants were routinely grown in the absence of antibiotic selection.

PCR testing of copy number. Two primers specific for the vector *attP* region (P2 and P3 [Table 2]) and two specific for the chromosomal *attB* region (P1 and P4 [Table 2]) were used to test integrants for ones with a single copy of the integrated plasmid at *att*λ. These primers were designed so that a nonintegrant was expected to yield a 231-bp PCR fragment with P1 and P4 (*attB* pair), a single-copy integrant was expected to yield a 327-bp PCR fragment with P1 and P2 (*attL* pair) and a 592-bp PCR fragment with P3 and P4 (*attR* pair), and a double (or higher-multiple) integrant was expected to yield the 327- and 592-bp PCR fragments of a single integrant and in addition a 698-bp PCR fragment with P2 and P3 (*attP* pair). Fragments of these sizes were obtained when nonintegrant, single-integrant, and double (or multiple)-integrant control strains were tested (data not shown).

PCR was performed directly, using bacterial colonies essentially as described elsewhere (3, 20). Cells were transferred with a glass capillary tube into 20 μl of lysis solution (1× PCR buffer [Promega] containing 2.5 mM $MgCl_2$, 0.1 mg of nuclease-free bovine serum albumin per ml, 0.05 mg of proteinase K per ml, 20 mM dithiothreitol, and 1.8 μM sodium dodecyl sulfate). These mixtures were incubated at 37°C for 30 min and then heated to 95°C for 5 min. Then 5 μl of lysed cells, 50 pmol of each primer P1 to P4, and 2 U of *Taq* DNA polymerase (Promega) were combined to form a 100-μl mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, and 200 μM each deoxynucleoside triphosphate. PCR was carried out for 25 cycles (denaturing for 1 min at 94°C, annealing for 1 min at 63°C, and extending for 1 min at 72°C).

Cell growth and enzyme assay. Cells were pregrown on minimal MOPS agar with the same carbon source but without an inducer, and isolated colonies were inoculated into 0.06% glucose–MOPS, 0.06% D-gluconate–MOPS, 0.06% fructose–MOPS, 0.1% glycerol–MOPS, or 0.1% potassium pyruvate–MOPS medium without or with 1.3 mM L-arabinose or 1.1 mM L-rhamnose and grown for 16 to 24 h. Such carbon-limited cultures yield highly reproducible values that are qualitatively similar to ones obtained for logarithmic-growth-phase cultures. β-Galactosidase and bacterial alkaline phosphatase (Bap) activities were measured as described elsewhere (43). β-Galactosidase units are nanomoles of *o*-nitrophenol made per minute at 28°C; Bap units are nanomoles of *p*-nitrophenol made per minute at 37°C.

RESULTS

An *E. coli* reporter system for studying the *Enterococcus faecium* VanS-VanR two-component regulatory system. Several transcriptional and translational fusions of the *Enterococcus faecium* BM4147 P_{vanH} promoter and the *vanH* and *vanR* translational start sequences to *E. coli lacZ* were constructed to determine whether VanR of *Enterococcus* is capable of regulating transcription in *E. coli*. The *lacZ* fusions were constructed in vitro and then recombined onto the chromosome of an appropriate host as described in Materials and Methods. The resultant recombinants have in single copy at the *lac* locus the entire *lacI* gene followed by multiple tandem copies of the *rrnB* terminator and the foreign promoter (P_{vanH} or P_{vanR}) driving *lacZYA* expression (17).

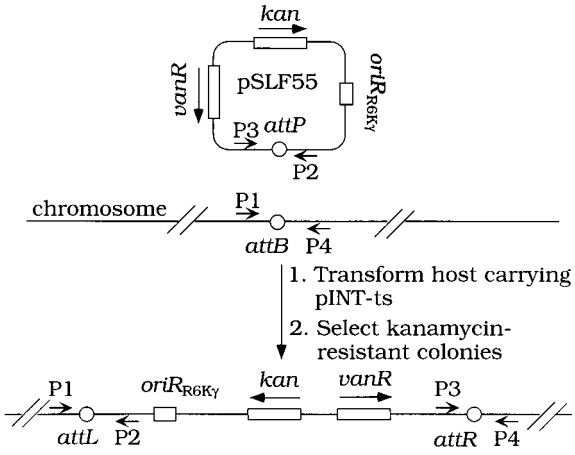

FIG. 3. Chromosomal integration of the conditionally replicative *attP* plasmid carrying P_{vanR}-*vanR*. Arrows show orientations of *kan* and *vanR*. Arrows labeled P1, P2, P3, and P4 show locations of the priming sites on the plasmid and chromosome. See text for details. The diagram is not to scale.

Most experiments used strains carrying *vanR* in single copy and were constructed with pSLF55, which expresses *vanR* from its native promoter (Fig. 2). pSLF55 is a derivative of the $oriR_{R6K\gamma}$ *attP* plasmid pSK49 (12). An $oriR_{R6K\gamma}$ *attP* plasmid requires the *trans*-acting Π protein (the *pir* gene product) for DNA replication; it integrates efficiently upon transformation of a non-*pir* host when Int is supplied in *trans* (Fig. 3). Indeed, the majority of recombinants contain the plasmid at *attB* in single copy, which was verified by PCR testing as described in Materials and Methods. Strains with *vanR* expressed from its native promoter in single copy were used because preliminary experiments showed that VanR activates transcription in the absence of its cognate kinase when synthesized from a multicopy plasmid or from the strong P_{tac} promoter even when in single copy (data not shown). Presumably, VanR when overproduced is activated (phosphorylated) nonspecifically by a noncognate kinase(s). Similar effects resulting from overproduction have also been observed for activation of PhoB in the absence of the kinases PhoR and CreC and acetyl phosphate synthesis (19).

The arabinose-regulated P_{araB} promoter was used for conditional synthesis of the kinases VanS and PhoR. Plasmids carrying various P_{araB}-*vanS* or P_{araB}-*phoR* fusions were constructed as described in Materials and Methods and then used to recombine each fusion onto the chromosome by allele replacement. The resultant recombinants contain *vanS* or *phoR* expressed from P_{araB} in place of *araBAD* at the *araCBAD* locus (13). Strains carrying these fusions in single copy were made in order to minimize aberrant regulatory effects due to overproduction of these kinases. Preliminary experiments also indicated that, as expected, strains with a single-copy P_{araB} fusion show tighter control by arabinose than ones with a similar fusion on a multicopy P_{araB} plasmid (8, 10).

TABLE 3. Activation of P_{vanH}-*lacZ* and P_{vanR}-*lacZ* transcription by VanR in *E. coli*

Strain[a]	*lacZ* fusion[b]	Regulator[c]	Kinase gene	β-Galactosidase sp act[d] (U/OD_{420})			
				With fructose as carbon source		With glycerol as carbon source	
				−Ara	+Ara	−Ara	+Ara
BW23928	P_{vanH}-*lacZ*(op)	VanR	None	28.5 ± 9.8	44.1 ± 8.9	69.3 ± 6.4	77.0
BW23441	P_{vanH}-*lacZ*(op)	None	P_{araB}-*phoR*$^+$	ND	63.0 ± 5.8	ND	66.1 ± 2.6
BW23477	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-*phoR*$^+$	38.0 ± 4.4	429 ± 57	65.7 ± 4.7	1113 ± 240
BW23666	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-*vanS*$^+$	51.1 ± 3.3	318 ± 72	102 ± 1	422 ± 87
BW23668	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-*vanS*$_{H164Q}$	50.7 ± 7.0	44.8 ± 17.5	55.5	90.3 ± 15.4
BW23665	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-′*phoR*	39.7 ± 5.9	65.1 ± 8.8	74.3 ± 12.4	125 ± 17
BW23667	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-′*vanS*	46.8 ± 3.5	44.7 ± 8.8	71.5 ± 10.2	60.6 ± 13.5
BW23664	P_{vanH}-*lacZ*(op)	VanR	P_{araB}-′*vanS*$_{H164Q}$	41.1 ± 3.9	44.5 ± 14.3	67.4 ± 10.5	76.0 ± 8.2
BW24025	P_{vanH}-*lacZ*(pr)1	VanR	P_{araB}-*vanS*$^+$	0.2 ± 0.0	8.9 ± 2.5	0.4 ± 0.1	9.7 ± 3.4
BW24019	P_{vanH}-*lacZ*(pr)1	VanR	P_{araB}-′*vanS*	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2
BW24086	P_{vanH}-*lacZ*(pr)2	VanR	P_{araB}-*vanS*$^+$	0.6 ± 0.2	30.6 ± 6.7	1.9 ± 0.1	36.9 ± 4.9
BW24088	P_{vanH}-*lacZ*(pr)2	VanR	P_{araB}-′*vanS*	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	1.0 ± 0.6
BW24066	P_{vanR}-*lacZ*(pr)	VanR	P_{araB}-*vanS*$^+$	10.1 ± 0.8	19.2 ± 3.1	9.4 ± 0.9	13.8
BW24065	P_{vanR}-*lacZ*(pr)	VanR	P_{araB}-′*vanS*	6.4 ± 3.1	5.2 ± 0.3	9.4 ± 1.7	14.1 ± 3.6

[a] Complete genotypes are given in Table 1.
[b] The P_{vanH}-*lacZ*(pr)1 and P_{vanH}-*lacZ*(pr)2 fusions have different amounts of upstream DNA (see Materials and Methods).
[c] VanR was synthesized from P_{vanR}-*vanR*$^+$.
[d] Cells were assayed following 16 h of growth in 0.06% fructose–or 0.1% glycerol–MOPS–2 mM P_i medium without (−Ara) or with (+Ara) arabinose. Specific activity is in units per cell OD_{420}. Mean values with standard deviations are given for three or more determinations. ND, not determined.

Individual strains have also various *phoBR*, *creBC*, *ackA*, and *pta* deletions. Most of these deletions are well defined, as they were made in vitro and recombined onto the chromosome by allele replacement as described in Materials and Methods. Strains with *phoBR* and *creBC* deletions were used to prevent interference by these chromosomally encoded response regulators and sensor kinases. Strains with Δ*ackA*, Δ*pta*, and Δ(*ackA pta*) mutations were used to test for effects of acetyl phosphate on activation of VanR or to prevent gratuitous activation of VanR by acetyl phosphate, as many response regulators including VanR are known to be activated (phosphorylated) by acetyl phosphate in vivo and in vitro (reviewed in reference 47). Phosphotransacetylase (the *pta* gene product) and acetate kinase (the *ackA* gene product) are responsible for acetyl phosphate synthesis. An *ackA* mutation results in accumulation of acetyl phosphate during growth on carbon sources metabolized via glycolysis; a *pta* mutation blocks acetyl phosphate synthesis during growth on glycolytic substrates; and a Δ(*ackA pta*) mutation blocks synthesis under all conditions (48).

Transcriptional activation by VanR in *E. coli*. All of the P_{vanH}- and P_{vanR}-*lacZ* fusions are expressed in *E. coli*, although there are substantial differences in their levels of expression (Table 3). All three P_{vanH}-*lacZ* fusions, but not the single P_{vanR}-*lacZ* fusion, also show higher expression when both VanR and VanS (or PhoR) are present. In these experiments, *vanR* is expressed from its own promoter, while *vanS* and *phoR* are expressed from the arabinose-inducible P_{araB} promoter. Since expression of P_{araB} is also governed by catabolite repression, we examined effects during growth on fructose and glycerol media. In general, similar effects were observed with both carbon sources. Hence, in the presence of arabinose, the amount of VanS or PhoR is not limiting due to catabolite repression. When differences are seen, higher activation results during growth on glycerol than on fructose, as expected. Under all conditions, the P_{vanH}-*lacZ* transcriptional fusion shows much higher expression than either of the P_{vanH}-*lacZ* translational fusions. The reason for this is unclear. The P_{vanH} transcriptional, but not the translational, fusion shows also substantial expression even in the absence of VanR. Nevertheless, activation of all three P_{vanH}-*lacZ* fusions by VanR always requires a kinase, as shown by the arabinose-dependent synthesis.

During growth on fructose, the P_{vanH}-*lacZ* transcriptional fusion strains synthesize, on average, ca. 42 U of β-galactosidase per cell OD_{420} (optical density at 420 nm) in the absence of a kinase (no arabinose [Table 3]). A similar level is also seen in the absence of VanR (data not shown). This fusion shows 8- to 10-fold-higher expression in the presence of VanS (BW23666) or PhoR (BW23477). Qualitatively similar results are seen during growth on glycerol media. Activation of VanR by VanS probably results from phosphorylation of VanR, because no activation results from the P_{araB}-*vanS*$_{H164Q}$ fusion (BW23668).

Although the synthesis levels are considerably lower, both P_{vanH}-*lacZ* translational fusions are also activated by VanR and VanS (compare values without and with arabinose for BW24025 and BW24086 [Table 3]). The expression of both P_{vanH}-*lacZ* translational fusions also requires VanR (data not shown). In these experiments, no activation of VanR results upon synthesis of the C-terminal kinase domains (′PhoR, ′VanS, and ′VanS$_{H164Q}$), regardless of the P_{vanH}-*lacZ* fusion tested, suggesting that these kinases are less active. The results also show that both VanS and PhoR activate VanR in the absence of a (known) signal. Apparently, activation of VanR by VanS is a consequence of its overproduction from P_{araB}. Activation of VanR by PhoR no doubt results from cross-reactivity and is therefore unlikely to respond to a specific signal.

The lower expression levels of the P_{vanH}-*lacZ* translational fusions may be due to inefficient translation initiation, an altered *lacZ* N terminus in these fusions, mRNA instability, or another difference. The predicted N terminus of these P_{vanH}-*lacZ* translational fusions is MTDPV, where V corresponds to residue 10 of β-galactosidase. The P_{vanH}-*lacZ* transcriptional and P_{vanH}-*lacZ*(pr)2 translational fusions have the same *vanS* upstream sequence; the P_{vanH}-*lacZ*(pr)1 translational fusion has a shorter upstream region (see Materials and Methods). The cause of the different expression levels is unknown.

We also tested for regulation of a P_{vanR}-*lacZ* translational

TABLE 4. Effect of VanS on activation of VanR by PhoR

Strain[a]	*van* gene	Heterologous kinase	P_i concn (mM) in medium	β-Galactosidase sp act (U/OD_{420})[b]	
				−Ara	+Ara
BW23734	P_{vanR}-*vanR*$^+$	None	2	50.8 ± 2.4	58.1 ± 2.6
BW23670	P_{vanR}-*vanR*$^+$	P_{araB}-*phoR*$^+$	2	56.1 ± 2.5	757 ± 29
BW23670	P_{vanR}-*vanR*$^+$	P_{araB}-*phoR*$^+$	0.1	51.8 ± 1.2	636 ± 168
BW23715	P_{vanR}-*vanRS*$^+$	None	2	56.0 ± 3.8	62.5 ± 4.6
BW23671	P_{vanR}-*vanRS*$^+$	P_{araB}-*phoR*$^+$	2	51.3 ± 4.2	62.4 ± 3.2

[a] Complete genotypes are given in Table 1.
[b] Cells were assayed after 16 h growth in 0.06% fructose–MOPS–2 mM P_i or 0.4% fructose–MOPS–0.1 mM P_i medium without (−Ara) or with (+Ara) arabinose. Values are expressed as in Table 3, footnote *d*.

fusion. In contrast to the P_{vanH}-*lacZ* fusions, no effect on expression of the P_{vanR}-*lacZ* translational fusion is apparent due to the presence of VanR alone or VanR and VanS together (compare values for BW24066 and BW24065 in Table 3). In both the presence and absence of VanR, P_{vanR} is expressed at a low level (data not shown). The small effects seen in Table 3 may be attributed to experimental fluctuation in these physiology experiments.

Inhibitory effect of VanS on activation of VanR by PhoR. VanS may also act as a P-VanR phosphatase, by analogy to PhoR and a number of other sensor kinases. Both in vitro and in vivo studies also suggest that VanS has a dual role as a kinase (and phosphotransferase) and phosphatase (1, 51). Therefore, we used our *E. coli* VanS-VanR reporter system to test whether VanS is a P-VanR phosphatase. If VanS is a P-VanR phosphatase, then VanS should interfere with activation of VanR by PhoR. However, since activation of VanR occurs in the absence of a signal when VanS is overproduced, it was necessary to avoid overproduction of VanS in order to test its phosphatase function. We therefore constructed reporter strains with the *vanR* or *vanRS* operon on the chromosome at the *rhaRSBAD* locus in place of *rhaBAD* (6). The strain with *vanRS* also has the rhamnose-regulated P_{rhaB} promoter upstream of P_{vanR} (see Materials and Methods).

In the *vanR* strains BW23734 and BW23670, the P_{vanH}-*lacZ* transcriptional fusion produces ca. 50 U of β-galactosidase per cell OD_{420} (Table 4). The otherwise isogenic *vanRS* strains BW23715 and BW23671 produce similar amounts of β-galactosidase. This amount increases about 14-fold upon induction of PhoR synthesis in the *vanR* strain BW23670. No further increase is evident when the same strain is subjected to P_i limitation, the normal signal for activation of PhoR (44). In contrast, no significant activation occurs upon induction of PhoR synthesis when VanS is present, as in the *vanRS* strain BW23671. Therefore, VanS interferes with activation of VanR by PhoR, probably because VanS acts as a P-VanR phosphatase.

An additional experiment was done to rule out a trivial explanation for the foregoing results. Since no activation of the P_{vanH}-*lacZ* fusion was ever seen in the *vanRS* strain BW23715, we considered that a mutation may have fortuitously arisen during its construction. To rule this out, we tested for the effect of rhamnose on activation because the *vanRS* operon lies immediately downstream of the P_{rhaB} promoter in BW23715. Consequently, rhamnose should induce VanR and VanS synthesis. As shown in Table 5, growth on rhamnose results in more than fourfold activation of the P_{vanH}-*lacZ* fusion in this strain. Therefore, the *vanRS* operon appears to be normal in BW23715, and VanS interferes with activation of VanR by PhoR in the absence of a signal.

Activation of VanR by acetyl phosphate. Reporter strains were also used to test for activation of VanR by acetyl phosphate and to determine whether VanS acts as a P-VanR phosphatase under these conditions. BW24386 and BW24387 accumulate acetyl phosphate due to an *ackA* mutation (48). The former strain has *vanR* alone; it produces β-galactosidase from the P_{vanH}-*lacZ* fusion at a high level (ca. 300 U per cell OD_{420} [Table 6]), thus showing that acetyl phosphate activates VanR in vivo. The latter has *vanRS*; it produces β-galactosidase at a lower level (ca. 50 U per cell OD_{420}) that is also characteristic for expression of this fusion in the absence of VanR. These data show that VanS also interferes with activation of VanR by acetyl phosphate, providing further evidence that VanS acts as a P-VanR phosphatase. These strains are also *phoB*$^+$ Δ*phoR* Δ*creC*. As expected, they synthesize Bap at a high level (ca. 400 U per cell OD_{420}). Similar strains produce <1 U of Bap per cell OD_{420} in the absence of PhoR, CreC, and acetyl phosphate synthesis (20, 48). We also showed that activation of VanR in these experiments is a consequence of acetyl phosphate synthesis by comparing the effects of Δ*pta*, Δ*ackA*, and Δ(*ackA pta*) mutations during growth on various carbon sources (data not shown).

In preliminary experiments, we tested for activation of VanR by acetyl phosphate in Pta$^+$ AckA$^+$ strains during growth on pyruvate and gluconate media. Growth on these carbon sources is expected to result in elevated acetyl phosphate levels (25, 48). Yet no activation of VanR was apparent under these conditions, even though the same cultures showed elevated Bap synthesis (data not shown). Apparently, activation of VanR requires greater amounts of acetyl phosphate than activation of PhoB. No experiment was done to determine whether activation of VanR by acetyl phosphate is a direct autophosphorylation reaction or an indirect one, as can occur for PhoB (20, 42).

TABLE 5. Activation of P_{vanH}-*lacZ* transcription upon induction of *vanRS* expression in *E. coli*

Strain[a]	Medium	β-Galactosidase sp act (U/OD_{420})[b]	
		−Rha	+Rha
BW23715	Fructose	58.2 ± 4.2	188 ± 6
BW23715	Glycerol	46.6 ± 0.3	197 ± 4

[a] BW23715 has the *vanRS* operon behind the rhamnose-regulated P_{rhaB} promoter. See Table 1 for complete genotype.
[b] Cells were assayed after 16 h of growth in 0.06% fructose (or 0.1% glycerol)MOPS–2 mM P_i medium without (−Rha) or with (+Rha) rhamnose. Values are expressed as in Table 3, footnote *d*.

TABLE 6. Effect of VanS on activation of VanR by acetyl phosphate

Strain[a]	*van* gene(s)	Sp act (U/OD_{420})[b]	
		β-Galactosidase	Bap
BW24386	P_{vanR}-*vanR*$^+$	313 ± 48	385 ± 20
BW24387	P_{vanR}-*vanRS*$^+$	49.4 ± 2.0	389 ± 6

[a] Complete genotypes are given in Table 1.
[b] Cells were assayed after 16 h growth in 0.06% glucose–MOPS–2 mM P_i medium. Values are expressed as in Table 3, footnote *d*.

Role of VanS conserved histidine on activation and deactivation of VanR. Residue H164 of VanS is its autophosphorylation site (51). In agreement, H164 is required for the in vivo activation of VanR by VanS (Table 3). We also examined whether H164 is required for deactivation (dephosphorylation) of P-VanR. To do this, we compared effects of VanS and $VanS_{H164Q}$ on activation of VanR by acetyl phosphate in *ackA* mutants. The reporter strains BW24267, BW24270, and BW24273 contain P_{araB}-*vanS*$^+$, P_{araB}-*vanS*$_{H164Q}$, and no fusion, respectively, at the *araCBAD* locus; they also carry the P_{vanH}-*lacZ* fusion and are *phoB*$^+$ Δ*phoR* Δ*creC*, which allows us to monitor Bap synthesis as a measure of acetyl phosphate accumulation.

Substantial activation of VanR by acetyl phosphate occurs in the control strain BW24273 (ca. 450 U per cell OD_{420} [Table 7]). Activation is significantly reduced in the VanS strain BW24267 when grown without arabinose (ca. 150 U per cell OD_{420}). These cultures were grown on fructose media. Under these conditions, a small amount of VanS synthesis may result from leakiness of P_{araB} causing (partial) dephosphorylation of P-VanR. No significant reduction is seen in the $VanS_{H164Q}$ strain BW24270 in the absence of arabinose, however.

In contrast, activation of VanR by acetyl phosphate is abolished (reduced ca. 7-fold) in the $VanS_{H164Q}$ strain upon induction with arabinose (ca. 60 and 430 U per cell OD_{420} for BW24270 and BW24273, respectively [Table 7]). The conserved H164 of VanS is therefore not required for dephosphorylation of P-VanR. Moreover, the addition of arabinose resulted in further activation of VanR by VanS. This is expected, as induction of VanS synthesis by arabinose also results in activation of VanR in the absence of acetyl phosphate (Table 3). Under all conditions, the same cultures produced similar amounts of Bap (Table 7), thus ruling out an indirect effect on acetyl phosphate synthesis in these experiments.

DISCUSSION

We developed an *E. coli* model system for studying the VanS-VanR two-component regulatory system of *Enterococcus faecium* BM4147 that is required for type A inducible vancomycin resistance. Our system uses *lacZ* fusions to P_{vanH} and P_{vanR}. Activation of P_{vanH}-*lacZ* transcriptional and translational fusions occurred when *vanR* was expressed from its native promoter and *vanS* was expressed from P_{araB}. Under similar conditions, no positive or negative regulation of P_{vanR} expression was apparent (Table 3). The absence of an effect on P_{vanR} expression in our system suggests that the particular P_{vanR}-*lacZ* fusion fails to mimic normal in vivo control or an unknown regulatory factor is not present.

Induction of VanS synthesis with arabinose in our P_{vanH}-*lacZ* reporter strains resulted in ca. 6- to 45-fold activation of VanR (Table 3), depending on the fusion. Under these conditions, activation occurs in the absence of a (known) signal; activation probably results because VanS is being overproduced. Likewise, activation of PhoB by its partner PhoR also occurs in the absence of a signal (i.e., in the presence of excess P_i) when PhoR is synthesized at high levels from P_{araB} (13). Interestingly, the fold activation of VanR by VanS in *E. coli* is similar in magnitude to that observed in *Enterococcus*. Arthur et al. (2) observed 19- and 38-fold induction ratios for expression of a P_{vanH}-*cat* fusion upon treatment with vancomycin. In contrast, the fold activation of VanR by VanS in a *Bacillus subtilis* reporter system (40) was substantially less, possibly because activation of VanR due to cross talk from nonpartner kinases (partially) masked activation by VanS. In the *B. subtilis* system, activation may have also resulted from overproduction of VanR.

Although VanR and VanS are required for high-level inducible vancomycin resistance (2), VanR alone has been shown capable of transcriptional activation in *Enterococcus*. It has therefore been proposed that VanS negatively controls VanR, presumably by dephosphorylation (1). Our studies show that both VanR and VanS are required for activation of P_{vanH}. We also showed that VanS, but not $VanS_{H164Q}$, activates VanR. It is thus reasonable to suggest that activation of VanR in the absence of VanS in *Enterococcus* is primarily due to cross talk from nonspecific histidine kinases. In this regard, we showed that both PhoR and acetyl phosphate are also capable of activating VanR in vivo. VanR was previously shown to be autophosphorylated by acetyl phosphate in vitro as well (15).

Other in vitro studies have also shown that VanS is an autokinase and that P-VanS efficiently transfers its phosphate group to VanR (51). These studies were carried out with a maltose-binding protein (MBP) fusion to the C-terminal cytosolic (M95 to S384) domain of VanS (denoted MBP-′$VanS_{M95\text{-}S384}$). It was therefore unexpected that only full-length VanS, but not ′VanS ($VanS_{M95\text{-}S384}$), activates VanR in vivo (Table 3). Possibly, ′$VanS_{M95\text{-}S384}$ adopts a different conformation than MBP-′$VanS_{M95\text{-}S384}$, preventing ′$VanS_{M95\text{-}S384}$ from phosphorylating VanR. Accordingly, ′$VanS_{M95\text{-}S384}$ may be inactive as a phosphotransferase toward VanR or more active as a P-VanR phosphatase. It is not especially surprising that alter-

TABLE 7. Effects of VanS and $VanS_{H164Q}$ on activation of VanR by acetyl phosphate

Strain[a]	Kinase	Sp act (U/OD_{420})[b]			
		β-Galactosidase		Bap	
		−Ara	+Ara	−Ara	+Ara
BW24273	None	452 ± 68	430 ± 55	380 ± 7	405 ± 30
BW24267	P_{araB}-*vanS*	146 ± 31	637 ± 62	397 ± 4	494 ± 16
BW24270	P_{araB}-*vanS*$_{H164Q}$	315 ± 72	58.4 ± 9.2	392 ± 16	393 ± 11

[a] Complete genotypes are given in Table 1.
[b] Cells were assayed after 16 h of growth in 0.06% fructose–MOPS–2 mM P_i medium without (−Ara) or with (+Ara) arabinose. Values are expressed as in Table 3, footnote *d*.

ation of the N terminus may affect its activity toward VanR. In the full-length protein, the N terminus is thought to regulate activity of the C-terminal kinase domain. An alternative possibility that ′$VanS_{M95\text{-}S384}$ is unstable in *E. coli* is unlikely; the same P_{araB}-′$vanS_{M95\text{-}S384}$ fusion results in the activation of mutant forms of PhoB (so-called $PhoB^{AR}$ proteins) under similar growth conditions (12). The ability of ′VanS to activate $PhoB^{AR}$ proteins, but not VanR, is compatible with the concept that partner kinase-response regulator interactions are regulated and that nonpartner interactions are unregulated. The inability of ′$PhoR_{M63\text{-}D431}$ to activate VanR is also understandable; the interaction of ′$PhoR_{M63\text{-}D431}$ with VanR is expected to be less efficient than the interaction of full-length PhoR with VanR because ′$PhoR_{M63\text{-}D431}$ is also less efficient than PhoR for activation of PhoB (13).

The mechanisms of response regulator dephosphorylation differ in various systems, requiring in some cases additional proteins. In *E. coli* chemotaxis, P-CheY dephosphorylation requires CheZ and not the kinase CheA (34). In *B. subtilis* sporulation, a separate protein, Spo0E, is the P-Spo0A phosphatase (28), while aspartyl phosphate phosphatases are also involved in dephosphorylation reactions of particular response regulators (30). In *E. coli* nitrogen regulation, dephosphorylation of P-NtrC (NR_I) requires both GlnB(P_{II}) and the kinase NtrB (NR_{II}). In this case, the conserved histidine of NtrB is not required for dephosphorylation (18). In *E. coli* osmoregulation, the conserved histidine of the Tar-EnvZ hybrid is required for P-OmpR dephosphorylation (53). A mutant EnvZ also accepts phosphate from P-OmpR, suggesting that dephosphorylation occurs via a back transfer reaction (7). However, two laboratories have now shown that particular mutations of the conserved histidine of EnvZ do not abolish dephosphorylation (16, 36). The conserved histidine of the kinase NarX is also not required for dephosphorylation of its partner response regulator (37).

Like many signaling kinases, VanS is probably a P-VanR phosphatase. This conclusion is supported by earlier in vitro studies. P-VanR is relatively stable (half-life of 820 ± 240 min); MBP-′$VanS_{M95\text{-}S384}$ accelerates decomposition of P-VanR ca. 6-fold (half-life of 120 min ± 40 min), indicating that VanS acts as a P-VanR phosphatase (51). The P-VanR phosphatase function is probably also responsible for the negative control of VanR by VanS in *Enterococcus* (1).

Our finding that VanR is activated by PhoR and acetyl phosphate in vivo provided an additional means for testing whether VanS is a P-VanR phosphatase. Indeed, we showed that VanS interferes with activation of VanR by PhoR and acetyl phosphate (Tables 4 and 6). Furthermore, we showed that the conserved histidine that is the autophosphorylation site of VanS (H164) is not required for this interference; $VanS_{H164Q}$ blocks activation of VanR by acetyl phosphate (Table 7).

Two mechanisms can account for how VanS interferes with activation of VanR by PhoR and acetyl phosphate (Fig. 4). By acting as a phosphatase, VanS may deactivate P-VanR by dephosphorylation. In this case, P-VanR dephosphorylation cannot require the conserved histidine because $VanS_{H164Q}$ carries out the same reaction. The dephosphorylation reaction therefore occurs by hydrolysis (Fig. 4A). Alternatively, VanS may cause inhibition by tightly binding VanR and thereby preventing access of PhoR or acetyl phosphate to the VanR phosphorylation site (Fig. 4B). Although we cannot rule out the second model, the former appears more likely as it is supported by in vitro studies (51). Nonetheless, the latter remains a formal possibility.

Our results clearly show that VanS has dual roles in the

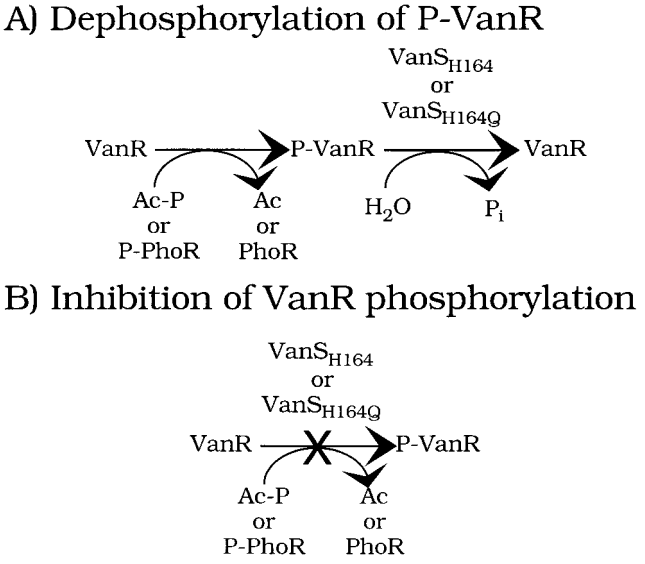

FIG. 4. Schemes for dephosphorylation of P-VanR and inhibition of VanR phosphorylation. See text. Ac-P, acetyl phosphate; Ac, acetate.

control of VanR in vivo. When synthesized at a low level from P_{vanR} or P_{araB} in the absence of arabinose, VanS has a negative role. Under these conditions, VanS acts like a P-VanR phosphatase. However, when synthesized at a higher level from P_{araB} in the presence of arabinose, or coexpressed with *vanR* from P_{rhaB} in the presence of rhamnose, VanS has an activator role. Under the latter conditions, VanS acts as a VanR kinase (Tables 3 to 7). This activation occurs in the apparent absence of a signal. Therefore, VanS adopts different conformations in vivo. In one conformation, its P-VanR phosphatase predominates; in the other, its VanR kinase predominates. Furthermore, the interconversion of VanS between these forms may mimic its normal control in response to vancomycin in *Enterococcus*. PhoR also acts as an apparent P-PhoB phosphatase or PhoB kinase under similar synthesis conditions (13), suggesting that an interconversion may be a general phenomenon for membrane-associated two-component phosphatase-kinase proteins. Understanding the basis of the interconversion may provide an insight into the control of transmembrane signaling kinases.

The interconversion of VanS between its two forms may be due to a change in its oligomeric state. Its P-VanR phosphatase may correspond to its monomeric (or low oligomeric) form, its VanR kinase may correspond to its dimeric (or higher oligomeric) form, or its P-VanR phosphatase may require the association of VanS with an unknown factor or specific membrane attachment site(s) that becomes limiting when VanS is present at high levels. Alternatively, its P-VanR phosphatase may require proper targeting of VanS to the membrane or folding of VanS within the membrane. This may occur only when VanS is at a low level. No compelling evidence or precedence now exists in favor of these or other possibilities. New biochemistry experiments are required to define the molecular basis of the switch between the P-VanR phosphatase and VanR kinase forms of VanS.

The use of an *E. coli* model system for studying vancomycin resistance provides both advantages and disadvantages. Our ability to examine both the VanR phosphotransferase and P-VanR phosphatase functions of VanS required the use of several reporter strains and mutants not readily available in other

bacteria. In some cases, it was also necessary to control synthesis levels in order to obtain meaningful results. Here again we benefited from the advantages of an *E. coli* model system. Type A vancomycin resistance of *Enterococcus faecium* BM4147 is inducible by vancomycin and the related glycopeptide antibiotic teicoplanin. The induction signal itself appears not to be a structural feature of vancomycin (21). Rather, induction may be due to accumulation of a cell wall precursor, possibly a membrane-bound lipid intermediate (4). Our present model system has not allowed us to test for effects of vancomycin or other glycopeptides on activation of VanS and VanR. As a gram-negative bacterium, *E. coli* is naturally resistant to these antibiotics due to the presence of an outer membrane. A number of vancomycin-sensitive *E. coli* mutants exist (22, 31, 33, 41), however. To overcome this problem, we have now also developed vancomycin-sensitive *E. coli* reporter strains that may be useful to search for signal molecules that activate the VanS-VanR two-component regulatory system. By using our reporter strains, it may also be possible to identify cell wall biosynthetic mutants causing activation or inhibition as an additional means for tracking this signaling pathway.

The VanS-VanR signal transduction pathway leading to activation of the *vanHAX* genes involves at least four steps: (i) detection of a signal; (ii) activation of the VanS autokinase, leading to formation of P-VanS; (iii) phosphotransfer to form P-VanR; and (iv) transcriptional activation of P_{vanH} by P-VanR. An inhibitor that blocks this pathway may interfere with any of these steps. By using our *E. coli* reporter strains, it should be possible not only to search for signal molecules that activate the system but also to identify inhibitors that prevent activation. Such an inhibitor may prove useful in combating clinical resistance to vancomycin.

ACKNOWLEDGMENTS

We thank individuals cited in the text for providing strains and plasmids.

This research was supported by NIH grant GM49338 to C.T.W. and NSF grant DMB9108005 to B.L.W. S.L.F. was supported by NIH postdoctoral fellowship GM16259. Initial studies were also supported by NIH grant GM35392 to B.L.W. The Dana Farber Cancer Institute Molecular Biology Core Facility is supported by NIH grants CA06516 and AI28691.

REFERENCES

1. **Arthur, M., F. Depardieu, G. Gerbaud, M. Galimand, R. Leclercq, and P. Courvalin.** 1997. The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn*1546* and related elements in the absence of induction. J. Bacteriol. **179:**97–106.
2. **Arthur, M., C. Molinas, and P. Courvalin.** 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **174:**2582–2591.
3. **Atlas, R. M., and A. K. Bej.** 1990. Detecting bacterial pathogens in environmental water samples by using PCR and gene probes, p. 399–406. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
4. **Baptista, M., F. Depardieu, P. Courvalin, and M. Arthur.** 1996. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **40:**2291–2295.
5. **Comess, K. M., L. M. Shewchuk, K. Ivanetich, and C. T. Walsh.** 1994. Construction of a synthetic gene for the metalloregulatory protein MerR and analysis of regionally mutated proteins for transcriptional regulation. Biochemistry **33:**4175–4186.
6. **Daniels, L. L., and B. L. Wanner.** Unpublished data.
7. **Dutta, R., and M. Inouye.** 1996. Reverse phosphotransfer from OmpR to EnvZ in a kinase$^-$/phosphatase$^+$ mutant of EnvZ (EnvZ.N347D), a bifunctional signal transducer of *Escherichia coli*. J. Biol. Chem. **271:**1424–1429.
8. **Fisher, S. L., W. Jiang, B. L. Wanner, and C. T. Walsh.** 1995. Cross-talk between the histidine protein kinase VanS and the response regulator PhoB: characterization and identification of a VanS domain that inhibits activation of PhoB. J. Biol. Chem. **270:**23143–23149.
9. **Fisher, S. L., S.-K. Kim, B. L. Wanner, and C. T. Walsh.** 1996. Kinetic comparison of the specificity of the vancomycin resistance kinase VanS for two response regulators, VanR and PhoB. Biochemistry **35:**4732–4740.
10. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
11. **Hakenbeck, R.** 1994. Resistance to glycopeptide antibiotics, p. 535–546. *In* A. Neuberger, L. M. M. van Deenen, J.-M. Ghuysen, and R. Hakenbeck (ed.), New comprehensive biochemistry, vol. 27. Bacterial cell wall. Elsevier Science B. V., Amsterdam, The Netherlands.
12. **Haldimann, A., M. K. Prahalad, S. L. Fisher, S.-K. Kim, C. T. Walsh, and B. L. Wanner.** 1996. Altered recognition mutants of the response regulator PhoB: a new genetic strategy for studying protein-protein interactions. Proc. Natl. Acad. Sci. USA **93:**14361–14366.
13. **Haldimann, A., and B. L. Wanner.** Unpublished data.
14. **Hasan, N., M. Koob, and W. Szybalski.** 1994. *Escherichia coli* genome targeting. I. Cre-*lox*-mediated in vitro generation of *ori* plasmids and their in vivo chromosomal integration and retrieval. Gene **150:**51–56.
15. **Holman, T. R., Z. Wu, B. L. Wanner, and C. T. Walsh.** 1994. Identification of the DNA-binding site for the phosphorylated VanR protein required for vancomycin resistance in *Enterococcus faecium*. Biochemistry **33:**4625–4631.
16. **Hsing, W., and T. J. Silhavy.** 1997. Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. J. Bacteriol. **179:**3729–3735.
17. **Jiang, W., A. Haldimann, and B. L. Wanner.** Unpublished data.
18. **Kamberov, E. S., M. R. Atkinson, P. Chandran, and A. J. Ninfa.** 1994. Effect of mutations in *Escherichia coli glnL* (*ntrB*), encoding nitrogen regulator II (NRII or NtrB), on the phosphatase activity involved in bacterial nitrogen regulation. J. Biol. Chem. **269:**28294–28299.
19. **Kim, S.-K., and B. L. Wanner.** Unpublished data.
20. **Kim, S.-K., M. R. Wilmes-Riesenberg, and B. L. Wanner.** 1996. Involvement of the sensor kinase EnvZ in the *in vivo* activation of the response regulator PhoB by acetyl phosphate. Mol. Microbiol. **22:**135–147.
21. **Lai, M. H., and D. R. Kirsch.** 1996. Induction signals for vancomycin resistance encoded by the *vanA* gene cluster in *Enterococcus faecium*. Antimicrob. Agents Chemother. **40:**1645–1648.
22. **Lazar, S. W., and R. Kolter.** 1996. SurA assists the folding of *Escherichia coli* outer membrane proteins. J. Bacteriol. **178:**1770–1773.
23. **Lee, T.-Y., K. Makino, H. Shinagawa, and A. Nakata.** 1990. Overproduction of acetate kinase activates the phosphate regulon in the absence of the *phoR* and *phoM* functions in *Escherichia coli*. J. Bacteriol. **172:**2245–2249.
24. **Linn, T., and R. St. Pierre.** 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. J. Bacteriol. **172:**1077–1084.
25. **McCleary, W. R., and J. B. Stock.** 1994. Acetyl phosphate and the activation of two-component response regulators. J. Biol. Chem. **269:**31567–31572.
26. **Metcalf, W. W., W. Jiang, L. L. Daniels, S.-K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZ*α for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1–13.
27. **Metcalf, W. W., and B. L. Wanner.** 1993. Mutational analysis of an *Escherichia coli* fourteen-gene operon for phosphonate degradation using Tn*phoA′* elements. J. Bacteriol. **175:**3430–3442.
28. **Ohlsen, K. L., J. K. Grimsley, and J. A. Hoch.** 1994. Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. Proc. Natl. Acad. Sci. USA **91:**1756–1760.
29. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73:**857–871.
30. **Perego, M., and J. A. Hoch.** 1996. Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **93:**1549–1553.
31. **Rida, S., J. Caillet, and J. H. Alix.** 1996. Amplification of a novel gene, *sanA*, abolishes a vancomycin-sensitive defect in *Escherichia coli*. J. Bacteriol. **178:**94–102.
32. **Rudolph, J., N. Tolliday, C. Schmitt, S. C. Schuster, and D. Oesterhelt.** 1995. Phosphorylation in halobacterial signal transduction. EMBO J. **14:**4249–4257.
33. **Russel, M.** 1994. Mutants at conserved positions in gene IV, a gene required for assembly and secretion of filamentous phages. Mol. Microbiol. **14:**357–369.

33a. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, vol. 1, 2, and 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

34. **Sanna, M. G., R. V. Swanson, R. B. Bourret, and M. I. Simon.** 1995. Mutations in the chemotactic response regulator, CheY, that confer resistance to the phosphatase activity of CheZ. Mol. Microbiol. **15:**1069–1079.
35. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
36. **Skarphol, K., J. Waukau, and S. A. Forst.** 1997. Role of His243 in the phosphatase activity of EnvZ in *Escherichia coli*. J. Bacteriol. **179:**1413–1416.
37. **Stewart, V., and R. S. Rabin.** 1995. Dual sensors and dual response regulators interact to control nitrate- and nitrite-responsive gene expression in

Escherichia coli, p. 233–252. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, D.C.
38. **Swanson, R. V., L. A. Alex, and M. I. Simon.** 1994. Histidine and aspartate phosphorylation: two-component systems and the limits of homology. Trends Biochem. Sci. **19:**485–490.
39. **Swartz, M. N.** 1994. Hospital-acquired infections: diseases with increasingly limited therapies. Proc. Natl. Acad. Sci. USA **91:**2420–2427.
40. **Ulijasz, A. T., A. Grenader, and B. Weisblum.** 1996. A vancomycin-inducible LacZ reporter system in *Bacillus subtilis*: induction by antibiotics that inhibit cell wall synthesis and by lysozyme. J. Bacteriol. **178:**6305–6309.
41. **Wandersman, C., and S. Létoffé.** 1993. Involvement of lipopolysaccharide in the secretion of *Escherichia coli* α-haemolysin and *Erwinia chrysanthemi* proteases. Mol. Microbiol. **7:**141–150.
42. **Wanner, B. L.** 1992. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. **174:** 2053–2058.
43. **Wanner, B. L.** 1994. Gene expression in bacteria using Tn*phoA* and Tn*phoA′* elements to make and switch *phoA* gene, *lacZ* (op), and *lacZ* (pr) fusions, p. 291–310. *In* K. W. Adolph (ed.), Methods in molecular genetics, vol. 3. Academic Press, Orlando, Fla.
44. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
45. **Wanner, B. L., and J. A. Boline.** 1990. Mapping and molecular cloning of the *phn* (*psiD*) locus for phosphonate utilization in *Escherichia coli*. J. Bacteriol. **172:**1186–1196.
46. **Wanner, B. L., and B.-D. Chang.** 1987. The *phoBR* operon in *Escherichia coli* K-12. J. Bacteriol. **169:**5569–5574.
47. **Wanner, B. L., W. Jiang, S.-K. Kim, S. Yamagata, A. Haldimann, and L. L. Daniels.** 1996. Are the multiple signal transduction pathways of the Pho regulon due to cross talk or cross regulation?, p. 297–315. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Co., Austin, Tex.
48. **Wanner, B. L., and M. R. Wilmes-Riesenberg.** 1992. Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in the control of the phosphate regulon in *Escherichia coli*. J. Bacteriol. **174:**2124–2130.
49. **Wilheim, M. P.** 1991. Vancomycin. Mayo Clin. Proc. **66:**1165–1170.
50. **Wilmes-Riesenberg, M. R., and B. L. Wanner.** 1992. Tn*phoA* and Tn*phoA′* elements for making and switching fusions for study of transcription, translation, and cell surface localization. J. Bacteriol. **174:**4558–4575.
51. **Wright, G. D., T. R. Holman, and C. T. Walsh.** 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry **32:**5057–5063.
52. **Wright, G. D., and C. T. Walsh.** 1992. D-Alanyl-D-alanine ligases and the molecular mechanism of vancomycin resistance. Acc. Chem. Res. **25:**468–473.
53. **Yang, Y., and M. Inouye.** 1993. Requirement of both kinase and phosphatase activities of an *Escherichia coli* receptor (Taz1) for ligand-dependent signal transduction. J. Mol. Biol. **231:**335–342.