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Identification of the DNA-Binding Site for the Phosphorylated VanR Protein Required for Vancomycin Resistance in *Enterococcus faecium*†

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ABSTRACT: The vancomycin resistance operon of *Enterococcus faecium* encodes a two-component regulatory system comprising VanS and VanR. *In vitro* experiments showed that about 5% of a labile phosphorylated VanR (P-VanR) was accumulated from ATP and a maltose-binding protein–VanS fusion protein (MBP–VanS). Alternatively, about an 8% abundance of P-VanR was produced with acetyl phosphate. In such incubations, gel shift experiments revealed that P-VanR selectively bound to a 254-bp DNA fragment that contains the *vanH* promoter for the *vanH, vanA,* and *vanX* structural genes. When VanS was added with a mole ratio for VanS:VanR of higher than 1:1, VanS competed with DNA for P-VanR and abolished the gel shift. P-VanR bound 500-fold more tightly to the *vanH* promoter region, with an estimated EC_{50} of 40 nM, than the unphosphorylated VanR. A second DNA fragment of 197 bp containing the proposed *vanR* promoter for the *vanR* and *vanS* regulatory genes also exhibited gel shift, but with much lower affinities. A mutant VanR(D53A) was shown to be incompetent for phosphorylation by phosphorylated MBP–VanS or by acetyl phosphate; however, it still bound DNA specifically, albeit with low affinity. DNase I footprinting by P-VanR revealed that a ca. 80-bp region was protected on the *vanH* promoter and a ca. 40-bp region was protected on the *vanR* promoter. The unphosphorylated VanR footprinted the same 80 bp on the *vanH* promoter, but only 20 bp on the *vanR* promoter. The markedly increased affinity of P-VanR for the *vanH* promoter over the *vanR* promoter and the doubling in size of the protected footprint (80-bp vs 40-bp) are consistent with the oligomerization of P-VanR on the *vanH* promoter. Analysis of the footprinted regions revealed a 12-bp sequence present in one copy in the *vanR* promoter and in two copies in the *vanH* promoter, which may serve as the consensus recognition site for P-VanR binding, transcriptional activation, and expression of vancomycin resistance.

Vancomycin is an antibiotic widely used for the treatment of Gram-positive bacterial infections (Wilhelm, 1991). It functions by binding to the cell wall (via the D-Ala-D-Ala moiety on the peptidoglycan chain terminal) and inhibits essential cell wall polymerization and transpeptidation, thereby inducing cell death [for reviews, see Barna and Williams (1984) and Reynolds (1989)]. Recently, Courvalin and co-workers isolated clinical strains of *Enterococcus faecium* BM4147 that are vancomycin-resistant and determined that five genes are required: *vanR*, *vanS*, *vanH*, *vanA*, and *vanX* (Figure 1) (Arthur et al., 1992a). Two gene products, VanH and VanA, are enzymes that confer the biochemical modification of the cell wall that is necessary for resistance. As predicted on the basis of sequence homology and verified by biochemical studies, VanH is a D-specific α-ketoacid reductase (Arthur et al., 1991; Bugg et al., 1991b), and VanA is a D-Ala-D-lactate depsipeptide ligase that uses the D-lactate generated by VanH (Dutka-Malen et al., 1990; Bugg et al., 1991a). Incorporation of D-Ala-D-lactate into the peptidoglycan chain in place of D-Ala-D-Ala reduces the affinity of vancomycin 1000-fold (Bugg et al., 1991b) and, thus, decreases its effectiveness as an antibiotic by an equivalent amount (Wright & Walsh, 1992; Messer & Reynolds, 1992). As of yet, the function of VanX is unknown.

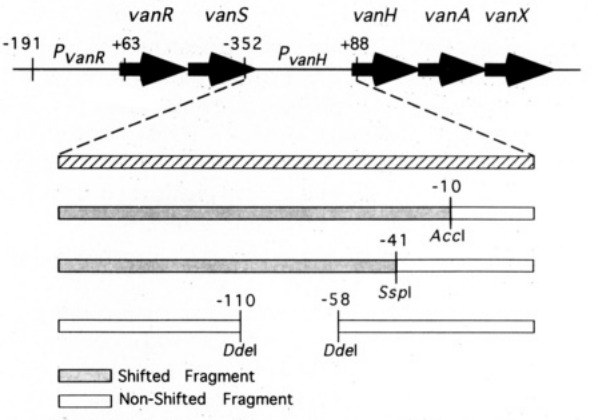

FIGURE 1: Vancomycin resistance operon and the *vanH* promoter fragments used for gel mobility shift assays. Base pairs are numbered relative to the mRNA start site of *vanH* and the proposed start site of *vanR*, respectively.

The other two gene products, VanR and VanS, are also necessary for vancomycin resistance. They have been shown, on the basis of sequence similarities (Arthur et al., 1992a) and biochemical studies (Wright et al., 1993), to comprise a two-component regulatory system, which is required for expression of the *vanH*, *vanA*, and *vanX* genes. Two-component regulatory systems are commonly used by bacteria to adapt to changes in their environment (i.e., nutrient limitation, chemotaxis, sporulation, virulence, and antibiotic resistance) [for reviews, see Bourret et al. (1991), Gross et al. (1989), Stock et al. (1992), Wanner (1992, 1993), and Parkinson (1993)]. Two-component regulatory systems usually consist of a sensor protein that is a histidine kinase (VanS)

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and a response regulator that is often, as in this case, a transcriptional activator (VanR).

Response regulators that are transcriptional regulators often contain two domains: a phosphorylation domain and a DNA-binding domain. The phosphorylation domain has an invariant aspartic acid residue that accepts the phosphoryl group from a phosphohistidine residue on the sensor. The DNA-binding domain recognizes a specific DNA sequence upstream of the target gene. In the well-characterized systems of EnvZ/OmpR (Aiba et al., 1989), NtrB/NtrC (Ninfa et al., 1987), PhoR/PhoB (Makino et al., 1989), and ComP/ComA (Roggiani & Dubnau, 1993), phosphorylation of the response regulator has been shown to increase DNA-binding affinity, possibly through the formation of higher oligomeric protein states and cooperative binding (Nakashima et al., 1991; Weiss et al., 1992).

In this report, VanR has been shown to act as a DNA-binding protein. It binds to DNA fragments containing the *vanH* and the proposed *vanR* promoter region. The binding affinity for both regions is increased dramatically by phosphorylation. *In vitro* footprinting experiments demonstrate sequence-specific DNA–protein interactions and reveal a possible consensus binding sequence. Addition of a maltose-binding protein–VanS fusion protein (MBP–VanS, hereafter referred to as VanS) to the P-VanR[1]/DNA mixture inhibits the formation of a P-VanR/DNA complex, indicating a possible VanS/P-VanR intermediate in this signal transduction pathway. The mutant VanR(D53A), in which the conserved Asp53 residue is changed to Ala, is incompetent for phosphorylation by phospho-VanS (P-VanS) or by acetyl phosphate; however, it still binds DNA by *in vitro* footprinting experiments with reduced affinity.

MATERIALS AND METHODS

Materials. Restriction endonucleases and polynucleotide kinase were from New England BioLabs (Beverly, MA). DNase I and acetyl phosphate were from Sigma (St. Louis, MO). [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]acetic anhydride (50 mCi/mmol), and [^{32}P]monopotassium phosphate (1000 mCi/mmol) were from New England Nuclear (Boston, MA), and all other reagents were reagent grade.

Plasmids and Strains. Plasmids for the overproduction of VanR (pTH1) and the MBP–VanS (pMal-VanS) were prepared by the expression cassette polymerase chain reaction (PCR) method (MacFerrin et al., 1990), as described previously (Wright et al., 1993). Site-directed mutagenesis was carried out by the Kunkel method (Kunkel, 1985). Uracil-containing ssDNA of M13mp19 with *vanR* was generated, and the D53A primer (5′-ACCTTGCAATATTGGCCAT-CATG-3′) was annealed. The dsDNA was synthesized with T4 DNA polymerase, ligated with T4 DNA ligase, and transformed into *Escherichia coli* XL1-Blue. The entire gene was sequenced to verify the presence of a single mutation. The mutated *vanR*(D53A) gene was excised with *Xba*I and *Hind*III and ligated to pKK223-3* (Zawadzke et al., 1991) to place it under the control of the *tac* promoter. This plasmid (pTH2) was transformed into *E. coli* W3110 for protein overproduction.

Purification of Proteins and Protein Phosphorylation Experiments. The wild-type VanR, mutant VanR(D53A), and VanS proteins were purified as described previously (Wright et al., 1993). The *in vitro* conditions for VanS autophosphorylation, purification of [^{32}P]VanS (phosphorylated VanS), and phosphoryl transfer between VanS and VanR or VanR(D53A) were also the same as described previously (Wright et al., 1993).

The phosphorylation of VanR by acetyl phosphate proceeded essentially as described (Lukat et al., 1992). Dilithium [^{32}P]-acetyl phosphate (approximately 0.075 Ci/mmol) was synthesized as described previously (Stadtman, 1957); however, much less LiOH solution was needed to adjust the pH to 7.5 than was indicated in that article. VanR (20 μM) was incubated at 37 °C in a reaction buffer (50 mM HEPES, pH 7.2, and 5 mM $MgCl_2$) containing 10, 25, or 50 mM [^{32}P]-acetyl phosphate. Aliquots were removed after 30 and 60 min, and the reactions were stopped by the addition of SDS sample buffer. The samples were analyzed by SDS–polyacrylamide gel electrophoresis (12.5% acrylamide) followed by staining with Coomassie brilliant blue and then two destaining steps. Bands corresponding to VanR were excised and counted. The percentage of VanR phosphorylation was calculated on the basis of the specific activity of [^{32}P]acetyl phosphate.

Acetylation of VanR by [^{3}H]Acetyl Phosphate. The possible acetylation of VanR by acetyl phosphate was studied with [^{3}H]acetyl phosphate (0.73 Ci/mol), which was synthesized like [^{32}P]acetyl phosphate except that [^{3}H]acetic anhydride was used. VanR (20 μM) was incubated at 37 °C in reaction buffer (50 mM HEPES, pH 7.2, and 5 mM $MgCl_2$) containing 0, 5, or 50 mM [^{3}H]acetyl phosphate (0.73 Ci/mol) for 60 min. The reactions were stopped by the addition of SDS sample buffer, loaded directly onto a 12% SDS–polyacrylamide gel, and separated by electrophoresis. The gel was stained with Coomassie brilliant blue, the bands corresponding to VanR were excised, and the ^{3}H radioactivity was counted.

DNA-Protein-Binding Assay. Gel mobility shift was utilized to identify the DNA-binding region(s). Three fragments were used: a 440-bp fragment containing the *vanH* promoter (−352 to +88), a 254-bp fragment also containing the *vanH* promoter (−191 to +63), and a 197-bp fragment containing the *vanR* promoter (−173 to +24). DNA fragments were obtained by PCR using the oligos 5′-CGAATTCAA-GAACACTG-3′ and 5′-AAGCTTTCTGTGAAAGGC-3′ for the 440-bp fragment, oligos 5′-CGCGGATCCGCGGG-GATGCCAATGGT-3′ and 5′-CGGAATTCCGGAAAG-CAATGATAACTAT-3′ for the 254-bp fragment, and oligos 5′-GAATTCTGTATTCCGCTA-3′ and 5′-CGCGGATC-CGCGCACATAAGTTTGCCCTTA-3′ for the 197-bp fragment. These fragments were purified with a 1.5% agrose gel, bound to DEAE paper, eluted in high-salt solution, and precipitated. The fragments were then 5′-end-labeled with polynucleotide kinase in the presence of [γ-^{32}P]ATP. The binding reaction contained 20 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM $CaCl_2$, 10% glycerol, 0.3 ng of radiolabeled DNA, 0.5 μg of salmon sperm DNA, and protein in a total volume of 15 μL. After a 15 min incubation on ice, reaction mixtures were loaded on a 5% nondenaturing polyacrylamide gel, which had been prerun overnight at 25 V in 45 mM Tris–borate buffer. Electrophoresis was performed at 4 °C with 160 V for 1 h. The gels were dried,

[1] Abbreviations: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EC_{50}, effective concentration for 50% response; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IC_{50}, effective concentration for 50% inhibition; MBP, maltose-binding protein; oligo, oligonucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; P-VanR, phosphorylated VanR; P-VanS, phosphorylated VanS; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

analyzed, and quantitated by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

DNase I Footprinting. The 254-bp and 197-bp probes were labeled with polynucleotide kinase (NEB, Beverly, MA) in the presence of [γ-^{32}P]ATP and digested with *Eco*RI or *Bam*HI, as appropriate. Binding reactions were performed as in the gel shift studies using 10 ng of probe with approximately 30 000 cpm for each reaction mixture. After binding, the solutions were warmed to 25 °C for 2 min, 2 pg of DNase I (Sigma) was added, and after 1 min, 90 μL of stop solution (150 mM sodium acetate and 10 μg of yeast tRNA per milliliter of ethanol) was added. The samples were chilled on dry ice for 15 min and then centrifuged at 25 °C for 5 min, and the pellet was rinsed with 150 μL of 95% ethanol (–20 °C). The samples were dried and then resuspended in 5 μL of loading buffer, heated to 95 °C, and then separated by electrophoresis on an 8% polyacrylamide gel containing urea. The gels were dried and exposed to Kodak X-OMAT-AR film. The guanine sequencing reaction was performed according to the Maxam–Gilbert chemical cleavage method (Maxam & Gilbert, 1980).

RESULTS

MBP–VanS, VanR, and VanR(D53A) Overproduction and Purification. MBP–VanS and VanR were overproduced in *E. coli* W3110 as described earlier (Wright et al., 1993). The plasmid for the mutant D53A (pTH2) was derived from pTH1 utilizing the Kunkel method of mutagenesis (Kunkel, 1985). This plasmid gave strong overproduction of the mutant VanR (ca. 4% soluble protein), which behaved exactly like the wild-type protein during the three-step purification. It yielded 23 mg of protein per liter. The mutant protein was ca. 85% pure, as judged by Coomassie blue staining, and comigrated on SDS–PAGE gels with the wild-type VanR.

Phosphorylation of VanR and VanR(D53A). It has been shown that other response regulator proteins are phosphorylated both by their partner sensor protein and by chemical phosphorylating agents such as acetyl phosphate and phosphoramidate (Lukat et al., 1992; Feng et al., 1992). Regulators phosphorylated by low molecular weight reagents can thereby be studied through DNA-binding experiments without the complication of an interacting sensor protein. Further, as shown below, the presence of VanS can cause problems because VanS competes with the DNA for binding to P-VanR. When VanR was incubated with [^{32}P]acetyl phosphate, a radioactive band corresponding to VanR in size was observed. This band appeared after a 30-min incubation with 50 mM [^{32}P]acetyl phosphate, and its intensity increased with time (data not shown). The amount of radiolabeled protein was determined by excising the band and counting the radioactivity. Approximately 8% of VanR was phosphorylated by using 50 mM acetyl phosphate. By comparison, about 15% of VanR was phosphorylated by using [^{32}P]VanS (Wright et al., 1993), although only 5% of VanR was phosphorylated by the [^{32}P]-VanS used in this work. As predicted, the mutant VanR-(D53A) was not phosphorylated by either [^{32}P]acetyl phosphate or [^{32}P]VanS, which is consistent with the phosphorylation of Asp53 in the wild-type protein.

Acetylation of VanR by [^{3}H]Acetyl Phosphate. Acetyl phosphate offers an easy and alternative way to phosphorylate VanR. However, acetyl phosphate as a mixed anhydride is not only a phosphorylating reagent but also a good acetylating reagent. During the phosphorylation reaction, a protein might be modified by acetylation with some effect on function, e.g., DNA binding. In order to address this potential problem, [^{3}H]acetyl phosphate was synthesized and incubated with VanR under the same conditions as those used for the phosphorylation reaction. When VanR was isolated by a SDS–PAGE gel, no significant ^{3}H radioactivity was detected (data not shown). From these results we concluded that acetylation is not a confounding problem for P-VanR preparation from acetyl phosphate.

DNA Interaction of VanR and VanR(D53A) with the vanH and vanR Promoter Regions. Courvalin and co-workers (Arthur et al., 1992b) showed that *in vivo* expression of the *vanH, -A,* and *-X* region required a 440-bp fragment (–352 to +88) containing the intergenic region between the *vanS* and *vanH* genes (Figure 1). To determine whether this region was recognized by VanR, a series of gel mobility shift experiments was carried out. Initial experiments with the 440-pp fragment and P-VanR showed a marked retardation of the DNA in the presence of a 250-fold excess of salmon sperm DNA, suggesting that the DNA interaction was specific. The 440-bp fragment was digested separately at position –10 and at position –41 (Figure 1), and in both cases the larger fragment was shifted by P-VanR. Digestion at positions –110 and –57 eliminated the mobility shift for either resulting radiolabeled fragment. This suggests that the interaction region is between –110 and –41. On the basis of these results, the 254-bp fragment from positions –191 to +63 (which was synthesized with PCR as described in Materials and Methods) was used for subsequent quantitative gel mobility shift experiments with VanR, phosphorylated VanR, and mutant VanR(D53A). It was also observed that a 197-bp PCR fragment just upstream of the *vanR* and *vanS* regulatory genes (–173 to +24) was specifically gel-shifted by VanR. These two fragments contain the *vanH* and proposed *vanR* promoter regions for *vanH/vanA* and *vanX/vanR/vanS* transcription, respectively.

P-VanR generated either from acetyl phosphate (lane 1) or from phosphorylated VanS (VanS/ATP) (lanes 2 and 3) induced a gel shift of the 254-bp *vanH* promoter fragment (Figure 2A). In lane 1, for a nominal 2.7 μM VanR, the P-VanR concentration was 0.22 μM (ca. 8% phosphorylation by acetyl phosphate). In parallel studies with [γ-^{32}P]ATP and VanS, about 5% of the VanR molecules were phosphorylated; thus, in lanes 2–4, the 4 μM VanR population contained about 0.20 μM P-VanR. In an attempt to increase the mole fraction of P-VanR formed from VanS/ATP, an increase in VanS levels actually led to suppression of the gel shift. As shown in Figure 2A, when VanS was raised from 1.1 to 2.1 μM, free DNA began to be detected, and by 6.7 μM VanS, only free DNA was observed (lane 4). This may be due to a simple competition between VanS and DNA for P-VanR or to dephosphorylation of P-VanR in the presence of VanS, although our previous study would suggest dephosphorylation to be too slow on this time scale (Wright et al., 1993). A control experiment for dephosphorylation was carried out with the same reaction conditions as those described for Figure 2A, except that [^{32}P]acetyl phosphate was used. The phosphorimaging analysis of VanR bands in an SDS–PAGE gel showed that there was no significant dephosphorylation of VanR in the presence of VanS under these conditions (data not shown). Therefore, the suppression of the gel shift with the increase of the VanS concentration is apparently due to an interaction between VanS and P-VanR. The inhibitory effect of VanS on DNA binding by P-VanR was quantitated as shown in Figure 2B. The P-VanR was produced by incubation with acetyl phosphate, after which the indicated amount of VanS was added. A very sharp dose response was

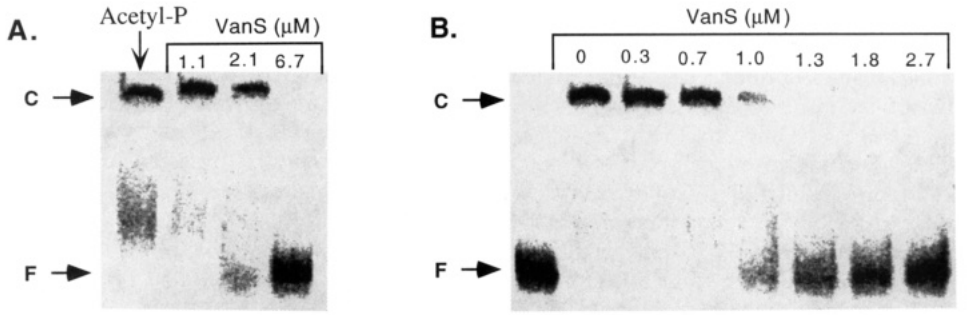

FIGURE 2: Gel mobility shift assays of binding reactions containing a fixed amount of ^{32}P-labeled *vanH* promoter fragment (0.3 ng) and various amounts of P-VanR. F: free *vanH* promoter probe; C: complex formed. (A) Lane 1: Phosphorylation of VanR (2.7 μM) by 50 mM acetyl phosphate. P-VanR was estimated as 0.22 μM. Lanes 2–4: Phosphorylation of VanR (4 μM) by increasing amounts of MBP–VanS/ATP, with the amount of VanS indicated above each lane. (B) Inhibition of P-VanR (0.22 μM) binding to the *vanH* promoter (0.3 ng) by increasing amounts of MBP–VanS. P-VanR was prepared by reaction with acetyl phosphate.

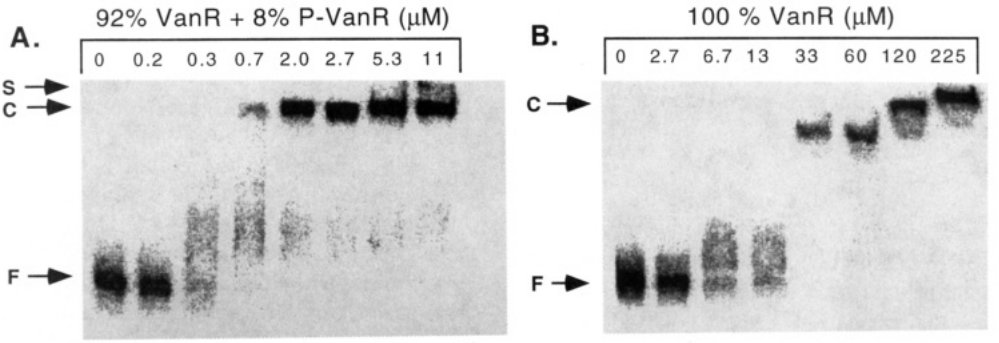

FIGURE 3: Gel mobility shift assays of binding reactions between the ^{32}P-labeled 254-bp *vanH* promoter fragment (0.3 ng) and increasing amounts of VanR prepared by incubation with 50 mM acetyl phosphate (A) or VanR alone (B), as indicated. F: free *vanH* promoter probe; C: complex formed; S: super-shifted complex.

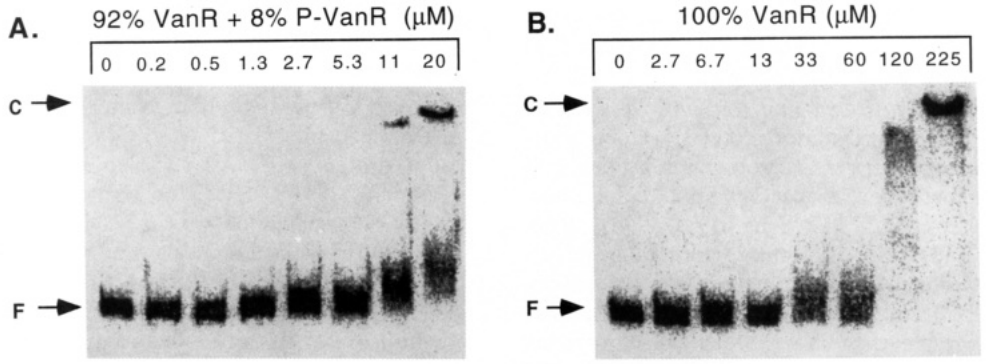

FIGURE 4: Gel mobility shift assays of binding reactions between the ^{32}P-labeled 197-bp *vanR* promoter fragment (0.3 ng) and increasing amounts of P-VanR prepared by incubation with 50 mM acetyl phosphate (A) or VanR alone (B), as indicated. F: free *vanR* promoter probe; C: complex formed.

reproducibly observed with an IC_{50} (effective concentration for 50% inhibition) of ca. 1 μM for VanS when the VanR total concentration was 2.7 μM, and that of P-VanR was estimated as 0.22 μM (ca. 8% phosphorylation). VanS likewise competed for the weaker binding (*vide infra*) of VanR to the DNA fragment containing the *vanH* promoter and exhibited an IC_{50} of 9 μM (data not shown). Given the clear inhibition by VanS in the gel shift assays, subsequent studies were conducted with P-VanR generated by incubation with acetyl phosphate.

The data in Figure 3A indicate that P-VanR shifts the 254-bp *vanH* promoter fragment with an estimated EC_{50} (effective concentration for 50% response) of 40 nM. The typical pattern of intermediate shifts as a smear makes the determination of the midpoint concentration for DNA shift problematic, but if we take 0.5 μM total VanR as an estimate with 8% of it as P-VanR, then (0.5) × (0.08) = 0.04 μM (Table 1). By comparison, a much higher concentration of unphosphorylated VanR is required for gel shift (Figure 3B). An estimated EC_{50} of 20 μM for VanR indicates that P-VanR binds the *vanH* promoter fragment about 500-fold more tightly than does VanR.

The second DNA fragment in the vancomycin resistance operon, the 197-bp *vanR* promoter fragment, shows much lower affinity for both P-VanR (Figure 4A) and free VanR (Figure 4B). An estimate of 20 μM for the 50% shift in Figure 4A leads to a calculation of 1.6 μM as the EC_{50} value for P-VanR (Table 1) on the *vanR* promoter. The unphosphorylated VanR shifts only at very high concentrations: ca. 100 μM as a crude EC_{50} (Figure 4B, Table 1). Thus, phosphorylation of VanR does reduce the estimated EC_{50} for the *vanR* promoter substantially (from 100 to 1.6 μM), but the *vanR* promoter binds 40-fold less well to P-VanR (1.6 vs 0.04 μM) than the *vanH* promoter. The VanR(D53A) mutant protein, defective in phosphorylation by both VanS and ATP or acetyl phosphate, was also tested in the gel shift assays. The mutant VanR(D53A) retained the low binding affinity

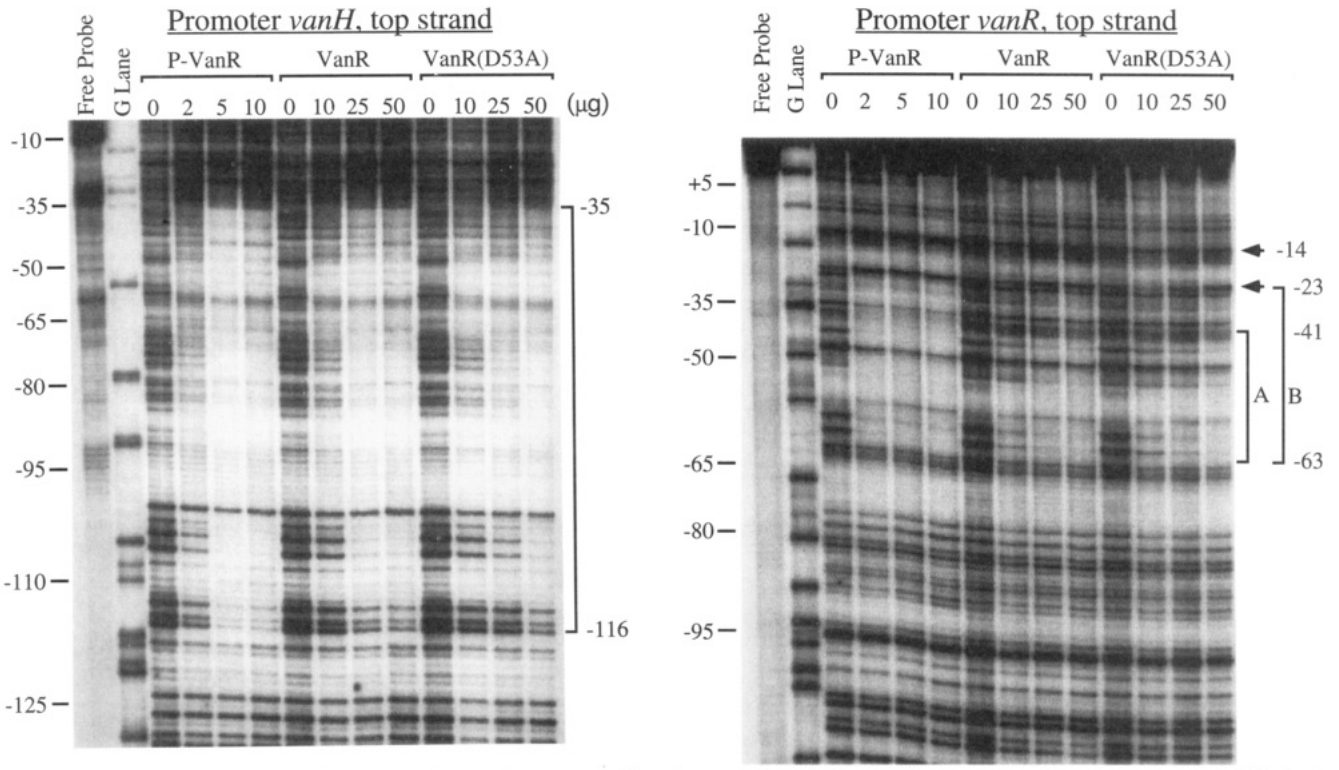

FIGURE 5: Footprinting analysis of VanR binding to the *vanH* (left) and *vanR* (right) promoter regions. The top strands are labeled for both promoters, and their base pairs are numbered relative to the start site of transcription of the *vanH* gene and the possible transcription start site of the *vanR* gene. The protected regions on each strand are shown by brackets. For the *vanR* promoter region, the bracket labeled A refers to the VanR protected region and the bracket labeled as B refers to the P-VanR protected region. The DNase I hypersensitive sites are indicated by arrows. For each strand, a series of amounts (in micrograms) of the indicated protein (i.e., P-VanR, VanR, VanR(D53A)) was used to demonstrate the relative affinities between them. As noted in the text, the P-VanR sample is only 8% phosphorylated. Also included are lanes with free probe (no DNase I), G sequencing ladder, and probe with DNase I (no protein). Only the top strands of the *vanH* and *vanR* promoter regions are shown here. The bottom strands also showed similar protected regions (data not shown), and the results are summarized in Figure 6.

Table 1: Binding Constants between DNA and P-VanR, VanR, or Mutant VanR(D53A)

	EC_{50} (μM)		IC_{50} (μM)
	promoter *vanH*	promoter *vanR*	VanS/*vanH*
P-VanR	0.040	1.6	1.0
VanR	20	100	9
VanR(D53A)	>50	>50	

of the unphosphorylated form of wild-type VanR, with a lower limit of $EC_{50} > 50$ μM (data not shown).

DNase I Protection of DNA by P-VanR, VanR, and VanR-(D53A). The gel mobility shift data strongly support the hypothesis that P-VanR specifically binds to two regions of DNA. To verify this and to determine the specific binding sites, *in vitro* footprinting experiments using DNase I and the 254-bp and 197-bp fragments containing the *vanH* promoter and the *vanR* promoter regions, respectively, were carried out as shown in Figure 5 and as summarized in Figure 6. P-VanR, VanR, and VanR(D53A) protected distinct regions on both strands of these DNAs.

The *vanH* promoter has a large (ca. 80 bp) region protected (–106 to –33 on the bottom strand and –116 to –35 on the top strand) by P-VanR, VanR, and VanR(D53A). The protected region for the *vanR* promoter is markedly different both in size and in nature of binding. P-VanR protects a region of DNA approximately one-half the size (ca. 40 bp): from –63 to –23 on the top strand and from –77 to –16 on the bottom strand. VanR and VanR(D53A) protect a smaller region (ca. 20 bp), approximately one-fourth of the protected region on the *vanH* promoter (–63 to –41 on the top strand and –77 to –55 on the bottom strand). For both DNA probes, P-VanR protects at an approximately 100-fold lower protein concentration than VanR, consistent with the mobility shift experiments. These results show that P-VanR and VanR specifically bind to both the *vanR* and *vanH* promoters and that phosphorylation not only increases the binding affinity but, in the case of the *vanR* promoter, also enlarges the protected region. In addition, sites at –23 and –10 in the *vanR* promoter region are hypersensitive to DNase I cleavage, suggestive of a distortion in the DNA helix. No hypersensitive site was observed within the *vanH* promoter fragment.

DISCUSSION

Recent studies of Arthur et al. (1992b) have shown, by using CAT reporter gene constructs to the *vanH* upstream region, that VanR was required for *in vivo* transcriptional activation of the *vanH*, *-A*, and *-X* genes. This and the sequence homology of VanR to two-component response regulators strongly suggested that VanR would bind DNA in a sequence-specific manner and might also bind with increased affinity upon phosphorylation, as has been observed for other response regulators. In the results presented herein, we show, through gel retardation and DNase I footprinting experiments, that P-VanR and VanR, albeit more weakly, specifically bind to the *vanH* promoter region identified by Arthur et al. (1992b). We also demonstrate that P-VanR and VanR bind specifically at a proposed *vanR* promoter region. The affinity constants of VanR for the *vanH* and *vanR* promoter regions are approximately 20 and 100 μM, respectively, and therefore little or no binding of the unphosphorylated form is expected *in vivo*. Phosphorylation of VanR increases the DNA affinity

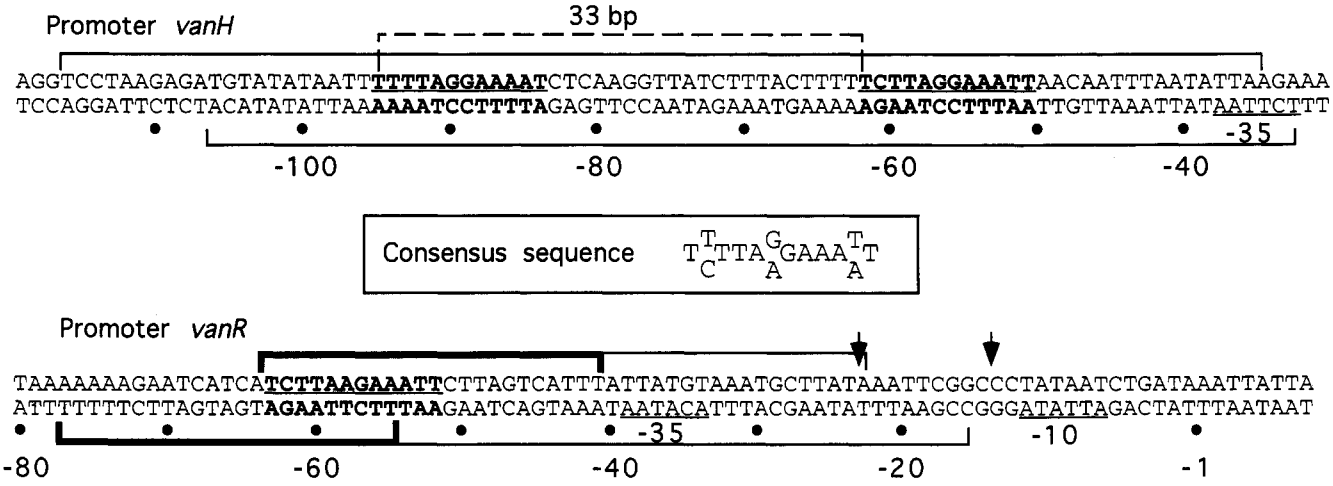

FIGURE 6: Footprinting of the promoter regions and the 12-bp consensus sequence. The sequences of the promoter regions for both the *vanH* and *vanR* DNA fragments are shown here, with the protected regions of both strands indicated by brackets. The hypersensitive sites are indicated by arrows, and the consensus sites are underlined and in boldface type. The double brackets for the *vanR* region indicate the difference in protected regions for VanR (bold bracket) and P-VanR. The sequences are numbered relative to the start sites of transcription, as discussed in the text.

dramatically by ca. 500-fold at the *vanH* promoter and by over 60-fold at the *vanR* site. The EC_{50} estimate of 40 nM at the *vanH* promoter makes it likely that this is a relevant site *in vivo*. It remains to be seen whether the weaker binding at the *vanR* promoter ($EC_{50} \approx 1.6$ μM) is important. For comparison, the approximate EC_{50} values are 14 and 1 nM for the unphosphorylated and phosphorylated forms of NtrC (Weiss & Magasanik, 1988), 30 and 1 μM for these forms of ComA (Roggiani & Dubnau, 1993), and 15 and 1.5 μM for these forms of OmpR, respectively (Aiba et al., 1989).

Further studies to quantitate the affinities for P-VanR more accurately will require the separation of P-VanR from VanR. To date, the kinetic lability of the aspartyl–phosphate linkages in the phosphorylated forms of response regulators has rendered this goal impractical, but the long half-life of P-VanR ($\approx$10 h) may make this a feasible goal (Wright et al., 1993). It should also be noted that the magnitude of the gel shift (Figures 3 and 4) of the DNA/protein complex is large in comparison to those of other DNA-binding proteins (Lane et al., 1992); however, this large shift is consistent with shifts seen for the other two-component response regulators, OmpR (Aiba et al., 1989) and ComA (Roggiani & Dubnau, 1993). These observations may be due to oligomerization of these proteins on the DNA and/or DNA bending. In addition, an even greater gel shift band (i.e., supershift, Figure 3A) was observed at the higher concentrations of P-VanR, which may be caused by the further oligomerization of P-VanR on DNA or by the aggregation of protein–DNA complex. Interestingly, in Figure 3B, the shift of labeled DNA increased with increasing of VanR concentration, which may indicate the different levels of oligomerization of VanR on DNA. All of these phenomena will require further investigations before any conclusion can be reached.

As we have shown, VanS competed for P-VanR, and a large excess of VanS completely eliminated the gel shift band with an IC_{50} around 1 μM (Figure 2B). This probably has no relevance for the P-VanR/*vanH* promoter interaction because the EC_{50} is low (ca. 40 nM), but it is on the same scale as the P-VanR/*vanR* promoter interaction and thus may play a physiological role. The interaction between VanS and P-VanR or VanR also indicates the existence of the VanS/P-VanR intermediate in this signal transduction pathway. It should be noted that VanS is a fusion protein, which may affect the VanS/P-VanR or -VanR interaction. Experiments are in progress to synthesize the cytosolic domain of VanS only as well as the full-length protein to determine the interaction coefficients for these proteins.

The region of the *vanH* promoter protected from DNase I digestion by P-VanR and VanR spans a large region of approximately 80 bp. This is an unusually large footprint. By comparison, OmpR and NtrC only protect 40 and 50 bp, respectively. On the other hand, the footprint for VirG covers ca. 80 bp (Roitsch et al., 1990). Although the DNA affinities vary dramatically, the sizes of the footprints for P-VanR and VanR do not vary greatly, as observed with the phosphorylated and unphosphorylated forms of OmpR (Aiba et al., 1989), NtrC (Weiss & Magasanik, 1988), and ComA (Roggiani & Dubnau, 1993). The footprint extends just upstream of the –35 region of the *vanH* promoter, in keeping with its role as a presumptive transcriptional activator of the *vanH*, *vanA*, and *vanX* genes.

The protected region of the *vanR* promoter spans a smaller region than the *vanH* promoter and is similar in size to the footprints of OmpR and NtrC on their cognate promoters. Intriguingly, the size of the protected region varies dramatically between P-VanR and VanR. VanR protects a 20-bp region that approaches the –35 region of the *vanR* promoter. P-VanR increases the length of the DNase I-protected region 2-fold by protecting an additional 20 bp that include the –35 promoter region and extend to approximately base –16.

The different footprints suggest the following interpretation. At the *vanH* promoter, VanR is an activator (Arthur et al., 1992b), and both phosphorylated and unphosphorylated forms bind just upstream of the –35 region. At the *vanR* promoter, VanR binds just upstream of the –35 promoter; however, the P-VanR-binding region includes the –35 region (Figure 6). If this affinity is high enough to function *in vivo*, P-VanR may repress VanR synthesis. As noted above, the +1 mRNA start site has been mapped for the *vanH* promoter, but not for the *vanR* promoter. The putative –10 (TAGACT) and –35 (TTAAGA) regions in the *vanH* promoter region have poor consensus to *E. coli* σ70 promoters (Hoopes & McClure, 1987) and no consensus with *Bacillus* promoter sites (σ54) (Collado-Vides et al., 1991). The putative –10 (TATAAT) and –35 (TTATGT) promoter elements for the *vanR* gene are closer to consensus for the σ70 promoters. This is consistent with

P-VanR acting as an activator for the *vanH* promoter and as a repressor for the *vanR* promoter.

Another indication that the transcriptional mechanism may be more similar to that of a σ70 promoter is the amino acid sequence homology of VanR with other response regulators (Parkinson, 1993). While the amino acid sequence homology of the phosphorylation domain in the N-terminal half of VanR, OmpR, PhoB, VirG, and NtrC is high, the C-terminal DNA-binding helix–turn–helix domains of VanR, OmpR, PhoB, and VirG diverge from that of NtrC. The explanation for this difference is thought to reflect activation at σ54 promoters by NtrC (and its homologs) and activation at σ70 promoters by VanR, OmpR, PhoB, and other proteins of this subfamily of response regulators.

By comparing the two regions of DNase I protection provided by VanR species at the *vanH* and *vanR* promoters, one observes a highly conserved sequence that strongly suggests a consensus binding sequence. As shown in Figure 6, the proposed consensus sequence is 12 bp long, and among these sequences, 9 of 12 nucleotides are conserved. There are two such sequences in the *vanH* promoter (–95 to –84 and –62 to –51) and one in the *vanR* promoter (–63 to –52) regions. The two sites in the *vanH* region are separated by 33 bp, which indicates that they would be on the same face of the DNA helix (10.5 bp per turn). Similarly, the two binding sites for NtrC (Weiss & Magasanik, 1988) and ComA (Roggiani & Dubnau, 1993) are also on the same side of the DNA helix, with 31-bp and 45-bp separation, respectively. In the case of NtrC, Weiss et al. proposed that the protein binding to the DNA was a cooperative process and that NtrC oligomerized on the DNA as a tetramer. This may also occur with VanR binding to DNA. VanR binds to the single consensus site on the *vanR* promoter and protects approximately 20 bp. Upon phosphorylation, the protected region increases 2-fold to 40 bp. When there are two consensus binding sites, as on the *vanH* promoter, the protected region increases to twice the length of the *vanR* protected region, 80 bp. VanR is a monomer in solution (Wright et al., 1993), but upon phosphorylation, it may dimerize and/or tetramerize and cooperatively bind to DNA, which may explain the 40-fold increase in P-VanR affinity for the *vanH* promoter over the *vanR* promoter. We are presently conducting experiments to test this hypothesis to determine whether the oligomerization of the phosphorylated VanR is essential to DNA binding.

In summary, we have shown that P-VanR is a site-specific DNA-binding protein at two sites within the five-gene *van* operon required for vancomycin resistance and that its affinity increases dramatically upon phosphorylation. We have also identified a possible consensus binding site of 12 bp within the DNA-binding regions. This work further defines the molecular events that lead to the clinically significant resistance of Gram-positive bacterial pathogens to vancomycin.

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