# Peptide Analogues of the VanS Catalytic Center Inhibit VanR Binding to Its Cognate Promoter†

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ABSTRACT: The dodecamer peptide SLCHDSVIGWEC, named E12, was selected from a combinatorial peptide library on the basis of its ability to bind to VanR, the two-component signal transduction response regulator which controls expression of vancomycin resistance in *Enterococcus faecium*. The binding of E12 was localized to the N-terminal, regulatory domain of VanR which contains Asp-55, the residue which accepts the phosphoryl group from His-164 in the activated VanS sensor kinase. E12, along with a related sequence SLAHDSIIGYLS, named E12.1, was found to inhibit the binding of VanR∼P to a DNA segment which corresponds to its cognate promoter P*vanH*. With a single gap, both E12 and E12.1 could be aligned with the octadecamer sequence YLAHDIKTPLTSIIGYLS, comprising Tyr-161 through Ser-178, of the catalytic center dimerization domain of VanS, a sequence with which VanR also normally interacts. Alanine substitution analysis of E12.1 identified six amino acids as indispensable for its ability to inhibit VanR∼P−P*vanH* DNA complex formation. A similar analysis of the corresponding amino acids in VanS showed a parallel dependence except for the substitutions Leu-162 → Ala and Gly-175 → Ala which interfered with the ability of E12.1 to compete with protein−DNA complex formation, but did not inhibit the ability of VanS to bind VanR. Our findings support a model in which E12 mimics the VanS phosphorylatable sequence with which the regulatory domain of VanR interacts, and thus functions as a "minimalist" analogue of VanS. Our results also indicate the usefulness of phage-displayed peptides as a general tool for mimicking the interacting faces of interacting proteins.

VanR and VanS comprise the respective transcriptional response regulator and sensor kinase of a two-component signal transduction (TCST)[1] system which regulates vancomycin resistance in *Enterococcus faecium* (*2*, *5*, *11*). Transcriptional activation of the *vanHAXYZ* gene cluster is the key event leading to the expression of resistance in induced cells. For general reviews of two-component signal transduction systems, see refs *22* and *25*. VanS, the sensor kinase which is responsive to the presence of vancomycin in the medium, undergoes a net increase in the level of phosphorylation of one of its residues, His-164, by ATP. VanS then transfers the phosphoryl group from His-164 of VanS to Asp-55 of VanR, the response regulator for vancomycin resistance, thereby increasing its affinity for P*vanH*, the promoter for transcription of the *vanHAXYZ* gene cluster. VanR∼P may bind cooperatively as a dimer to P*vanH* where, by analogy to OmpR (*12*), it recruits RNA polymerase to initiate transcription of the *vanHAXYZ* gene cluster.

Because of the importance in understanding the molecular basis of vancomycin resistance, it is of great interest to map the region of protein−protein interaction between VanS and VanR and to examine the consequences of their interaction. To learn what the minimal region for specific interaction might be, we utilized a phage-display combinatorial peptide library to select peptide ligands to VanR. In the study presented here, we identify several such peptides and characterize the interaction of one of them with VanR.

## MATERIALS AND METHODS

*Bacterial Strains, Plasmids, Phage, and PCR Primers.* Replicative materials and PCR primers that were used are summarized in Table 1.

*Plasmids and Cloning.* Glutathione *S*-transferase (GST) fusion proteins GST−VanR (complete VanR sequence), GST−VanR regulatory domain (Met-1−Ser-124), GST−DNA binding domain (Ser-123−TAA-231), GST−VanSc (Arg-93−TAA-283 stop), and GST−PhoB (complete PhoB sequence) were constructed in plasmid pGEX-2TK (Amersham Pharmacia Biotech, Piscataway, NJ) by a modification of procedures described by Wright et al. (*32*). Four PCR primers were used to prepare each gene cassette by the "melt-anneal" method described previously (*30*). For each set of four PCR primers, the respective amplimer pairs were denatured, annealed, and ligated. The resultant cassette, with *Eco*RI and *Bam*HI cohesive ends, was cloned using pGEX-2TK DNA, previously digested with *Eco*RI and *Bam*HI restriction endonucleases. The unfractionated reaction mix-

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[1] Abbreviations: GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; RD, regulatory domain; DBD, DNA-binding domain; TAE, Tris-acetate-EDTA; HPLC, high-performance and/or -pressure liquid chromatography; VanSc, VanS soluble cytoplasmic domain; TCST, two-component signal transduction; VanR, vancomycin resistance response regulator protein; VanR∼P, phosphorylated VanR; VanS, vancomycin resistance sensor kinase protein; VanS∼P, phosphorylated VanS; P*van*H, promoter upstream of *vanH*.

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Table 1: Plasmids, Bacterial Strains, and PCR Primers

| plasmid, bacterial strain, or PCR primer | description | ref or source |
|---|---|---|
| plasmid | | |
| pGEX2TK | a *tac* promotor inducible GST fusion expression vector | Pharmacia Biotech |
| pAU301 | pGEX2TK with VanRa cloned into *Bam*HI and *Eco*RI sites and N-terminally fused to GST | this work |
| pAU302 | pGEX2TK with the VanS cytoplasmic domain cloned into *Bam*HI and *Eco*RI sites and N-terminally fused to GST | this work |
| pAU303 | pGEX2TK with PhoB cloned into *Bam*HI and *Eco*RI sites and N-terminally fused to GST | this work |
| pAU304 | pGEX2TK with the VanR regulatory domain cloned into *Eco*RI and *Bam*HI sites and N-terminally fused to GST | this work |
| pAU305 | pGEX2TK with the VanR DNA binding domain cloned into *Eco*RI and *Bam*HI sites and N-terminally fused to GST | this work |
| strain | | |
| *E. coli* BL21(DE3) | transformable recipient strain used for expression of VanS and VanR−GST | Novagen |
| *E. coli* JM109 | transformable recipient strain for primary plasmid cloning | *21* |
| *E. coli* EAU301 | *E. coli* BL21(DE3) with pAU301 plasmid used for expression of VanR−GST | this work |
| *E. coli* EAU302 | *E. coli* BL21(DE3) with pAU302 plasmid used for expression of VanS−GST | this work |
| *E. coli* EAU303 | *E. coli* BL21(DE3) transformed with plasmid pAU303, used for expression of PhoB−GST | this work |
| *E. coli* EAU304 | *E. coli* BL21(DE3) transformed with plasmid pAU304, used for expression of VanR(RD)−GST | this work |
| *E. coli* EAU305 | *E. coli* BL21(DE3) transformed with plasmid pAU305, used for expression of VanR(DBD)−GST | this work |
| *E. faecium* A634 | VanA reference strain, original source of VanRa and VanSa DNA | *23* |
| primer | | |
| 15981 | VanR, forward 1 (5′ GAT CCA TGA GCG ATA AAA TAC TTA TTG) | this work |
| 15983 | VanR, forward 2 (5′ CAT GAG CGA TAA AAT ACT TAT TG) | this work |
| 15984 | VanR, reverse 1 (5′ AAT TCT TAT TTT TCA ATT TTA TAA CCA AC) | this work |
| 15982 | VanR, reverse 2 (5′ CTT ATT TTT CAA TTT TAT AAC CAA C) | this work |
| 20285 | VanSc, forward 1 (5′ GAT CCC GCG TCA TGC TTT CAA AAT TC) | this work |
| 20287 | VanSc, forward 2 (5′ CCG CGT CAT GCT TTC AAA ATT C) | this work |
| 20288 | VanSc, reverse 1 (5′ AAT TTT AGG ACC TCC TTT TAT CAA CCA AG) | this work |
| 20286 | VanSc, reverse 2 (5′ TTA GGA CCT CCT TTT ATC AAC CAA G) | this work |
| 26611 | PhoB, forward 1 (5′ GAT CCA TGG CGA GAC GTA TTC TGG TCG) | this work |
| 26612 | PhoB, forward 2 (5′ CAT GGC ACG TAT TCT GGT CG) | this work |
| 26613 | PhoB, reverse 1 (5′ AAT TTA AAA GCG GGT TGA AAA ACG ATA TC) | this work |
| 26614 | PhoB, reverse 2 (5′ TAA AAG CGG GTT GAA AAA CGA TAT C) | this work |
| 20289 | VanH promotor, forward (5′ CGC GGA TCC GCG GGG ATG CCA ATG GT) | this work |
| 20290 | VanH promotor, reverse (5′ CGG AAT TCC GGA AAG CAA TGA TAA CTA T) | this work |
| 25949 | VanR(DBD), forward 1 (5′ GAT CCG TTA TCG TCC ACT CCG GC) | this work |
| 25950 | VanR(DBD), forward 2 (5′ CGT TAT CGT CCA CTC CGG C) | this work |
| 25951 | VanR(RD), reverse 1 (5′ AAT TCT TAA TTT TCG TTC TGC TCC TTT AC) | this work |
| 25952 | VanR(RD), reverse 2 (5′ CTT AAT TTT CGT TCT GCT CCT TTA C) | this work |
| 26490 | 18-mer, SLAHDIKTPLTSIIGYLS *Xho*I−*Xba*I cassette, sense strand (5′ TCG AGC TTG GCG CAC GAT ATT AAA ACG CCC CTT ACA TCC ATT ATC GGT TAT TTG AGC T) | this work |
| 26491 | 18-mer, SLAHDIKTPLTSIIGYLS *Xho*I−*Xba*I cassette, antisense strand (5′ CTA GAG CTC AAA TAA CCG ATA ATG GAT GTA AGG GGC GTT TTA ATA TCG TGC GCC AAG C) | this work |

ture of ligation products was used to transform *Escherichia coli* JM109 cells by electroporation, followed by selection for ampicillin resistance. Cells carrying plasmids with inserts were screened by colony PCR. Plasmids specifying the desired fusion constructs were introduced into *E. coli* BL21 $\lambda$ DE3 cells for protein overexpression.

*Overexpression and Purification of GST Fusion Proteins.* The respective GST fusion proteins were purified on a 1 L scale according to instructions provided by the supplier. The resultant GST−VanR and GST−PhoB proteins were cleaved on the column of glutathione−agarose by incubation with thrombin (2 units) for 4 h at room temperature, and concentrated by centrifugal ultrafiltration (Centricon 10 concentrator, Amicon, Bedford, MA). The resultant VanR preparation, free of GST, was stored in GST elution buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% glycerol] at −80 °C. VanSc−GST was eluted from the column in the same buffer supplemented with 10 mM reduced glutathione, concentrated, stored similarly, and used as the intact GST fusion protein.

*Amplification and 5′-End-Labeling of P*$v$*anH DNA.* A 254 bp DNA fragment containing the P$v$*anH* promotor region (residues −191 to 63) was obtained by PCR as described by Holman et al. (*14*) using a boiled cell preparation of *En. faecium* A634 as the template source. Amplified P$v$*anH* promotor DNA was 5′-end-labeled with [$\gamma$-$^{32}$P]ATP (specific activity of 200 Ci/mol) and phage T4 polynucleotide kinase. The reaction mixture was incubated for 30 min at 37 °C, and unincorporated ATP was removed by centrifugal gel filtration (Sephadex G-50 spin column, Amersham Pharmacia Biotech).

*Phosphorylation of VanR with Acetyl Phosphate.* VanR was phosphorylated as described previously (*14*). The reaction was performed in a total volume of 100 $\mu$L which contained 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 50 mM lithium acetyl phosphate, and 9 $\mu$M VanR. After incubation for 1 h at 37 °C, the reaction mixture was used directly as a source of VanR∼P, or stored at −80 °C and used within 1 month.

*Gel Shift Analysis of VanR∼P Binding to P*$v$*anH DNA.* VanR−P$v$*anH* complexes were quantified by gel shift analysis as described by Holman et al. (*14*). Complexes were formed in a total volume of 15 $\mu$L which contained 4 pmol of VanR∼P, which was preincubated with the appropriate

effector peptides (as aqueous solutions) for 10 min at room temperature in 20 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 0.1 mM $Na_2EDTA$, 0.5 mM $CaCl_2$, 10% glycerol, and 0.5 $\mu$g of salmon sperm DNA. To start the reaction, $^{32}$P-labeled P*vanH* promotor probe DNA (0.65 ng in 1 $\mu$L) was added, and the reaction mixture was incubated on ice for 15 min. The resultant VanR∼P−P*vanH* complexes were analyzed by nondenaturing PAGE (*28*). Gels were cast in TAE buffer, and electrophoresis was performed in the same buffer at 155 V for 45 min. Gels were then dried on Whatman 3MM filter paper, and exposed to a phosphorimager screen overnight for analysis of DNA−protein complexes.

*Phosphotransferase Assay.* VanSc−GST (4 pmol) was labeled for 60 min at room temperature in a 5 $\mu$L volume containing, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 100 mM KCl. The reaction was initiated by addition of 1 $\mu$L of 200 $\mu$M [$\gamma$-$^{32}$P]ATP which contained 1 $\mu$Ci. After 60 min, the 5 $\mu$L reaction mixture was added to a 15 $\mu$L reaction mixture containing 80 pmol of PhoB and 2.5 mM PMSF all in the same buffer, with the appropriate peptide at a final concentration of 1 mM. Reactions were stopped by the addition of 10 $\mu$L of SDS gel loading buffer, and the resultant mixture was fractionated by SDS−PAGE. Phosphoproteins were assessed autoradiographically with a phosphorimager, as previously described.

*Phage Display.* Phage display was performed using the methods described by Sparks et al. (*29*). Microtiter plate wells were coated with purified VanR and used to select binding phage from a library displaying combinatorial $X_{10}C$ peptides, i.e., 10 random NNK codons followed by a Cys codon, TGC, in the eleventh position (N is A, G, C, or T, and K is G or T). After three rounds of selection, the binding of individual phage clones to the VanR target protein was assessed by phage-ELISA using an anti-M13 phage monoclonal antibody−horseradish peroxidase conjugate (Pharmacia). Ninety-six phage plaques were picked from the third round of selection and tested for their ability to bind to VanR and to BSA, by phage ELISA, to determine levels of specific and background binding, respectively.

For inserting determinants for desired peptide sequences into phage M13 DNA, oligonucleotides encoding the defined peptide sequences were cloned into the *Xho*I and *Xba*I sites within gene III of bacteriophage M13. The resulting recombinant phage were then tested for binding to various protein targets, immobilized within microtiter plate wells, by phage ELISA (*29*).

*Sequence Analysis of Combinatorial Peptides.* Of the 96 chosen phage plaques, 25 isolates with a relative selectivity for VanR relative to BSA greater than a factor of 4 (by phage ELISA) were selected for DNA sequence analysis. The deduced peptide sequences were further analyzed by alignment with the amino acid sequence of VanS using the program MultAlin (*6*).

*Chemical Synthesis of Combinatorial Peptides.* Peptides were chemically synthesized by Chiron Technologies (Raleigh, NC) and Research Genetics (Huntsville, AL). They were purified by the supplier using HPLC, and further analyzed by mass spectrometry.

*Construction of Alanine Substitution Mutants of VanSc.* Alanine substitutions were incorporated into the VanS soluble intracellular domain construct, GST−VanSc, by overlap-extension PCR (*15*). Two paired sets of primers were used for each mutant construction. The first pair consisted of a 5′-forward primer encoding the N-terminus of the VanSc construct and a counterpart reverse primer, which encoded the alanine-substituted E12 peptide homologue. The second pair consisted of a 5′-forward primer encoding the complementary alanine-substituted E12 peptide homologue and a counterpart reverse primer, which encoded the C-terminus of VanSc. The two resultant amplimers were gel-purified, denatured, annealed, and extended with *Pfu* DNA polymerase. The full-length construct was then amplified using the forward and reverse VanSc primers. The resultant amplimer was cloned in plasmid pGEX-2TK for high-level expression in *E. coli*. The resultant mutant construct was overexpressed as a GST fusion protein and purified for binding experiments.

Table 2: Peptide Sequences Obtained by Phage Display[a]

| Phage number | VanR/BSA | Peptide Sequence |
|---|---|---|
| A04 | 1.08 | RGEMCMAGEGAC |
| E06 | 12.94 | SAKSCNPLKKHC |
| A09 | 5.39 | SARVEICGWWEC |
| D05 | 4.93 | SARVEICGWWEC |
| F08 | 4.70 | SARVEICGWWEC |
| D09 | 8.02 | SDSGWEGCWGWC |
| A10 | 9.13 | SEFSAWASDPGCSRAKMKRSRPTRPPRVAKEVSELSVC |
| A08 | 4.81 | SEHDLATKATMCSRANKDTTNQKSTSRGRAGRQRQAQC |
| E01 | 1.26 | SFLSCSKFDRLC |
| B08 | 10.28 | SLCHDSVIGWEC |
| E09 | 13.15 | SLCHDSVIGWEC |
| E12 | 11.44 | SLCHDSVIGWEC |
| F10 | 12.13 | SLCHDSVIGWEC |
| G08 | 11.09 | SLCHDSVIGWEC |
| H11 | 11.28 | SLCHDSVIGWEC |
| C05 | 1.64 | SPQTANVCGGLC |
| C07 | 10.85 | SRIKVICSGESC |
| F07 | 5.53 | SRPCSGFGPGDC |
| H01 | 1.34 | SRVGHYMVCPDC |
| A11 | 5.94 | SSPMWLQGAVTY |
| G01 | 6.16 | SVFFSRCSGDGC |
| E04 | 4.14 | SVWGSCPDAWWC |
| C06 | 1.11 | SWIMSAAWDRF |
| H10 | 5.02 | SWRPMCEGLGTC |
| F03 | 7.29 | SYGGSLYFCKNC |

[a] Summary of phage display peptide sequences obtained in this study. The $X_{10}C$ library was screened for phage which bound to VanR-coated wells, as described. Individual plaques were amplified by growth on a bacterial cell host. For each plaque, phage binding to VanR- and to BSA-coated wells was assessed by phage ELISA. VanR/BSA, i.e., a measure of specific binding to nonspecific binding, was calculated for each peptide. The DNA sequences and deduced amino acid sequences were determined for the 25 peptides which bound with the highest affinity. One peptide with the sequence SLCHDSVIGWEC occurred six of 25 times and was used for further studies. Four phages with the lowest affinity (VanR/BSA < 1.5) were included for use as negative controls.

## RESULTS

*Screening for Peptides Which Bind to VanR.* Microtiter plate wells, coated with purified VanR, were used to select peptides from a phage library displaying combinatorial peptides on capsid protein III. After three rounds of screening and amplification of the library, 96 phage plaques were chosen for further characterization by phage ELISA. Of these, 20 phage exhibited ≥4-fold more binding to VanR than to BSA, i.e., an unrelated target. These phage were selected for DNA sequencing and determination of the amino acid sequences of their respective inserts (Table 2). One of the peptide sequences shown, E12, SLCHDSVIGWEC,

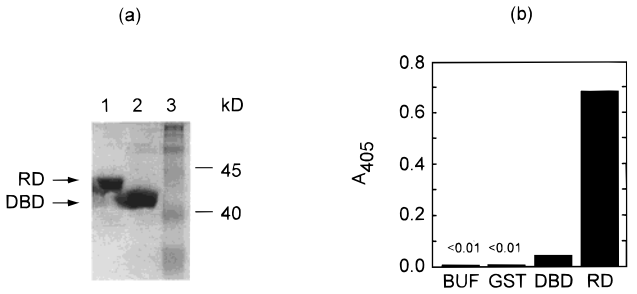


FIGURE 1: Binding of phages bearing the E12 peptide to the separated regulatory and DNA-binding domains of VanR. (a) Coomassie blue-stained analytical PAGE separation of GST fused to the VanR regulatory domain (lane 1) or VanR DNA-binding domain (lane 2). Protein standards, labeled 40 and 45 kD, are shown in lane 3. (b) Phage ELISA using microtiter plate wells coated with GST fused to the VanR regulatory and DNA-binding domain. Wells were tested for their ability to bind M13 phage bearing peptide E12. The wells were coated using buffer (BUF), GST (GST), GST−DNA binding domain fusion (DBD), or GST−regulatory domain (RD). The phage binding activity readout at 405 nM was based on the colorimetric measurement of peroxidase activity and was proportional to the level of binding of the peroxidase phage-specific monoclonal antibody congugate. The predominant binding activity was seen to the RD well. The level of binding to the DBD well was less than 10% of that seen for the RD sample, while negligible binding to uncoated or to GST-coated wells was seen.

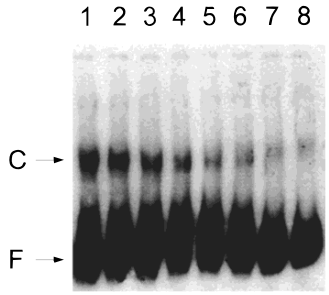


FIGURE 2: Peptide E12 concentration dependence for inhibition of VanR−P*vanH* DNA complex formation. The binding of VanR to P*vanH* DNA reduces the electrophoretic mobility of the DNA. Addition of peptide E12 reverses the mobility shift with an $IC_{50}$ of 3 $\mu$M. Lanes 1−8 contained 0, 1, 2, 5, 10, 20, 50, and 100 $\mu$M E12, respectively. "C" indicates the VanR−P*vanH* DNA complex that formed; "F" indicates uncomplexed $^{32}$P-labeled P*vanH* DNA.

occurred 6 out of 25 times and bound reproducibly to the cloned regulatory domain of VanR preferentially, as shown in Figure 1, and was selected for further study.

*Effect of Peptide E12 on VanR−PvanH Complex Formation.* Peptide E12 was chemically synthesized in the disulfide form and tested for its ability to inhibit the interaction between a $^{32}$P-labeled DNA segment of the P*vanH* promoter and VanR. Results shown in Figure 2 indicate that E12 inhibited the interaction between VanR and P*vanH* with an $IC_{50}$ value of 3 $\mu$M. As control, the peptide Ac-LSGVIAT-OMe was tested and found to be inactive in the promoter fragment binding assay (data not shown).

*Similarity between E12 and the Catalytic Center of VanS.* When we used the program MultAlin (*6*) to compare E12 with VanS, we found a match with the 18-amino acid sequence, residues Tyr-161−Ser-178, by introducing a six-amino acid gap in E12 (Figure 3). This match suggested that E12 was acting as an analogue of the VanS sequence Tyr-161−Ser-178. E12 was therefore used as a lead peptide to design and test whether more precise peptide mimics of the VanS catalytic center might be more effective at inhibiting

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SLCHD......SVIGWEC     E12 gapped
YLAHDIKTPLTSIIGYLS     VanS Tyr-161 to Ser-178
SLAHD......SIIGYLS     E12.1 gapped
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FIGURE 3: Amino acid sequences of E12 and E12.1, compared with VanS. The amino acid sequences of peptides E12 and E12.1 are compared at Tyr-161−Ser-178 of the VanS catalytic center. It is necessary to introduce a six-amino acid gap into E12 and E12.1 corresponding to IKTPLT to align them with VanS.

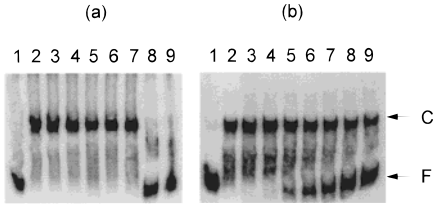


FIGURE 4: Inhibition of VanR∼P−PvanH DNA complex formation and comparison of the effect of peptides E12.1 and E12. (a) The comparative effects of peptides E12.1 and E12 on VanR∼P−[$^{32}$P]-P*vanH* DNA complex formation are shown as a function of added peptide E12.1: lane 1, $^{32}$P-labeled P*vanH* DNA alone, control; lane 2, Complete, without the competing peptide; lanes 3−9, Complete with 1, 3, 10, 30, 100, 300, and 1000 $\mu$M E12, respectively. (b) The comparative effects are shown as a function of added peptide E12: lane 1, $^{32}$P-labeled P*vanH* DNA alone, control; lane 2, Complete, without the competing peptide; lanes 3−9, Complete with 10, 20, 50, 100, 200, 500, and 1000 $\mu$M E12, respectively.

the binding of VanR to P*vanH*. A modified peptide, E12.1, was designed, based on E12, which more closely resembled the sequence of VanS. Peptide E12.1 incorporated the pentapeptide sequence SLAHD and the heptapeptide sequence SIIGYLS of VanS, but not the central six residues, IKTPLT, as shown. In addition, the two Cys residues of E12 were replaced with Ala and Ser, simplifying the synthesis of Ala-substituted variant peptides used in later experiments. The Ser residue which corresponds to Tyr-161 was derived from the sequence of M13.

In testing E12.1, we compared its activity with that of E12 under conditions which utilize the physiologically active form of VanR, VanR∼P, as well. In comparing E12 binding to VanR (Figure 1) with binding to VanR∼P, we found a >100-fold difference, i.e., an $IC_{50}$ of 3 $\mu$M, as shown in Figure 2, versus the value of >1 mM shown in Figure 4. E12.1 was tested for its ability to inhibit VanR∼P−[$^{32}$P]P*vanH* complex formation as the target. The results shown in Figure 4 indicate that the $IC_{50}$ for peptide E12.1 was 100 $\mu$M, a >10-fold increase over that of E12 for inhibiting VanR∼P binding to [$^{32}$P]P*vanH* (*9*, *10*), suggesting that the closer similarity between E12.1 and the catalytic center of VanS might be responsible for the tighter binding.

*Alanine Substitution Analysis of Peptide E12.1 with Respect to VanR∼P−PvanH Complex Formation.* We next tested the hypothesis that the inhibitory activity of E12.1 toward VanR∼P−P*vanH* complex formation may be due to its resemblance to VanS by evaluating the contribution of the residues within E12.1 toward inhibiting the interaction between VanR∼P and P*vanH* DNA. A set of 12 individual Ala-substituted variants of E12.1 were chemically synthesized and tested. The results shown in Figure 5, and listed in Table 3, indicate that of the 12 alanine-substituted peptides, six inhibited the VanR∼P-induced mobility shift of the P*vanH* DNA probe, while five had no effect. The test of E12.1 with Ala substituted for Asp-165 (lane 7) was left

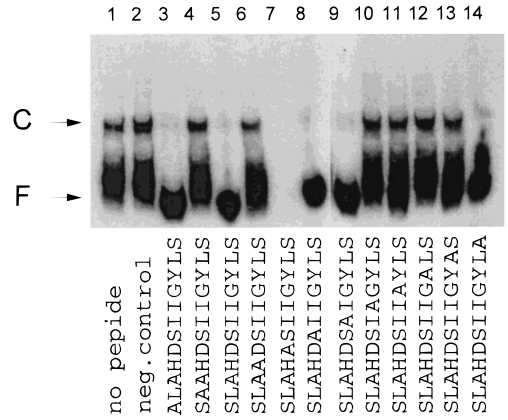


FIGURE 5: Alanine substitution analysis of peptide E12.1 and the effect on VanR−P*vanH* DNA complex formation. A set of 12 peptides was chemically synthesized in which Ala was systemtically substituted at each position of peptide E12.1. Peptides were each tested for their ability to reverse the mobility shift of P*vanH* DNA induced by VanR∼P. Results are collated in Table 3 for comparison with effects of Ala substitution on other activities that are tested: lane 1, peptide dilution buffer control; lane 2, unrelated peptide control; lane 3, Ser-161 → Ala; lane 4, Leu-162 → Ala; lane 5, Ala-163 (unsubstituted); lane 6, His-164 → Ala; lane 7, Asp-165 → Ala; lane 8, Ser-172 → Ala; lane 9, Ile-173 → Ala; lane 10, Ile-174 → Ala; lane 11, Gly-175 → Ala; lane 12, Tyr-176 → Ala; lane 13, Leu-177 → Ala; lane 14, Ser-178 → Ala.

Table 3: Activity of Alanine-substituted Peptides[a]

| | | CAN PEPTIDE DISRUPT COMPLEX? (Fig. 5) | CAN VANSc DISRUPT COMPLEX? (Fig. 7) |
|---|---|---|---|
| VanS | YLAHDIKTPLTSIIGYLS | | |
| E12.1 | SLAHD.....SIIGYLS | | |
| Tyr-161 | A----.....------- | YES | nd |
| Leu-162 | -A---.....------- | NO | YES |
| Ala-163 | --A--.....------- | YES | YES |
| His-164 | ---A-.....------- | NO | nd |
| Asp-165 | ----A.....------- | nd | nd |
| Ser-172 | -----.....A------ | YES | YES |
| Ile-173 | -----.....-A----- | YES | nd |
| Ile-174 | -----.....--A---- | NO | NO |
| Gly-175 | -----.....---A--- | NO | YES |
| Tyr-176 | -----.....----A-- | NO | nd |
| Leu-177 | -----.....-----A- | NO | nd |
| Ser-178 | -----.....------A | YES | YES |

[a] Effect of alanine substitution on the activity of peptide E12 and related VanS constructs. The 18-mer VanS sequence, Tyr-161−Ser-178, is included for reference together with the sequence of peptide E12.1. The location of the gap is indicated by a six-character-long series of periods. The figure number which is summarized in each of two columns is listed.

blank because the substituted peptide was insoluble in the buffer used for the assay. These observations indicate that for five of 11 substituted peptides (lanes 3, 5, 8, 9, and 14) that were tested, the amino acid could be replaced with Ala without a loss of activity, suggesting that these amino acids were not critical for inhibitory activity and, more importantly, that the other six substitutions (lanes 4, 6, and 10−13) were important for activity.

*Inhibition of VanR∼P−PvanH Complex Formation by VanSc and Its Amino Acid-Substituted Variants.* VanSc, the 189-amino acid soluble cytoplasmic domain of VanS (Arg-93−TAA-283 stop), has been reported to inhibit the binding of VanR∼P to P*vanH* (*9*, *10*). This observation raises the following question. To what extent do the amino acid

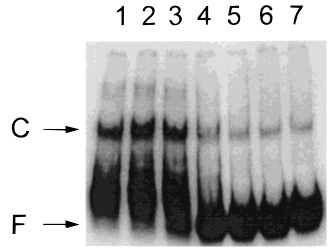


FIGURE 6: Effect of VanSc on VanR∼P−P*vanH* DNA complex formation and concentration dependence. VanSc−GST was tested as a function of concentration for the ability to reverse the binding reaction between VanR∼P and P*vanH*. The $IC_{50}$ for the reaction was 200 nM. The GST−VanSc construct at a concentration of 1 $\mu$M was used in the succeeding experiment to saturate VanR with VanSc, enabling an apparent all-or-none assay: lanes 1−7, 0, 0.063, 0.125, 0.250, 0.50, 1.0, and 2.0 $\mu$M VanSc added, respectively. "C" indicates the VanR−*PvanH* DNA complex that formed; "F" indicates uncomplexed $^{32}$P-labeled P*vanH* DNA.

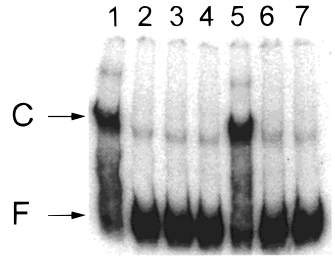


FIGURE 7: Alanine substitution analysis of VanSc and the effect on VanR∼P−P*vanH* DNA complex formation. Ala substitutions were made genetically in the cloned gene for VanSc which was tested for the ability of the construct (as the corresponding GST fusion) to inhibit VanR∼P−P*vanH* DNA complex formation. Five substitutions were selected as a signature of the effect, for comparison with the in vitro mobility shift data. Ala substitutions Leu-162 → Ala and Gly-175 → Ala resulted in a loss of peptide activity; activity was retained by GST−VanSc which was substituted at the other positions. The results are listed in Table 3 for comparison with effects of Ala substitution on other activites that were tested: lane 1, no VanSc added; lane 2, wild-type VanSc; lane 3, VanSc Leu-162 → Ala; lane 4, VanSc Ser-172 → Ala; lane 5, VanSc Ile-174 → Ala; lane 6, VanSc Gly-175 → Ala; lane 7, VanSc Ser-178 → Ala.

sequences of E12.1 and VanSc contribute equally to their ability to inhibit VanR∼P−P*vanH* complex formation? A set of six GST−VanSc Ala-subtituted variants (which included the "wild-type" VanSc) were therefore constructed. The variants incorporated the same amino acid changes present in the corresponding Ala-substituted E12.1 peptide set shown in Figure 5. The concentration dependence for wild-type VanSc was first determined. The results shown in Figure 6 indicate that GST−VanSc inhibited VanR binding to P*vanH* with an $IC_{50}$ value of 200 nM. A higher concentration of VanSc (1 $\mu$M) was therefore used in testing the inhibition of VanR∼P−P*vanH* complex formation by the Ala-substituted variants of VanSc because it made the experimental determinantion of activity into an all-or-none assay. The set of five parallel Ala-substituted analogues of VanSc was next tested to determine the extent to which they share the same amino acid specificity with the phosphorylatable center of VanSc in their ability to inhibit VanR∼P−P*vanH* complex formation. The results shown in Figure 7 and summarized in Table 3 indicate no loss of activity, i.e., of the ability of the alanine-substituted VanSc to inhibit VanR∼P−P*vanH* complex formation for five of six constructs tested, including the wild type. There were two

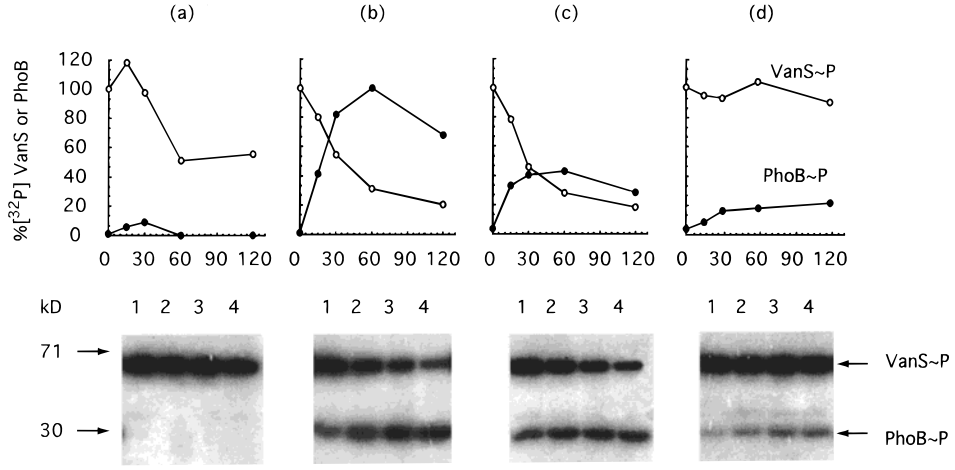


FIGURE 8: Inhibition of phosphoryl transfer from VanS∼P to PhoB by peptide E12.1. Four picomoles of VanSc−GST was labeled in the presence of [$\gamma$-$^{32}$P]ATP for 60 min at room temperature and then added to 80 pmol of PhoB. Data were taken at 15, 30, 60, and 120 min, shown in lanes 1−4, respectively. Reaction conditions were as follows. In panel a there was no PhoB and no peptide added, in panel b PhoB but no peptide added, in panel c PhoB plus 1 mM E12.1 I174A added, and panel d PhoB plus 1 mM E12.1 added.

exceptions noted when comparing the inhibitory action of E12.1 with that of VanSc. The amino acid substitutions Leu162 → Ala and Gly175 → Ala were active for inhibition of VanR∼P−P*vanH* complex formation in the E12.1 background, but were not active when present in the longer VanSc construct. This suggests additional complexity in the interaction between VanSc and VanR∼P.

*Phage Display of Lead Peptides.* We previously attempted to test the ability of a chemically synthesized peptide corresponding to residues Tyr-161−Ser-178 but were unable to do so because of its low solubility. We therefore synthesized a DNA cassette which specificed the VanS 18-mer and inserted it into a vector for reverse-engineered phage surface display. Because of the amino acid sequence of the vector adjacent to the insert, Tyr-161 was replaced with Ser.

The resultant phage construct was tested along with E12 by phage ELISA for the ability to bind to GST−VanR-coated microtiter plate wells versus GST alone. The amounts bound, after normalization for background binding to GST, were similar for both constructs (data not shown). Thus, we suspect that the E12 peptide mimics a region of VanS that binds to VanR.

*Specificity of Chemically Synthesized E12.1 as an Inhibitor of Phosphoryl Transfer from P∼VanS to PhoB.* Our previous studies showed that CpdA could perturb VanR function and inhibit phosphoryl transfer. We now demonstrate that peptide E12.1 can do the same. Since the affinity of VanS for VanR is in the nanomolar range (*10*), we used a weakened but nevertheless specific interaction, namely, between VanS and PhoB. VanS∼P was incubated with PhoB, and phosphoryl transfer was assessed. The results shown in Figure 8 indicate that 1 mM E12.1 inhibited phosphoryl transfer (panel d), but that the I174A Ala substitution variant did not (panel c). For comparison, see lane 10 of Figure 5 and lane 5 of Figure 7, where the I174A variant was shown to be inactive in inhibiting the gel shift reaction (ability of VanR to bind to PvanH DNA) in both the peptide and full-length VanSc forms, respectively. This further supports the interpretation that a peptide as small as E12.1 can specifically compete with and, therefore, mimic VanS. This also demonstrates that I174 could play an important role in VanR−VanS binding in vitro.

## DISCUSSION

*Peptide Ligands.* Through the use of phage display, we discovered several different peptides which selectively bound to immobilized VanR. The amino acid sequence of one peptide in particular, E12, occurred most often in the screen, and with a consistently high ratio for selectively binding to *VanR*; it appeared to mimic VanS, both functionally and structurally. E12 contains the dipeptide sequence His-Asp in a context which suggests that it is an analogue of the dipeptide sequence His-164-Asp-165 at the site of VanS autophosphorylation. Interestingly, the dipeptide in the form of either His-Asp or His-Glu appears to be a structural feature that is common to nearly all two-component kinases. Other peptides that were obtained in this study and shown in Table 2 were listed to give an idea of the distribution of structures that one obtains by the use of phage display. They were not tested further in this study.

Fisher et al. (*9*) prepared a C-terminal truncation library of VanS and observed that a 79-mer construct ["VanS(M95−I174)"] could inhibit the phosphorylation of the response regulator PhoB by VanSc ["VanS(M95−Ser384)"]. In the studies presented here, we tested this reaction and additionally used a different assay, VanR∼P−P*vanH* complex formation, and were able to show activity of a minimalist 12-amino acid peptide, which contains the putative phosphorylatable His-164.

A modified sequence, E12.1, based on E12 which more precisely matched the amino acid sequence of VanS was found to inhibit P∼VanR−P*vanH* complex formation more effectively than E12. Apparently, the conformational constraint on E12 imposed by the disulfide bridge formed between the two Cys residues does not contribute to the peptide's binding to VanS. Rather, the presence of a pair of cysteines in the peptide sequence is more likely due to selective pressure for the phage to display even numbers of cysteines in the peptide (*18*).

The impetus for these studies was to use phage display to generate the necessary combinatorial complexity that will ensure us a specific ligand for our target; however, we do not expect a perfect match between E12 and VanR because the library sample which contains about $10^8$ phage could not

possibly cover the theoretical $20^{10}$ sequences that would be represented by a complete $X_{10}C$ library. Our results suggest that the match may not only include a discontinuity in the sequence but also need not be perfect as long as certain critical amino acid residues identified in this study are present.

*Sequence Requirements for Inhibition of VanR−P*v*anH Complex Formation.* In analysis of our peptides, we observed that peptides E12 (and E12.1) inhibit the ability of VanR to bind to a DNA fragment of the P*vanH* promoter. In ELISA experiments, in which we tested the binding of phage displaying the E12 sequence to N- and C-terminal halves of the *VanR* protein, we observed preferential binding to the N-terminal segment (Figure 1). Thus, the ability of the E12 peptide to inhibit the binding of the C-terminal half of *VanR* to DNA is likely due to allosteric changes in the C-terminal domain caused by the peptide binding to the N-terminal half. A precedent for this form of allosteric regulation can be found in the case of the mammalian transcription factor elk-1 in which phosphorylation of the C-terminal transcriptional activation domain by MAP kinases stimulates DNA binding by the N-terminal domain with resultant transcriptional activation (*34*).

Conversely, the E12 peptide ligand could act to inhibit dimerization of the phosphorylated form of VanR after binding to VanS. In the EnvZ−OmpR two-component signal transduction system, OmpR∼P has been reported to bind to its promoter as a dimer whose formation is facilitated by its phosphorylation (*1*), and vice versa (*12*). Thus, peptide ligands could act indirectly on the interaction between VanR and P*vanH* DNA by inhibiting the dimerization of the phosphorylated form of VanR.

*E12.1 Inhibits both Response Regulator Binding and Phosphoryl Transferase Activity.* Previous studies (*9*, *10*), as well as results described above, have shown that VanSc can inhibit the VanR−P*vanH* interaction, presumably by interacting with VanR∼P, with a $K_d$ of 30 nM, much in agreement with our measurement ($IC_{50}$ = 200 nM). It was also noted that a dodecamer peptide which resembles a part of VanS competes in the same reaction, suggesting that a part of the specificity of the reported interaction between VanR and VanS resides in a short subsequence of VanS (*9*, *10*).

The results obtained with two of the five mutants in the VanSc protein constructs (Figure 7) did not correlate with results obtained with the parallel peptide construct, possibly because they are not involved with inhibition of complex formation. We believe that since the VanS protein probably interacts with VanR at more places than the peptide, these two amino acids could be less critical for binding in the whole protein than in the peptide, which lacks the additional interactive amino acids. The difference in the $IC_{50}$ between the protein and the peptide for causing dissociation of VanR∼P and P*vanH* could be explained by this hypothesis. To test for inhibition of VanSc phosphotransferase activity, knowing the large difference in peptide and protien binding, we used PhoB, another response regulator.

It was shown previously that VanSc can phosphorylate PhoB, albeit at a reduced rate (*10*). Beacuse of the high affinity of kinases for their cognate response regulators, we used this noncognate cross-talk interaction to test for specific inhibition of phosphotransferase activity of VanSc. The E12.1 at 1 mM inhibited the phosphotransferase activity of VanSc toward PhoB, whereas the Ala-substituted peptide, E12.1 I174A, did not (Figure 8). Our findings support the hypothesis that E12.1 acts as a surrogate of the kinase sequence of VanS and in so doing inhibits both the DNA binding activity of VanR and the phosphorylation activity of VanSc toward a response regulator.

It would be of interest to test whether a comparable 18-mer peptide based on other kinases may serve as a model for the construction of phages that will bind to their respective cognate response regulators, possibly across a wider range of cognate kinase−response regulator combinations. Such constructs could be made directly by inserting a chemically synthesized DNA determinant casette into a phage display vector (*29*), or into a plasmid for display on *E. coli* alkaline phosphatase (*33*). The use of such constructs in a ligand displacement assay for drug-lead discovery would be much simpler than scanning gel shifts as described above. Indeed, a recent report (*17*) describes progress in drug-lead discovery using this approach.

*Inhibitors of Bacterial Signal Transduction.* The selective binding of combinatorial peptide ligands to components of bacterial signal transduction systems in vitro suggests directions for the discovery of ligands which might be similarly active in vivo. Since TCST systems have been found in bacteria, plants, and lower eukaryotes (yeasts and fungi), but none have yet been found in animal cell systems, bacterial signal transduction might provide useful targets at which to aim for the discovery of new classes of anti-infective agents. Indeed, one such agent, glycerol monolaurate, has been described (*27*), presumably acting through the bacterial cell membrane to inhibit the activation of vancomycin resistance by *vanRS*, although the precise molecular mechanism has not been proven experimentally.

Several classes of kinase inhibitors that are active in vitro have been described (*3*, *4*, *7*, *8*, *13*, *20*); however, complete inhibition of any one kinase may be circumvented by cross-talk with noncognate kinases. Moreover, Hilliard et al. (*13*) have also noted that some agents reported to inhibit His kinases also inhibited either membrane integrity, uridine, or amino acid incorporation, at comparable concentrations, and therefore were not as selective as was originally thought.

Gene inactivation studies suggest that the response regulator might be a more suitable target for antimicrobial agents. The example of VncR−VncS is instructive. Novack et al. (*24*) demonstrated mutational inactivation of VncS as the basis of vancomycin tolerance in clinical isolates of *Streptococcus pneumoniae*. In this case, genetic ablation of kinase function actually provoked cells to become tolerant of vancomycin. This observation implied the formation of VncR∼P in these cells, presumably due to cross-talk with other noncognate kinases (*32*). The inactivation of *S. pneumoniae* VncR by site-specific recombination-mediated gene disruption is lethal, presumably by reducing the level of synthesis of an antagonist of an endolytic enzyme system (*24*). In related studies, Lange et al. (*19*) reported that viable transformants could not be obtained for two out of 13 *S. pneumoniae* response regulators which they attempted, similarly, to inactivate. Finally, our earlier characterization of VanR as the selective target of CpdA action (*31*) suggests that selective inhibitors of response regulators await synthesis and or discovery.

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