Regulated interactions between partner and non-partner sensors and response regulators that control glycopeptide resistance gene expression in enterococci

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Transcription of the *vanA* and *vanB* glycopeptide resistance gene clusters is regulated by the VanRS and VanR_BS_B two-component regulatory systems, respectively. Histidine to glutamine substitutions were introduced at positions 164 of VanS and 233 of VanS_B to prevent autophosphorylation of the sensor kinases and transfer of the phosphate groups to the VanR and VanR_B response regulators. VanSH₁₆₄Q and VanS_BH₂₃₃Q abolished activation of VanR and VanR_B by host kinases. The phosphatase activity of VanS_BH₂₃₃Q was negatively modulated by vancomycin whereas VanSH₁₆₄Q prevented transcription of the resistance genes under all growth conditions. Cross-talk was detected between VanR_B and VanS in a *vanS_B* null mutant. VanR is required for activation of promoters *P_R* and *P_H* allowing transcription of the regulatory (*vanRS*) and resistance (*vanHAXYZ*) genes, respectively. Under non-inducing conditions, activation of VanR by cross-talk was blocked by the presence of a multicopy plasmid carrying *P_H*. Presence of the high-affinity VanR-binding sites of the regulatory region of *P_H* on the multicopy vector probably sequestered VanR, thereby preventing autoactivation of the *P_R* promoter. Under such circumstances, stimulation of the host kinase by glycopeptides or moenomycin was required for expression of the resistance genes.

Keywords: glycopeptide resistance, peptidoglycan, two-component regulatory system, cross-talk, enterococci

INTRODUCTION

Acquired resistance to the glycopeptide antibiotics vancomycin and teicoplanin in enterococci is due to production of peptidoglycan precursors ending in the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) instead of the dipeptide D-Ala-D-Ala (Arthur *et al.*, 1996b; Walsh *et al.*, 1996). Resistance by this mechanism is mediated by two related gene clusters, *vanA* and *vanB*, that encode a dehydrogenase and a ligase for synthesis of D-Ala-D-Lac and two DD-peptidases for elimination of peptidoglycan precursors ending in D-Ala (Fig. 1) (Bugg *et al.*, 1991; Arthur *et al.*, 1998). Transcription of the *vanA* and *vanB* clusters is regulated by the VanRS and VanR_BS_B two-component regulatory systems (Arthur *et al.*, 1992; Evers & Courvalin, 1996). Phosphorylation of

the VanR response regulator enhances the affinity of the protein for the regulatory regions of the *P_R* and *P_H* promoters and allows transcription of the regulatory (*vanRS*) and resistance (*vanHAXYZ*) genes, respectively (Holman *et al.*, 1994; Arthur *et al.*, 1997). Similarly, VanR_B is required for activation of the *P_{RB}* and *P_{YB}* promoters located upstream from the regulatory and resistance genes of the *vanB* cluster (Evers & Courvalin, 1996; Silva *et al.*, 1998). The level of phosphorylation of VanR and VanR_B is modulated by the kinase and phosphatase activities of the VanS and VanS_B sensors, respectively (Wright *et al.*, 1993; Arthur *et al.*, 1997; Baptista *et al.*, 1997). The response regulators can also be activated by kinases encoded by the host chromosome in the absence of their partner sensors (Arthur *et al.*, 1997; Silva *et al.*, 1998; Baptista *et al.*, 1999).

Activation of the *P_R* and *P_H* promoters by VanR is inducible by vancomycin, teicoplanin and moenomycin in wild VanA-type strains and constitutive in *vanS* null

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Fig. 1. Map of the *vanA* and *vanB* gene clusters. The percentage of identical amino acids between homologous proteins is indicated. Each cluster contains two promoters for transcription of the regulatory (P_R for *vanRS* and P_{R_B} for *vanR_BS_B*) and resistance (P_H for *vanHAXYZ* and P_{Y_B} for *vanY_BWH_BBX_B*) genes (Evers & Courvalin, 1996; Holman *et al.*, 1994; Arthur *et al.*, 1997; Silva *et al.*, 1998).

Regulation	D-Ala-D-Lac synthesis	Hydrolysis of precursors ending in D-Ala
Response regulator VanR VanR _B 33%	D-Lac dehydrogenase VanH VanH _B 67%	D,D-di-peptidase VanX VanX _B 71%
Kinase VanS VanS _B 19%	D-Ala:D-Lac ligase VanA VanB 76%	D,D-carboxypeptidase VanY VanY _B 30%

Table 1. Characteristics of VanR and VanR_B activation by various kinases

Sensor	Phenotype	Putative signal for the sensor	Reference
Activation of VanR by:			
VanS	Inducible by vancomycin, teicoplanin and moenomycin	Accumulation of lipid II	Handwerger & Kolokathis (1990)
VanSH ₁₆₄ Q	Not inducible since the sensor acts as a phosphatase under all conditions	Not applicable	This work
Host kinase	Constitutive or inducible by vancomycin, teicoplanin and moenomycin depending upon the relative copy numbers of P_H and P_R	Accumulation of lipid II	This work; Arthur <i>et al.</i> (1997)
Activation of VanR_B by:			
VanS _B	Inducible by vancomycin	Interaction with the drug	Baptista <i>et al.</i> (1996)
VanS _B H ₂₃₃ Q	Inducible by vancomycin (loss of kinase activity)	Interaction with the drug	This work
VanS _B A ₁₆₇ S	Inducible by vancomycin and teicoplanin (extension of specificity)	Interaction with the drugs	Baptista <i>et al.</i> (1999)
VanS _B T ₂₃₇ M	Constitutive (loss of phosphatase activity)	Not applicable	Baptista <i>et al.</i> (1999)
Host kinase	Inducible by vancomycin, teicoplanin and moenomycin	Accumulation of lipid II	Baptista <i>et al.</i> (1999)

mutants (Arthur *et al.*, 1997) (Table 1). Glycopeptides and moenomycin inhibit the transglycosylation reaction by different mechanisms and are structurally unrelated, indicating that induction by these antibiotics is unlikely to involve direct interaction of the VanS sensor with the drugs (Handwerger & Kolokathis, 1990). Since moenomycin and glycopeptides block incorporation of lipid intermediate II into nascent peptidoglycan, accumulation of this precursor may be the signalling stimulus for VanS (Baptista *et al.*, 1996).

Expression of the *vanB* cluster is inducible by vancomycin only in wild VanB-type strains and inducible by vancomycin, teicoplanin and moenomycin in *vanS_B* null mutants (Table 1) (Evers & Courvalin, 1996; Baptista *et al.*, 1999). Activation of VanR_B by cross-talk leads to clonal variation under non-inducing conditions (Baptista *et al.*, 1999). Amino acid substitutions in the N-terminal sensing domain of VanS_B allow induction by teicoplanin but not by moenomycin (Baptista *et al.*,

1997; Baptista *et al.*, 1999). Stimulation of VanS_B may involve direct interaction with the antibiotic since this would account for the fact that antibiotics with the same mode of action but different structures are not all inducers (Baptista *et al.*, 1999). The T₂₃₇M substitution in VanS_B impairs the phosphatase activity of the sensor and leads to constitutive expression of the resistance genes (Baptista *et al.*, 1999).

In this report, site-directed mutagenesis was used to introduce glutamine instead of histidine residues at the putative autophosphorylation site of VanS (H₁₆₄) and VanS_B (H₂₃₃). Based on previous analyses, the resulting proteins, VanSH₁₆₄Q and VanS_BH₂₃₃Q, were expected to be unable to activate the response regulators by phosphorylation but to retain phosphatase activity (Haldimann *et al.*, 1997; Hsing & Silhavy, 1997). The *in vivo* activity of VanSH₁₆₄Q and VanS_BH₂₃₃Q was determined based on comparison of the regulation of the *vanX* and *vanX_B* DD-di-peptidase genes in strains har-

bouring various alleles of *vanS* and *vanS_B* (Table 1). The role of inducible expression of the *vanR* and *vanS* genes in the regulation of the *vanA* gene cluster was also investigated.

METHODS

Strains and growth conditions. The origin and properties of the *Enterococcus faecalis* strains and plasmids used are described in Table 2. *E. faecalis* JH2-2 and BM4110 are

derivatives of strain JH2, resistant to fusidic acid and rifampicin or to streptomycin, respectively. Strains were grown in brain heart infusion (BHI) broth and agar (Difco) at 37 °C. Spectinomycin (60 µg ml⁻¹), gentamicin (32 µg ml⁻¹) and erythromycin (10 µg ml⁻¹) were added to the media to prevent loss of plasmids derived from pAT78, pAT392 and pIP836, respectively.

Plasmid construction. Plasmid DNA isolation, digestion with restriction endonucleases (Boehringer Mannheim), amplification of DNA by PCR with *Pfu* DNA polymerase (Strata-

Table 2. Characteristics and origin of the plasmids and strains used

Plasmid or strain	Origin and relevant characteristics*	Source or reference
Plasmids		
pAT29	Shuttle vector (Sp ^r)	Trieu-Cuot <i>et al.</i> (1990)
pAT78	Derivative of pAT29 carrying the <i>cat</i> reporter gene	Arthur <i>et al.</i> (1992)
pAT392	Derivative of pAT29 carrying the <i>P₂</i> promoter and the <i>aac(6')</i> - <i>aph(2')</i> gene (Gm ^r)	Arthur <i>et al.</i> (1994)
pAT90	Derivative of pAT78 harbouring a <i>P_H</i> - <i>cat</i> transcriptional fusion	Arthur <i>et al.</i> (1992)
pAT418	Derivative of pAT78 harbouring a <i>P_R</i> - <i>cat</i> transcriptional fusion	Arthur <i>et al.</i> (1997)
pAT626	Replacement of the <i>P₂</i> promoter of pAT392 by <i>P_R</i>	This work
pAT627	Replacement of the <i>P₂</i> promoter of pAT392 by <i>P_H</i>	This work
pAT628	<i>vanS</i> gene cloned under the control of the <i>P₂</i> promoter of pAT392	This work
pAT629	<i>vanSH</i> ₁₆₄ Q gene cloned under the control of the <i>P₂</i> promoter of pAT392	This work
pAT630	<i>vanS_BH</i> ₂₃₃ Q gene cloned under the control of the <i>P₂</i> promoter of pAT392	This work
pAT631	<i>vanS_B</i> gene cloned under the control of the <i>P₂</i> promoter of pAT392	This work
pIP836	Self-transferable plasmid harbouring a wild-type <i>vanB</i> gene cluster (Em ^r)	Baptista <i>et al.</i> (1999)
pIP842	Derivative of pIP836 harbouring the <i>vanS_BY</i> ₁₂ ter mutation	Baptista <i>et al.</i> (1999)
<i>E. faecalis</i> strains		
JH2-2	Fus ^r Rif ^r derivative of JH2	Jacob & Hobbs (1974)
BM4110	Str ^r derivative of JH2	Courvalin & Carlier (1986)
BM4311	<i>P_RvanRS</i> inserted into the chromosome of JH2-2	Arthur <i>et al.</i> (1992)
BM4312	<i>P₂vanR</i> inserted into the chromosome of JH2-2	Arthur <i>et al.</i> (1997)
BM4332	<i>P_RvanRP_HvanHAXcat</i> gene cluster inserted into the chromosome of JH2-2	Arthur <i>et al.</i> (1997)
BM4375	<i>P_RvanR</i> inserted into the chromosome of JH2-2	This work
BM4396	pIP836(<i>vanS_B</i>) in BM4110	Baptista <i>et al.</i> (1999)
BM4399	pIP842(<i>vanS_BY</i> ₁₂ ter) in BM4110	Baptista <i>et al.</i> (1999)
BM4406	pIP842(<i>vanS_BY</i> ₁₂ ter) and pAT630(<i>P₂vanS_BH</i> ₂₃₃ Q) in BM4110	This work
BM4407	pIP842(<i>vanS_BY</i> ₁₂ ter) and pAT628(<i>P₂vanS</i>) in BM4110	This work
BM4408	pIP842(<i>vanS_BY</i> ₁₂ ter) and pAT629(<i>P₂vanSH</i> ₁₆₄ Q) in BM4110	This work

*Em, erythromycin; Fus, fusidic acid; Gm, gentamicin; Rif, rifampicin; Sp, spectinomycin; Str, streptomycin; ter, termination.

gene), ligation of DNA fragments with T4 DNA ligase (Pharmacia Biotech), Southern hybridization, nucleotide sequencing with T7 DNA polymerase (Pharmacia) and transformation of *Escherichia coli* JM83 with recombinant plasmids were performed by standard methods (Ausubel *et al.*, 1987).

For the construction of pAT628(P_2 vanS), the *vanS* gene of pAT80(P_R vanRSP_HHAXcat) (Arthur *et al.*, 1992) was amplified with oligodeoxynucleotides NA1 and CA1, digested with *SacI* and *XbaI*, and cloned under the control of the P_2 promoter of the shuttle vector pAT392. Primer NA1 (5'-GCACGAGCTCTTGAAAGGAGACAGGAGCATGGTTATAAAATTGAAA) contained a *SacI* site (italicized), a ribosome-binding site (RBS; underlined) and 18 bases of *vanS* including the ATG translation initiation codon (underlined). Primer CA1 (5'-TAAGTCTAGATTAGGACCTCCTTTTAT) comprised a recognition site for *XbaI* (italicized) and 17 bases of the 3' end of *vanS* including the stop codon (underlined).

Plasmid pAT629(P_2 vanSH₁₆₄Q) was obtained by cloning the *vanSH*₁₆₄Q gene under the control of the P_2 promoter of pAT392. The *vanSH*₁₆₄Q gene was constructed by two sequential amplifications leading to the replacement of the CAC codon specifying His at position 164 by a CAG glutamine codon. In the first step, two overlapping portions of *vanS* were independently amplified using primers NA1 plus N164 and C164 plus CA1. Primer N164 (5'-CTGCGCCAAGTACA-TAAC) was complementary to the *vanS* coding strand upstream from codon 164 and contained a CTG triplet (underlined) that was complementary to a CAG glutamine codon for replacement of the CAC histidine codon at position 164. Primer C164 (5'-GTTATGTACTTGGCGCAGGATAT-TAAACGGCCCT) contained 18 bases complementary to the sequence of primer N164 (underlined) and 17 bases of *vanS* downstream from codon 164. For the second step, the amplicons obtained with NA1 plus N164 and C164 plus CA1 were purified (Microspin S-400 HR; Pharmacia), mixed, denatured (10 min, 95 °C) and annealed (30 min, 50 °C). Primers NA1 and CA1 were added and the *vanSH*₁₆₄Q gene was amplified. The amplicon was digested with *SacI* and *XbaI* and cloned into pAT392(P_2) digested with the same enzymes generating pAT629(P_2 vanSH₁₆₄Q).

For the construction of pAT630(P_2 vanS_H₂₃₃Q), the *vanS*_H₂₃₃Q gene was obtained by two sequential amplifications and cloned under the control of the P_2 promoter of pAT392 using *SacI* and *XbaI* as described for pAT629(P_2 vanSH₁₆₄Q). Briefly, the 5' portion of *vanS*_B was amplified using primers NB1 and N233. NB1 (5'-GCACGAGCTCTTGAAAGGAGACAGGAGCATGGAAAGAAAAGGGAT) contained a *SacI* site (italicized), an RBS (underlined) and 17 bases of *vanS*_B including the ATG translation initiation codon (underlined). N233 (5'-CTGAGAGGCTGCCGCAAA) was complementary to codons 228–233 of *vanS*_B with a G to C base substitution (underlined) for replacement of CAC (His) by CAG (Gln) at codon 233. The 3' portion of *vanS*_B was amplified with primers C233 and CB1. Primer C233 (5'-TTTGGCGCAGCCTCTCAGGAGT-TAAAAACGCCCAT) contained 18 bases complementary to the sequence of primer N233 (underlined) and 17 bases of *vanS*_B downstream from codon 233. Primer CB1 (5'-AGTGTCTAGATTATAGTGTGATGTGG) comprised a recognition site for *XbaI* (italicized) and 17 bases of the 3' extremity of *vanS*_B including the stop codon (underlined). The amplicons obtained with NB1 plus N233 and C233 plus CB1 were purified, annealed and the *vanS*_H₂₃₃Q gene was

amplified with primers NB1 and CB1. For construction of pAT631(P_2 vanS_H), *vanS*_H was amplified using primers NB11 and CB1, the amplicon was digested with *SacI* plus *XbaI* and cloned into pAT392 digested with the same enzymes. Primers NB11 (5'-GCACGAGCTCTTGAAAGGAGACAGGAGCATGCATCACCATCACCATCACGAAAGAAAAGGGAT-TTT) contained a *SacI* site (italicized), an ATG translation initiation codon preceded by an RBS (underlined), 6 His codons (italicized) and 17 bases of *vanS*_H downstream from the initiation codon (underlined).

Plasmid pAT626(P_R) was constructed by replacing the *EcoRI*–*SacI* fragment of pAT392 carrying the P_2 promoter by an *EcoRI*–*SacI* fragment harbouring the P_R promoter. The latter fragment was obtained by amplification of the P_R promoter of pAT80(P_R vanRSP_HHAXcat) using primers R1 and R2 as described previously (Arthur *et al.*, 1997).

Plasmid pAT627(P_H) was constructed in two steps. The P_H promoter was amplified by PCR using DNA of pAT87(P_H vanHAXcat) (Arthur *et al.*, 1992) as a template and oligodeoxynucleotides H1 and 'sequencing 17-mer' (Amersham) as primers. Primer H1 (5'-ATGAGAGC-TACAGTAATGCCGATGTTATT) contained a *SacI* site (italicized) and 20 bases complementary to codons 2–8 of the *vanH* gene. The 'sequencing 17-mer' primer was complementary to the sequence of the vector upstream from the *EcoRI* site. The amplicon was digested with *EcoRI* plus *SacI* and cloned into pAT29 digested with the same enzymes. In the second step, the *SacI*–*SphI* fragment of pAT392 carrying the *aac*(6')-*aph*(2'') gentamicin resistance gene was cloned downstream from P_H . The nucleotide sequence of the inserts obtained by PCR was redetermined.

Strain construction. *E. faecalis* BM4375 was obtained by inserting a copy of the *vanR* gene under the control of the P_R promoter into the chromosome of JH2-2 using the suicide vector pAT113 (Trieu-Cuot *et al.*, 1991). Integrative plasmid pAT615(P_R vanR) (Arthur *et al.*, 1997) was introduced into *E. faecalis* JH2-2/pAT145(Km^r Int-Tn) by electroporation and clones resulting from chromosomal integration of pAT615 mediated by the Int-Tn integrase were selected on agar containing erythromycin (10 µg ml⁻¹). Spontaneous loss of pAT145(Km^r Int-Tn) was obtained by subculturing transformants for approximately 30 generations in antibiotic-free medium. Total DNA of a clone, designated BM4375(P_R vanR), was digested with *EcoRI* and *HindIII* and analysed by Southern hybridization using pAT615-labelled DNA as a probe. The data obtained (not shown) indicated the presence of a single chromosomal copy of pAT615(P_R vanR).

For construction of BM4406, BM4407 and BM4408, plasmids pAT630(P_2 vanS_H₂₃₃Q Gm^r), pAT628(P_2 vanS Gm^r) and pAT629(P_2 vanSH₁₆₄Q Gm^r), respectively, were introduced into *E. faecalis* BM4110 by electroporation (Cruz-Rodz & Gilmore, 1990) and transformants were selected on agar containing gentamicin (32 µg ml⁻¹). Plasmid pIP842(*vanS*_BY₁₂ter Em^r) was introduced into the resulting transformants by filter mating using BM4399 as a donor. Transconjugants were selected on agar containing gentamicin (32 µg ml⁻¹) and erythromycin (10 µg ml⁻¹).

Enzyme assays. For preparation of cytoplasmic extracts, 8 ml of an overnight culture were added to 100 ml broth containing vancomycin, teicoplanin or moenomycin at various concentrations and bacteria were grown until the optical density at 600 nm reached 0.7. The cells were harvested by centrifugation, washed in 0.1 M phosphate buffer pH 7.0, resuspended in the same buffer, treated with lysozyme and lysed

by sonication (Arthur *et al.*, 1996a). The lysate was centrifuged for 45 min at 100 000 *g* and the supernatant was assayed for VanX and VanX_B DD-di-peptidase activity using D-Ala-D-Ala as the substrate and D-amino acid oxidase coupled to peroxidase for the indicator reactions (Arthur *et al.*, 1996a). The supernatant was also assayed for chloramphenicol acetyltransferase activity as previously described (Arthur *et al.*, 1992).

RESULTS AND DISCUSSION

Phosphatase activity of VanS_BH₂₃₃Q

Determination of VanX_B DD-di-peptidase activity indicated that expression of the resistance genes was inducible by vancomycin, teicoplanin and moenomycin in BM4399, which contained a translation stop at codon 12 of *vanS_B* (Table 3). Thus, VanR_B was activated by a heterologous kinase as previously described for *vanS_B* null mutants (Table 1; Baptista *et al.*, 1999). In contrast, expression of *vanX_B* was inducible by vancomycin only in BM4396 and BM4406, which produced VanS_B and VanS_BH₂₃₃Q, respectively (Table 3). The wild-type sensor acted as a phosphatase in the absence of vancomycin since production of VanS_B reduced expression of *vanX_B* under non-inducing conditions and prevented induction by teicoplanin and moenomycin. VanS_B may act as a kinase in the presence of vancomycin although the increase in *vanX_B* expression associated with production of VanS_B was small. VanS_BH₂₃₃Q had phosphatase activity since it also prevented expression of *vanX_B* in media containing teicoplanin, moenomycin or no antibiotic. Expression of *vanX_B* was inducible by vancomycin, revealing that the phosphatase activity of VanS_BH₂₃₃Q was inhibited by this antibiotic, allowing activation of VanR_B by the heterologous kinase. Thus, the phosphatase activity of VanS_B was regulated independently from the reaction of autophosphorylation at the histidine residue position 233. Fig. 2 presents a working model for the phosphatase and kinase activities of VanS_B and VanS_BH₂₃₃Q.

Phosphatase activity of VanSH₁₆₄Q

E. faecalis BM4332(*P_RvanRP_HvanHAX*) harbours a chromosomal copy of *vanR* under the control of the *P_R* promoter and of the *vanHAX* operon under the control of the *P_H* promoter (Fig. 2d). The *P_RvanRP_HvanHAX* cluster was generated by a deletion internal to *vanS* (Arthur *et al.*, 1997). Expression of *vanX* was constitutive in BM4332(*P_RvanRP_HvanHAX*), indicating that VanR was activated by a heterologous kinase in the absence of VanS (Arthur *et al.*, 1997). High-level expression of *vanX* did not require stimulation of the heterologous kinase by glycopeptides. Likewise, insertional inactivation of *vanS* in the *vanA* gene cluster carried by a natural plasmid was previously shown to lead to high-level constitutive activation of the *P_R* and *P_H* promoters (Arthur *et al.*, 1997).

As expected, introduction of plasmid pAT392(*P₂*) into BM4332(*P_RvanRP_HvanHAX*) did not modify the regulation of *vanX* (Table 4). Production of VanS in BM4332(*P_RvanRP_HvanHAX*)/pAT628(*P₂vanS*) restored inducible expression of the resistance genes (Table 4). Thus, VanS acted as a phosphatase under non-inducing conditions and prevented activation of VanR by the heterologous kinase (Fig. 2b). The negative control mediated by VanS was suppressed by glycopeptides and reduced by moenomycin, leading to induction by these antibiotics (Table 4). This observation indicates that the phosphatase activity of VanS was inhibited by these antibiotics (Fig. 2b). VanS may act as a kinase under inducing conditions but this activity could not be detected since activation of VanR by cross-talk leads to transcription of *vanX* at its maximum level as previously described (Arthur *et al.*, 1997). Production of VanSH₁₆₄Q in BM4332(*P_RvanRP_HvanHAX*)/pAT629(*P₂vanSH164Q*) prevented expression of *vanX* under all growth conditions (Table 4). Thus, VanSH₁₆₄Q acted as a phosphatase and this activity was not modulated by glycopeptides or moenomycin (Fig. 2b). This observation suggests that the phosphatase activity of VanS

Table 3. Control of VanR_B by various sensors

Results are expressed as VanX_B activity under the inducing conditions indicated. Data are means ± SD obtained from a minimum of three independent extracts.

Strain	Van sensor	VanX _B activity (nmol min ⁻¹ mg ⁻¹)			
		Uninduced	Vancomycin (8 µg ml ⁻¹)	Teicoplanin (0.5 µg ml ⁻¹)	Moenomycin (0.04 µg ml ⁻¹)
BM4399	None	21 ± 4	140 ± 28	270 ± 120	40 ± 13
BM4396	VanS _B	3 ± 1	270 ± 63	5 ± 2	1 ± 0
BM4406	VanS _B H ₂₃₃ Q	1 ± 0	82 ± 9*	2 ± 0	1 ± 0
BM4407	VanS	470 ± 130	640 ± 74	610 ± 130	170 ± 54
BM4408	VanSH ₁₆₄ Q	28 ± 12	110 ± 72	130 ± 17	19 ± 7

* Induction was performed with 4 µg vancomycin ml⁻¹ since BM4406 does not grow in the presence of 8 µg vancomycin ml⁻¹.

Fig. 2. Models for positive and negative controls of VanR and VanR_B by various sensors. Phosphorylation and dephosphorylation of the response regulators are thought to be responsible for positive and negative control, respectively, of the transcription level of the resistance genes, although the kinase and phosphatase activities of the sensors were not determined. RR, VanR_B or VanR response regulator; P, phosphate. (a) Activation of VanR_B and VanR by cross-talk. Previous analyses indicated that VanR and VanR_B are activated by heterologous kinase(s) encoded by the host chromosome in *vanS* and *vanS_B* null mutants (see Introduction). This mode of VanR_B regulation leads to clonal variation under non-inducing conditions and to induction by vancomycin (Vm), teicoplanin (Te) and moenomycin (Mo). VanR is constitutively activated by cross-talk in *vanS* null mutants of wild-type VanA strains. However, cross-talk leads to induction by Vm, Te and Mo if the *P_H* promoter is carried by a multicopy plasmid (see below, Fig. 2d). (b) Phosphatase activity of VanS_B and VanS. The Van sensors were considered to act as phosphatases if the levels of *vanX* and *vanX_B* expression were significantly reduced in comparison to those in isogenic *vanS* or *vanS_B* null mutants grown in the same conditions. According to this criterion, VanS_B and VanS_BH₂₃₃Q dephosphorylated VanR_B and the phosphatase activity of both sensors was inhibited if medium contained Vm. VanS and VanSH₁₆₄Q dephosphorylated the cognate response regulator VanR. Inhibition of the phosphatase activity by Vm, Te and Mo was only detected for the wild-type VanS sensor. VanR_B and VanR were not dephosphorylated by the heterologous sensors VanS_B and VanS_B, respectively. (c) Kinase activity of VanS_B and VanS. The Van sensors were considered to act as kinases if the levels of expression of *vanX* and *vanX_B* were increased in comparison to those in isogenic *vanS* or *vanS_B* null mutants grown in the same conditions. According to this criterion, VanR_B was activated (phosphorylated) by VanS_B in the presence of Vm and by VanS in all conditions. Phosphorylation of VanR by VanS_B or VanS could not be detected in the expression systems studied since activation of VanR by cross-talk led to transcription of *vanX* at its maximum level. (d) Model for regulation of *vanX* in

Table 4. Activation of VanR in derivatives of BM4332(P_R vanRP_HvanHAX) harbouring various plasmids

Results are expressed as VanX activity under the inducing conditions indicated. Data are means $\pm$ SD obtained from a minimum of three independent extracts.

Plasmid	Characteristics	VanX activity (nmol min ⁻¹ mg ⁻¹)			
		Uninduced	Vancomycin (1 μ g ml ⁻¹)	Teicoplanin (0.5 μ g ml ⁻¹)	Moenomycin (0.05 μ g ml ⁻¹)
None		370 $\pm$ 50	410 $\pm$ 27	260 $\pm$ 27	100 $\pm$ 20
pAT392	P_2^*	290 $\pm$ 40	250 $\pm$ 21	280 $\pm$ 27	98 $\pm$ 18
pAT628	P_2 vanS	5 $\pm$ 1	190 $\pm$ 31	280 $\pm$ 55	18 $\pm$ 4
pAT629	P_2 vanSH ₁₆₄ Q	2 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 3	5 $\pm$ 3
pAT631	P_2 vanS _B	320 $\pm$ 10	330 $\pm$ 23	280 $\pm$ 23	240 $\pm$ 97
pAT630	P_2 vanS _B H ₂₃₃ Q	390 $\pm$ 43	380 $\pm$ 28	350 $\pm$ 42	330 $\pm$ 14
pAT627	P_H	4 $\pm$ 1	67 $\pm$ 34	130 $\pm$ 41	32 $\pm$ 4
pAT626	P_R	330 $\pm$ 11	320 $\pm$ 23	250 $\pm$ 8	130 $\pm$ 19

* The P_2 promoter is constitutive and originates from the *aphA-3* kanamycin resistance gene of enterococcal plasmid pJH1 (Trieu-Cuot *et al.*, 1985; Arthur *et al.*, 1997).

cannot be regulated independently from the reaction of autophosphorylation at H₁₆₄. Alternatively, the H₁₆₄Q substitution may have indirectly impaired signal transduction.

Cross-talk between the VanRS and VanR_SS_B systems

Production of VanS_B or VanS_BH₂₃₃Q did not modify expression of *vanX* in BM4332(P_R vanRP_HvanHAX) (Table 4). Thus, VanS_B did not prevent activation of VanR by the heterologous kinase, indicating that the sensor of the *vanB* cluster was unable to dephosphorylate the heterologous VanR response regulator encoded by the *vanA* cluster (Fig. 2b). Activation of VanR by VanS_B could not be tested in this system since *vanX* was constitutively expressed at a high level in the *vanS* null mutant BM4332(P_R vanRP_HvanHAX) (Table 4 and Fig. 2c). In *E. coli*, deletion of the *phoBR*, *creBC* and *ackA-pta* loci prevents activation of VanR by cross-talk (Silva *et al.*, 1998). However, activation of VanR by VanS_B could not be tested since VanS_B was locked in a phosphatase state in this host. In addition, induction by glycopeptides could not be studied since the drugs do not penetrate the *E. coli* outer membrane.

In comparison to the *vanS_B* null mutant BM4399, VanX_B activity was higher in BM4407, which contained

pAT628(P_2 vanS), suggesting that VanS activated VanR_B (Table 3). VanS stimulated expression of *vanX_B* under all growth conditions. VanS and the heterologous kinase were previously shown to be stimulated by the same antibiotics and to mediate low-level expression of the resistance genes in the absence of inducers (Table 1; Baptista *et al.*, 1999). Thus, the two kinases may contribute to activation of VanR_B in BM4407 (Fig. 2c). As expected, production of VanSH₁₆₄Q did not increase expression of *vanX_B* (BM4408 in Table 3). Previous attempts to detect activation of VanR_B by VanS in *E. coli* were unsuccessful since VanR_B was constitutively activated by cross-talk in spite of deletions of the *phoBR*, *creBC* and *ackA-pta* loci (Silva *et al.*, 1998). The VanS sensor was locked in an activator (kinase) state in this host. In contrast, VanS displayed phosphatase activity in *E. faecalis* in the absence of glycopeptides in the growth medium (Table 3 and Fig. 2b).

Activation of VanR in derivatives of BM4332 harbouring the P_R and P_H promoters on multicopy plasmids

Introduction of the multicopy plasmid pAT627 containing P_H in BM4332(P_R vanRP_HvanHAX) prevented expression of the chromosomal copy of *vanX* under non-inducing conditions (Table 4). Synthesis of VanX

BM4332(P_R vanRP_HvanHAX)/pAT627(P_H). VanR stimulates its own synthesis since binding of the phosphorylated form of VanR (P-VanR) to P_R activates transcription of *vanR*. In BM4332(P_R vanRP_HvanHAX), the amplification loop compensates for inefficient phosphorylation of VanR in the absence of a stimulus for the host kinase(s). However, the amplification loop does not function in BM4332(P_R vanRP_HvanHAX)/pAT627(P_H) since P-VanR preferentially binds to the high-affinity P_H promoter located on plasmid pAT627. Expression of *vanX* is inducible by glycopeptides and moenomycin since stimulation of the host kinase by these antibiotics is required for efficient phosphorylation of VanR and synthesis of this protein.

Table 5. *trans*-activation of the P_H and P_R promoters by VanR in various hosts

Results are expressed as chloramphenicol acetyltransferase activity under the inducing conditions indicated. Data are means $\pm$ SD obtained from a minimum of three independent extracts.

Host	Plasmid	Chloramphenicol acetyltransferase activity (nmol min ⁻¹ mg ⁻¹)			
		Uninduced	Vancomycin (0.5 µg ml ⁻¹)	Teicoplanin (0.25 µg ml ⁻¹)	Moenomycin (0.05 µg ml ⁻¹)
JH2-2	pAT78(<i>cat</i>)	230 $\pm$ 110	220 $\pm$ 79	310 $\pm$ 130	160 $\pm$ 170
	pAT418(P_R <i>cat</i>)	830 $\pm$ 520	380 $\pm$ 160	550 $\pm$ 340	500 $\pm$ 13
	pAT90(P_H <i>cat</i>)	150 $\pm$ 49	150 $\pm$ 22	360 $\pm$ 170	430 $\pm$ 120
BM4311(P_R <i>vanRS</i>)	pAT78(<i>cat</i>)	450 $\pm$ 240	290 $\pm$ 70	230 $\pm$ 120	560 $\pm$ 320
	pAT418(P_R <i>cat</i>)	370 $\pm$ 97	3700 $\pm$ 1000	4300 $\pm$ 1400	1600 $\pm$ 330
	pAT90(P_H <i>cat</i>)	160 $\pm$ 95	2000 $\pm$ 620	7900 $\pm$ 2600	2000 $\pm$ 480
BM4312(P_2 <i>vanR</i>)	pAT78(<i>cat</i>)	120 $\pm$ 66	100 $\pm$ 19	29 $\pm$ 140	89 $\pm$ 30
	pAT418(P_R <i>cat</i>)	4400 $\pm$ 1200	5300 $\pm$ 1500	10000 $\pm$ 1300	9300 $\pm$ 3000
	pAT90(P_H <i>cat</i>)	10000 $\pm$ 1600	9900 $\pm$ 2700	15000 $\pm$ 560	22000 $\pm$ 5900
BM4375(P_R <i>vanR</i>)	pAT78(<i>cat</i>)	250 $\pm$ 42	430 $\pm$ 330	790 $\pm$ 320	520 $\pm$ 62
	pAT418(P_R <i>cat</i>)	9200 $\pm$ 1400	10000 $\pm$ 1800	8900 $\pm$ 880	4900 $\pm$ 640
	pAT90(P_H <i>cat</i>)	790 $\pm$ 240	1800 $\pm$ 480	3600 $\pm$ 1000	5100 $\pm$ 1000

was inducible by vancomycin, teicoplanin and moenomycin, indicating that stimulation of the heterologous kinase by these antibiotics was required for activation of the resistance genes located in the chromosome. Notably, induction occurred with the same antibiotics in *vanS_B* null mutants (Table 1), raising the possibility that VanR and VanR_B may be activated by the same host kinase. The P_R promoter of plasmid pAT626 had no effect on the regulation of the *vanX* gene of BM4332(P_R *vanR* P_H *vanHAX*).

trans-activation of the P_R and P_H promoters

We previously compared the regulation of the P_R and P_H promoters in *E. faecalis* JH2-2 and derivatives harbouring a chromosomal copy of *vanR* and *vanS* under the control of P_R (BM4311) or of *vanR* alone under the control of the heterologous promoter P_2 (BM4312) (Arthur *et al.*, 1992, 1997). In this study, we extended this analysis to a novel host, BM4375(P_R *vanR*), that contained a chromosomal copy of *vanR* under the control of P_R . Plasmids pAT418(P_R *cat*) and pAT90(P_H *cat*) containing a *cat* reporter gene transcriptionally fused to P_R and P_H were introduced into these three hosts. The P_H and P_R promoters were not active in JH2-2 and inducible by glycopeptides and moenomycin in BM4311(P_R *vanRS*) (Table 5). The P_H promoter of pAT90(P_H *cat*) was constitutive in BM4312(P_2 *vanR*) and inducible by glycopeptides and moenomycin in BM4375(P_R *vanR*). Thus, activation of VanR required stimulation of the heterologous kinase by glycopeptides or moenomycin if P_H was present on a multicopy plasmid and *vanR* was expressed under the

control of P_R (Fig. 2a). In contrast, expression of *vanR* under the control of P_2 in BM4312(P_2 *vanR*) or of P_R in BM4375(P_R *vanR*) led to constitutive activation of the P_R promoter of pAT418(P_R *cat*).

A model for inducible expression of the resistance genes in *vanS* null mutants harbouring P_H on a multicopy plasmid

Comparison of wild-type and *vanS* null mutants indicated that activation of VanR by cross-talk is very efficient since production of VanS does not increase transcription initiation at P_R and P_H (Arthur *et al.*, 1997). This observation is surprising since phosphorylation of response regulators by heterologous kinases is not expected to be efficient (Fisher *et al.*, 1995; Hoch & Silhavy, 1995). Two factors were thought to contribute to the accumulation of the phosphorylated form of VanR (VanR-P) in the absence of VanS (Arthur *et al.*, 1997). First, hydrolysis of phospho-VanR in the absence of VanS is remarkably slow *in vitro* ($t_{1/2}$ = 10–12 h) (Wright *et al.*, 1993). Second, the amplification loop resulting from activation of P_R by VanR (Fig. 2d) may compensate for inefficient phosphorylation of the regulator (Arthur *et al.*, 1997). However, activation of VanR required stimulation of the heterologous kinase by glycopeptides or moenomycin in BM4332(P_R *vanR* P_H *vanHAX*)/pAT627(P_H) and in BM4375(P_R *vanR*)/pAT90(P_H *cat*) (Tables 4 and 5). We propose that P_H , present on multicopy plasmids in these strains, competed with the chromosomal copy of P_R for limiting amounts of VanR-P in the absence of a stimulus for the heterologous kinase (Fig. 2d). If VanR-P preferentially

binds to the plasmid-borne P_H promoter, the concentration of VanR-P may not be sufficient for activation of the chromosomal P_R promoter and synthesis of VanR in the absence of induction. In agreement with this model, VanR-P displays a 40-fold higher affinity for P_H than for P_R (Holman *et al.*, 1994). Activation of VanR in BM4375(P_{RvanR}) was not affected by the presence of P_R on a multicopy plasmid (Table 5) indicating that the difference in the affinity of VanR-P for the two promoters was critical for the inducible phenotype. Inducible activation of VanR in the absence of VanS was only detected in strains harbouring P_R in the chromosome and P_H on a multicopy plasmid (Tables 4 and 5) (Arthur *et al.*, 1997). Thus, the difference in the relative concentration of the two promoters was also critical. Finally, stimulation of the heterologous kinase was only required if *vanR* was expressed under the control of P_R since the P_H promoter of pAT90(P_Hcat) was inducible in BM4375(P_{RvanR}) but constitutive in BM4312($P_{\delta vanR}$) (Table 5).

ACKNOWLEDGEMENTS

This work was supported in part by a Bristol-Myers-Squibb Unrestricted Biomedical Research Grant in Infectious Diseases.

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Received 12 January 1999; revised 19 April 1999; accepted 22 April 1999.