REVIEW ARTICLE

Sebastián Méndez-Álvarez [1,2]
Xiomara Pérez-Hernández [1]
Félix Claverie-Martín [1]

[1] Molecular Biology Laboratory, Research Unit,
Nuestra Señora de Candelaria Hospital,
Santa Cruz de Tenerife, Spain
[2] Department of Cellular Biology and
Microbiology, University of La Laguna,
Santa Cruz de Tenerife, Spain

Glycopeptide resistance in enterococci

Correspondence to:
Félix Claverie-Martín. Laboratorio de Biología
Molecular. Unidad de Investigación.
Hospital Ntra. Sra. de Candelaria.
Ctra. Del Rosario s/n.
38010 Santa Cruz de Tenerife. Spain.
Tel: +34-922602938.
Fax: +34-922600562.
E-mail: claverie@hcan.rcanaria.es

Summary The selective pressure resulting from the extensive use of antibiotics over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. Consequently, we are now at serious risk of suffering intractable, life-threatening infections. The progressive emergence and rapid dissemination of resistance to glycopeptides, the last resort for treating nosocomial infections with enterococci resistant to usual antibiotics, constitute one of the most dramatic examples of such resistance. Enterococci are normal human commensals, but are also a frequent cause of nosocomial urinary tract infections and nosocomial bacteremia. *Enterococcus faecalis* causes 80 to 90% of human enterococcal infections, while *Enterococcus faecium* accounts for most of the remainder. During the last decade, our understanding of the genetics and biochemical basis of resistance to glycopeptides has increased greatly. Furthermore, the application of molecular methods for the diagnosis of glycopeptide-resistant enterococci has provided new insights into the epidemiology of enterococcal infections.

Key words *Enterococcus* spp. · Glycopeptide resistance · Vancomycin · Teicoplanin · Resistance genes

Introduction

When the first generation of antibiotics appeared in the 1940s, geneticists suggested that, taking into account the low frequency of appearance of spontaneous mutants found in nature, the emergence of resistance during therapy was unlikely. However, the fact that bacteria are easily able to store and exchange genetic information, coupled with the high pressure resulting from the indiscriminate use of antibiotics, has determined the existence and rapid spread of different resistance mechanisms. Such mechanisms limit possible therapies and lead to clinical complications that may end in death. For those reasons, bacterial resistance to antibiotics has become a major threat to the human population. In Spain, the development of antibiotic resistance in community-acquired bacterial pathogens and its relation to antibiotic use and consumption have been of both national and international concern [7]. Risk analysis has been suggested as a suitable practice in order to find guidelines for minimizing the development of antibiotic resistance [8]. The progressive emergence and rapid dissemination of resistance to glycopeptide antibiotics is one of the most dramatic example of such types of resistance [52]. The glycopeptide antibiotics vancomycin and teicoplanin (Fig. 1) are considered to be the last resort for treating a variety of serious infections caused by Gram-positive bacteria, such as enterococci, methicillin-resistant *Staphylococcus aureus* (i.e. MRSA) and *Clostridium difficile*. Although enterococci are part of the normal microbiota of the gastrointestinal tract in humans and other animals, under certain circumstances they are able to produce nosocomial infections [34]. In fact, enterococci are now the second most common pathogens isolated from hospital-acquired infections. Vancomycin has been used heavily in the last two decades, resulting in the emergence of enterococci resistant to glycopeptide antibiotics. Vancomycin-resistant enterococci (VRE) are being increasingly isolated from clinical specimens, and infections caused by VRE are sometimes untreatable by currently available antibiotics.

Resistance to vancomycin was first reported in England in 1988. In the USA, it rose from 0.3% of the hospital isolates sent to the Center for Disease Control and Prevention (Atlanta, GA) in 1989 to 15.4% in 1997. Also in the USA, the percentage of resistant isolates from intensive care units rose from 0.4 to 23.4 % [21, 45]. In contrast to the USA, the prevalence rates of VRE in European hospitals are low. A multicenter study of resistance in enterococci showed that the rate of resistance to glycopeptides in Spain was 1.8% for
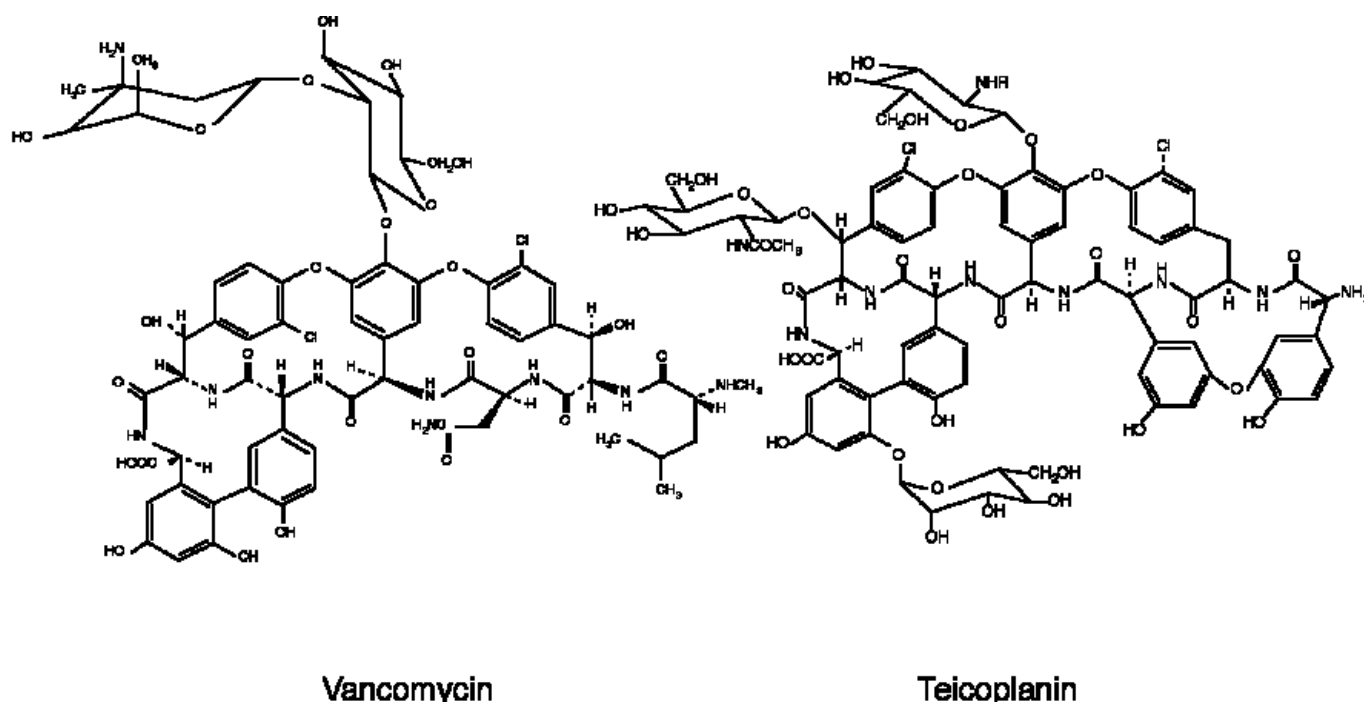
Vancomycin   **Teicoplanin**

Fig. 1 Structure of vancomycin and teicoplanin

vancomycin and 1% for teicoplanin [12]. However, Spain has been recognized by the scientific community as one of the countries with the highest rates of bacterial resistance to antibiotics, which indicates that controlling the emergence of new types of resistance is required [7]. Furthermore, the apparently low prevalence of VRE in Spanish hospitals as well as in hospitals in other European countries may be due to the inability of the diagnostic laboratories to detect all VRE [2].

The most worrying features of resistance to vancomycin are: (1) the lack of synergy when vancomycin is combined with aminoglycosides, (2) the high frequency of simultaneous resistance to several antibiotics and (3) the putative risk of spread to more virulent bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. The rapid emergence and spread of VRE has become a serious concern to both physicians and patients. The emergence of resistance among enterococci threatens to make vancomycin obsolete in the treatment of infections caused by these bacteria [5, 54]. Resistance is observed most commonly in *Enterococcus faecium*, which is often resistant to aminoglycosides and β-lactams, and to a lesser extent in other *Enterococcus* spp*., including *E. faecalis* [34].

Several authors have reviewed glycopeptide resistance in enterococci [5, 21, 54, 55]. Over the last five years, our understanding of the molecular basis of resistance has increased considerably, new types of acquired glycopeptide resistance have appeared, and several VRE outbreaks have occurred. In this review, we provide an update on different aspects of

glycopeptide resistance, and briefly describe new molecular techniques for the rapid, accurate diagnosis of VRE.

The cell wall of Gram-positive eubacteria

The strength of the cell wall of eubacteria depends mainly on the peptidoglycan component. The chemical composition of that peptidoglycan is very similar in Gram-negative and in Gram-positive bacteria. The peptidoglycan layer comprises two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and a group of amino acids, usually consisting of L-alanine, D-glutamic acid, D-alanine, and either diaminopimelic acid (DAP) or another amino acid, which is L-lysine in the case of most Gram-positive cocci.

The peptidoglycan is a multilayered structure in which the glycan chains formed by sugars are connected by peptide cross-links formed by amino acids (Fig. 2). This cross-linking is essential for the full strength of the peptidoglycan structure, and occurs to varying degrees which are characteristic in different bacteria. There are almost 100 known peptidoglycan types, and the greatest variation occurs in the interbridge. Any of the amino acids present in the tetrapeptide can also occur in the interbridge; besides, other amino acids can be found. In Gram-positive eubacteria, cross-linkage is usually established by a peptide interbridge, the kinds and numbers of cross-linking amino acids varying from organism to organism.
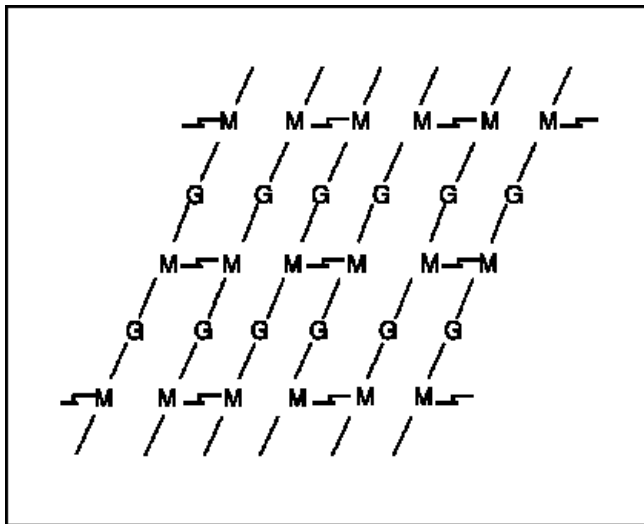
Fig. 2 Overall structure of the enterococcal peptidoglycan. The diagram depicts several glycan chains cross-linked to one another. G: N-acetylglucosamine; M: N-acetylmuramic acid. Thick lines represent interbridges

In *S. aureus*, the best-studied Gram-positive eubacteria, each interbridge peptide consists of five glycine molecules connected by peptide bonds (Fig. 3A). Alternatively, the interbridge of many staphylococci strains consists of glycine associated with alanine and/or serine. In *E. faecalis* the cross-bridge between D-Ala and L-Lys consists of one or two alanine molecules (Fig. 3B) [16]. In other enterococci, such as *E. faecium,* the cross bridge contains D-aspartate (D-Asp) or D-asparagine (D-Asn) as the sole amino acid [9, 15]. Changes in the interbridge or in the tetrapeptide lead to several types of antibiotic resistance found in different bacteria. However, acquired resistance to glycopeptides 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-alanyl-D-alanine (D-Ala-D-Ala) found in susceptible bacteria. Variations in the composition of the dipeptide explain the existence of different types of resistance to glycopeptides— e.g. *E. gallinarum* synthesizes peptidoglycan precursors ending in D-alanyl-D-serine (D-Ala-D-Ser) (Fig. 3C) [44].

De Jonge et al. [16] have studied the muropeptide compositions of isogenic vancomycin-susceptible and -resistant *E. faecalis* strains. The peptidoglycan of the susceptible strain contains pentapeptides as stem peptides, whereas the peptidoglycan of the isogenic resistant strain is composed of muropeptides with tetrapeptide stem peptides. The resistant strain synthesizes peptidoglycan precursors terminating in D-Ala-D-Lac rather than D-Ala-D-Ala; however, no lactate-containing muropeptides are detected in its peptidoglycan [9, 16].
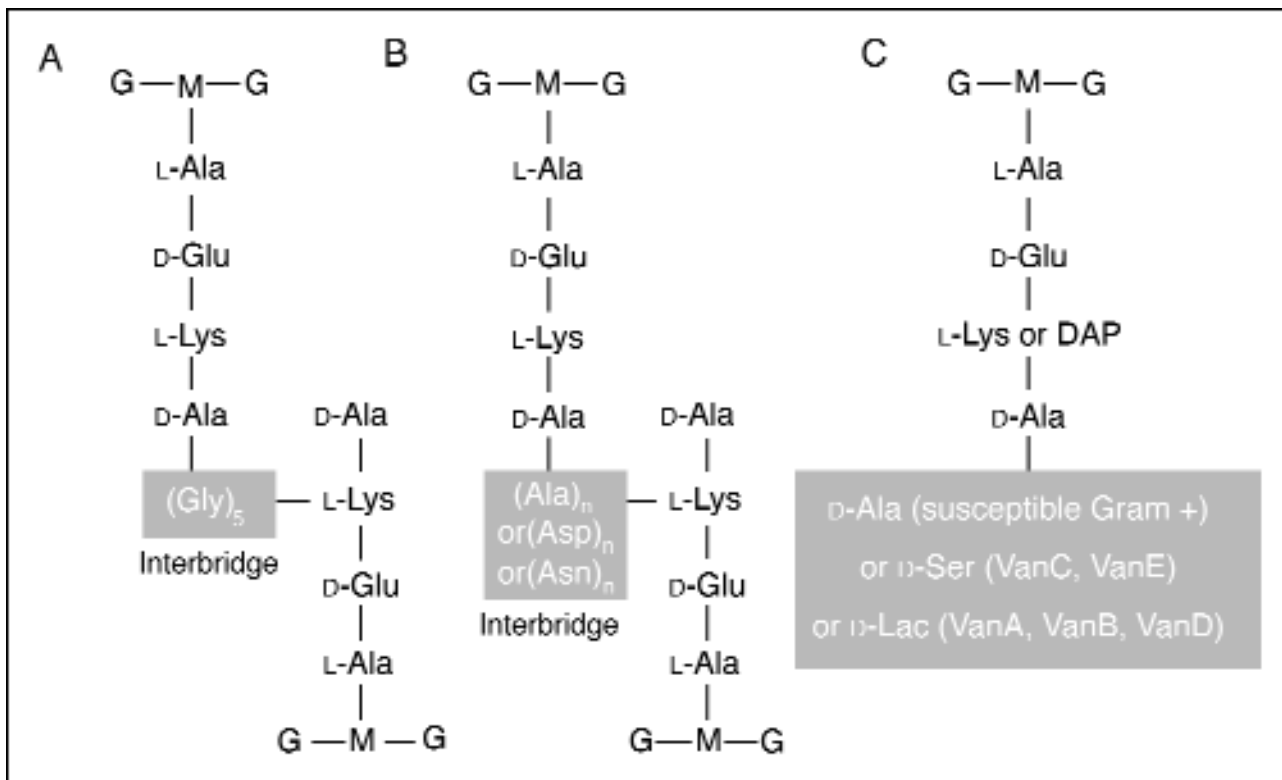

Fig. 3 (A) Glycine interbridge in *Staphylococcus aureus*. (B) Interbridge in glycopeptide resistant enterococci. (C) Variations in the composition of the peptidoglycan precursor

De Jonge et al. suggested that either lactate-terminating precursors are not incorporated into peptidoglycan of the resistant strain or that the lactate residues are removed from peptidoglycan during synthesis. The degree of cross-linkage observed in both vancomycin-resistant and -susceptible *E. faecalis* strains was the same, indicating that transpeptidation was not affected.

Glycopeptide antibiotics: Mechanism of action

Although more than 200 glycopeptides have been identified, only vancomycin and teicoplanin have been developed for clinical application thus far [54]. A third glycopeptide, avoparcin, has been used extensively in agricultural applications and in veterinary medicine in Europe [21]. The potential agricultural applications of a fourth agent, actaplanin, have been studied, but this glycopeptide has not yet been marketed. A fifth agent, ristocetin, appeared promising in early clinical trials, but development was stopped because of its toxicity. The glycopeptides are complex structures that consist of a multiring peptide core (the aglycone component) containing six peptide linkages, an unusual triphenyl ether moiety, and sugar molecules that are attached at various sites (Fig. 1). In vancomycin, the aglycone component is made up of seven amino acids, joined together to give a heptapeptide. The side chains of several of the amino acids are crosslinked, resulting in a closely-packed structure. Two sugars are attached to the side chain of amino acids [36]. As with the other glycopeptides, vancomycin is produced by several *Actinomyces* species isolated from soil samples, but recently, Nicolau et al. [36] achieved total synthesis in the laboratory. The latest achievement by Nicolau's group is the conversion of vancomycin aglycone to vancomycin by adding two sugars. This is a significant piece of work because it demonstrates that selective addition of modified sugars to the vancomycin aglycone is possible in principle. It has been established that the sugars of glycopeptides are important in promoting their activity, and so vancomycin with modified sugars would have enhanced activity against both VRE and MRSA [36].

The molecular target of glycopeptide antibiotics is the D-Ala-D-Ala terminus of the N-acetyl-muramyl-pentapeptide subunit of the growing cell wall in Gram-positive bacteria. By binding to this terminal dipeptide, glycopeptide antibiotics interfere with proper cell wall formation, which results in cell death [54]. Teicoplanin, a lipid-containing glycopeptide, has the same mechanism of action as vancomycin, but its action might be enhanced by its ability to be anchored in the membrane.

Many Gram-positive pathogens including streptococci, staphylococci, clostridia and corynebacteria are susceptible in vitro to vancomycin and teicoplanin. However, some Gram-positive bacteria (*Enterococcus casseliflavus, E. flavescens, E. gallinarum* and many lactobacilli) are intrinsically resistant to glycopeptide antibiotics [5]. Gram-negative bacteria are, in general, resistant to glycopeptides because these antibiotics are unable to cross the outer cell envelope.

Resistance mechanisms: phenotypes and genes involved in resistance

In vancomycin susceptible enterococci, the dipeptide D-Ala-D-Ala synthesized by D-Ala:D-Ala ligases (Ddl) is added to a tripeptide precursor to form a pentapeptide precursor [54]. The D-Ala-D-Ala terminus is the target of vancomycin, which only binds after the transfer of cytoplasmic muramyl-pentapeptide to the lipid and further transfer across the membrane. Once vancomycin binds to the target, the utilization of the pentapeptide precursor for cell-wall synthesis is blocked. However, enterococci have developed a sophisticated mechanism to overcome glycopeptide antibiotics. Glycopeptide-resistant enterococci evade the action of vancomycin by modifying the antibiotic's molecular target: D-Ala-D-Ala, to D-Ala-D-Lac or D-Ala-D-Ser [10, 20]. Vancomycin binds these peptidoglycan precursors with greatly reduced affinity [10]. At least five glycopeptide resistance phenotypes (VanA, VanB, VanC, VanD and VanE) can be distinguished in enterococci on the basis of the level and inducibility of resistance to vancomycin and teicoplanin (Table 1). VanA and VanB are the most common resistance phenotypes.

VanA-type glycopeptide resistance is characterized by acquired resistance to high levels of both vancomycin and teicoplanin; resistance is induced by either vancomycin or teicoplanin. VanA-type resistance has been described for *Enterococcus faecalis, E. faecium, E. durans, E. raffinosus, E. hirae, E. avium* and *E. gallinarum* [13, 32, 40, 47, 48, 54]. VanB-type resistance is characterized by acquired resistance to various concentrations of vancomycin but not to teicoplanin; resistance is induced only by vancomycin and not by teicoplanin. VanB-type glycopeptide resistance has been described for *E. faecalis, E. faecium, E. gallinarum* and *E. raffinosus* [13, 28, 32]. The genes encoding the VanA and VanB phenotypes are carried on transposons Tn*1546* and Tn*1547*, respectively, which may be found on plasmids or inserted on the chromosome [5]. The *vanA* and *vanB* operons contain seven genes (Fig. 4). The functions of the proteins encoded by six of these genes have been studied extensively [see review in ref. 5]. Genes *vanS* ($vanS_B$) and *vanR* ($vanR_B$) encode a two-component signal-transducing regulatory system required for the induction of resistance genes in response to the presence of glycopeptide antibiotics in the medium. VanA- and VanB-type strains are therefore characterized by the inducible expression of resistance genes. The *vanA* or *vanB* and *vanH* ($vanH_B$) genes enable enterococci to synthesize peptidoglycan precursors terminating in D-Ala-D-Lac, rather than in D-Ala-D-Ala [1, 25, 31]. *vanA* and *vanB* encode ligases which ligate D-Lac to D-Ala, and *vanH* ($vanH_B$) encodes a dehydrogenase which synthesizes D-Lac from pyruvate. The *vanX* ($vanX_B$) and *vanY* ($vanY_B$) genes encode a DD-dipeptidase and a DD-carboxypeptidase, respectively, which eliminate the production of peptidoglycan precursors ending in D-Ala-D-Ala [43, 44]. The DD-dipeptidase cleaves the D-Ala-D-Ala dipeptide

Table 1 Types of glycopeptide resistance in *Enterococcus* spp.

Type[1]	Expression	Location of genes	MIC (µg/ml)		Transferable by conjugation	Termination of peptidoglycan precursor	Species
			Vancomycin	Teicoplanin			
VanA	Inducible	Chromosome and plasmids (Tn*1546*)	>128	>64	+	D-Ala-D-Lac	*E. faecium* *E. faecalis* [2]
VanB	Inducible	Chromosome and plasmids (Tn*1547*)	16–1024	<2	+	D-Ala-D-Lac	*E. faecium* *E. faecalis* [2]
VanC	Constitutive	Chromosome	4–16	0.5–1	–	D-Ala-D-Ser	*E. gallinarum* *E. casseliflavus* *E. flavescens*[3]
VanD	Inducible or constitutive[4]	Chromosome	64–128	4	–	D-Ala-D-Lac	*E. faecium* *E. faecalis*
VanE	Inducible	ND[5]	16	0.5	–	D-Ala-D-Ser	*E. faecalis*

[1]All glycopeptide-resistance types are acquired, except VanC which is an intrinsic property of *E. gallinarum*,
E. casseliflavus and *E. flavescens*. For references see text.
[2]VanA and VanB resistance types have also been found in other *Enterococcus* spp. (see text for details).
[3]*E. casseliflavus* and *E. flavescens* are now considered the same species [27].
[4]Constitutive in *E. faecalis* BM4339 [41]; inducible in *E. faecium* A803, A804, and A902 [38].
[5]ND, not determined.

from the pentapeptide precursor, and the DD-carboxypeptidase, a Zn^{2+}-dependent enzyme, removes the C-terminal D-Ala [4, 5]. The *vanA* operon contains another open reading frame, *vanZ*, which encodes a protein that confers low-level teicoplanin resistance by an unknown mechanism. On the other hand, the *vanB* operon contains *vanW*, but the function of the corresponding protein is unknown [22].

The VanC phenotype is characterized by constitutive low-level resistance to vancomycin and susceptibility to teicoplanin, and is an intrinsic property of most *E. gallinarum, E. casseliflavus* and *E. flavescens* isolates [20, 54]. This type of resistance requires three proteins: VanC and $VanXY_C$, which catalyze the synthesis of D-Ala-D-Ser and which eliminate precursors ending in D-Ala-D-Ala, and VanT, a membrane-bound racemase for production of D-Ser [42, 20, 35, 3]. The nucleotide sequences of the *vanC-1* (*E. gallinarum*), *vanC-2* (*E. casseliflavus*), and *vanC-3* (*E. flavescens*) have been published [20, 35]. There is extensive homology between the
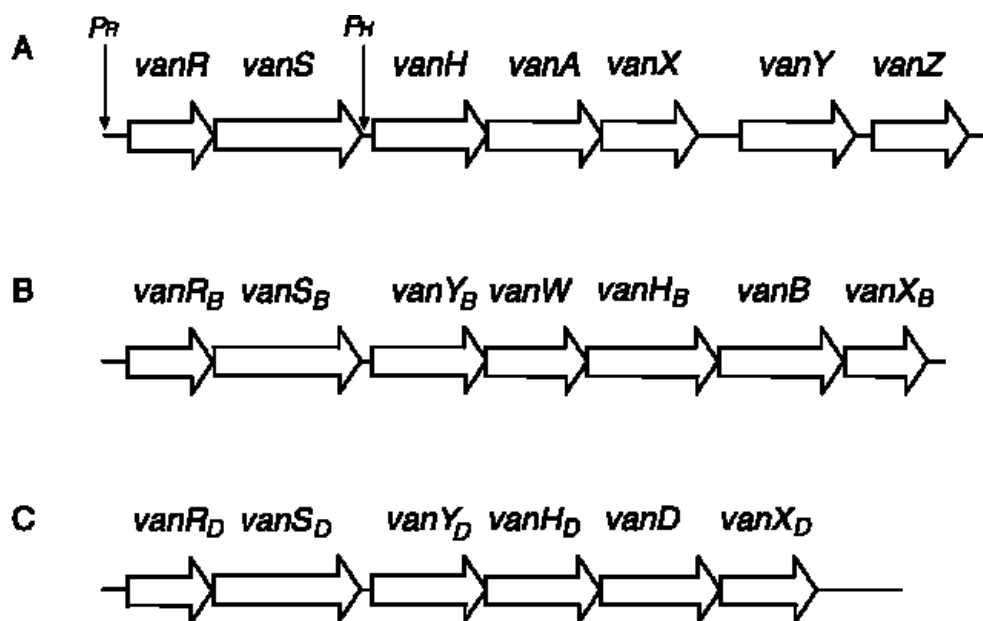

Fig. 4 Schematic representation of the *vanA* (A), *vanB* (B), and *vanD* (C) operons. P_R and P_H are the promotors controlling the gene expression

vanC-2 and *vanC-3* genes. In fact, in a recent study, *E. casseliflavus* and *E. flavescens* were found to be a single species, and the term "*E. casseliflavus*" was recommended to be retained as the species denomination [27]. The *vanC* gene products are structurally related to D-Ala:D-Ala ligases and to D-Ala:D-Lac ligases [23]. Apart from *vanC*, these strains also contain the chromosomal *ddl* gene that encodes the D-Ala:D-Ala ligase [35]. Unlike the genes determining the VanA- and VanB-type resistances, the *vanC* genes are located exclusively on the bacterial chromosome. The *vanC-1* gene product synthesizes D-Ala-D-Ser that replaces the D-Ala-D-Ala terminus in peptidoglycan precursors [39, 44]. Insertional inactivation of *vanC-1* in *E. gallinarum* caused reversion to vancomycin susceptibility, indicating that *vanC-1* is necessary for expression of vancomycin resistance [20]. The peptidoglycan precursors of the insertional mutant end in D-Ala-D-Ala. The gene downstream from *vanC-1* encodes VanXY$_c$, which has both DD-dipeptidase and DD-carboxypeptidase activities [42]. The VanXY$_c$ protein is similar to VanY in an overlap of 158 amino acids, and contains consensus sequences for binding zinc, stabilizing the substrate and catalyzing hydrolysis, present in both VanX (VanX$_B$) and VanY (VanY$_B$). Unlike VanX, VanXY$_c$ has very low dipeptidase activity against D-Ala-D-Ser, and unlike VanY, it has no activity against peptidoglycan precursors ending in D-Ala-D-Ser [42]. The function of VanXY$_c$ seems to be the hydrolysis of D-Ala-D-Ala and the removal of C-terminal D-Ala from peptidoglycan precursors ending in D-Ala-D-Ala. *E. gallinarum* also produces VanT, a membrane-bound serine racemase which catalyses conversion of L- to D-Ser [3]. Insertional inactivation of the *vanC-1* gene has a polar effect on the expression of *vanXY$_C$* and *vanT* genes.

E. gallinarum isolates that carry both the *vanA* and *vanC-1* genes have been described [40]. Besides, the *vanC-1* gene has been detected in some strains that, under phenotypic analysis, appeared to be *E. faecalis* and *E. faecium* [40; Pérez-Hernández X, Méndez-Álvarez S, Claverie-Martín F, unpublished data].

Recently, two new types of acquired glycopeptide resistance, designated as VanD and VanE, have been described in *E. faecium* and *E. faecalis* strains, respectively [24, 41]. These two types of resistance are uncommon because only a few isolates have been identified. The VanD phenotype is characterized by resistance to intermediate levels of vancomycin and to low levels of teicoplanin (Table 1), and is a result of synthesis of D-Ala-D-Lac-terminating peptidoglycan precursors [41]. The VanD phenotype has been found in *E. faecium* BM4339 and in three other *E. faecium* isolates [38, 41].

In *E. faecium* BM4339, the VanD phenotype is expressed constitutively [41], whereas in three other isolates the vancomycin resistance is induced by vancomycin [38]. The genes determining the VanD phenotype appear to be located on the chromosome and are not transferable to other enterococci [38, 41]. This could explain the scarcity of recognized VanD

strains in contrast to the widespread and high-prevalence VanA and VanB strains.

The *vanD* gene cluster contains six genes: *vanR$_D$*, *vanS$_D$*, *vanRY$_D$*, *vanH$_D$*, *vanD* and *vanX$_D$* (Fig. 4C) [11]. The deduced VanH$_D$ dehydrogenase, VanD ligase, and VanX$_D$ DD-dipeptidase are highly similar to the corresponding proteins encoded by the vanA and vanB operons. The deduced VanY$_D$ protein is homologous to penicillin-binding proteins that display DD-carboxypeptidase activity. No genes homologous to *vanZ* and *vanW* from the *vanA* and *vanB* operons, respectively, have been found in the *vanD* gene cluster [11]. The D-Ala:D-Ala ligase of *E. faecium* BM4339 is not functional due to a frameshift mutation in the chromosomal *ddl* gene. However, the growth of BM4339 does not depend on the presence of glycopeptides, because the expression of the *vanD* genes in this strain is constitutive [41].

VanE-type resistance has been described in one only strain of *E. faecalis*, BM4405, which is resistant to low levels of vancomycin and susceptible to teicoplanin [24]. Resistance in BM4405 is inducible by vancomycin. A gene with homology to *vanC* has been partially characterized. This is consistent with the fact that strain BM4405 synthesizes peptidoglycan precursors that end in D-Ala-D-Ser. A serine racemase activity is present in the membrane fraction of BM4405. Therefore, VanE-type resistance is due to synthesis of peptidoglycan precursors ending in D-Ala-D-Ser. It is unknown whether the resistance genes are located on the chromosome or in plasmids.

Regulation of glycopeptide resistance

The presence of vancomycin or teicoplanin induces the expression of the *vanA* operon in VanA-type resistant enterococci. Induction takes place at the transcriptional level by a two-component regulatory system, VanR-VanS [reviewed in ref. 54]. The presence of the glycopeptide in the medium results in autophosphorylation of a histidine residue on the VanS sensor. The phosphoryl group is transferred to an aspartate residue on the response regulator VanR. The phosphorylated VanR binds to the *vanR$_P$* and *vanH$_P$* promoter regions, and activates transcription of the vancomycin resistance genes (Fig. 4A). As with other response regulators, phosphorylation of VanR increases its affinity for the promoter region, which is essential for activation of transcription [26, 53].

The regulatory mechanism in VanB strains must be different because, in contrast to the *vanA* operon, the VanR$_B$-VanS$_B$ system mediates promoter activation only in the presence of vancomycin. VanB-type constitutive variants harbor mutations in the *vanS$_B$* sensor gene. These mutations are thought to impair dephosphorylation of the VanR$_B$ regulator [6].

The VanR$_D$ and VanS$_D$ proteins from *E. faecium* BM4339 contain the characteristic amino acids involved in protein phosphorylation, as well as the motifs conserved in response regulators and protein kinases [11]. Casadewall and Courvalin

[11] have suggested that the constitutive phenotype of *E. faecium* BM4339 may be a result of mutations located near the putative autophosphorylation site, and which are known to alter the phosphatase activity of VanS$_B$ [6]. Alternatively, the signal recognition properties of VanS$_D$ may be impaired, resulting in phosphorylation of VanR$_D$, even in the absence of vancomycin.

Origin of glycopeptide resistance in enterococci

The origin of the genes encoding high-level resistance to glycopeptides in enterococci is unknown. It has been postulated that, under the pressure of increased glycopeptide usage both in agriculture and in clinical practice, the vancomycin resistance genes present in other microorganisms of the environment may have been transferred to enterococci [45]. Both the intrinsically vancomycin-resistant lactic acid bacteria (*Leuconostoc*, *Pediococcus*, and *Lactobacillus*) and the glycopeptide-producing microorganisms are potential reservoirs. Alternatively, the genes may have originated through mutations in homologous genes within enterococci or other bacteria. *Leuconostoc mesenteroides* produces a D-Ala:D-Lac ligase and a D-lactate dehydrogenase, although its ligase differs notably from that of enterococci in a region that is critical for catalysis. Marshall et al. [29] have cloned three genes encoding homologues of VanH, VanA (VanB) and VanX from both *Streptomyces toyocaensis* and *Amycolatopsis orientalis*, two organisms which produce glycopeptide antibiotics. Furthermore, the arrangement of the *vanH*, *vanA* (*vanB*), and *vanX* genes in these organisms is identical to the arrangement found in VRE. These results indicate that glycopeptide-producing organisms use a resistance mechanism that is similar to that found in VRE, and suggest that they have been the source of resistance genes in VRE. However, not all the vancomycin resistance genes may have been acquired in a single transfer from another organism. There is very strong evidence that the basic mechanism evolved in one of the producer organisms, but the regulatory system could easily have come from another Gram-positive bacterium. Accordingly, the G+C contents of the VRE *vanH*, *vanA* (*vanB*), and *vanX* genes are 5–10% higher than those of the adjacent *vanR*, *vanS*, *vanY*, and *vanZ* (or *vanW*) [29]. It seems therefore, that glycopeptide-producing organisms with different G+C contents than *S. toyocaensis* and *A. orientalis* genes may have served as donors of the *vanHA(B)X* gene cluster. Alternatively, bacteria that do not produce glycopeptide may have acquired the glycopeptide resistance genes as a defense mechanism, and eventually they may have passed them to enterococci.

It has been hypothesized also that a gene resembling the vancomycin resistance genes in enterococci might have conferred vancomycin resistance to *Bacillus popiliae* [46]. Since biopesticides containing spores of *B. popiliae* have been used for more than fifty years in the United States for agricultural purposes, this bacterium might have served as donor of the vancomycin resistance gene.

Epidemiology

Enterococci have emerged in recent years as pathogens in a growing number of severe nosocomial infections. Especially worrying is the increased number of VRE. Knowledge of the epidemiology of VRE is essential to prevent it from spreading. Note that the epidemiology of enterococci resistance to vancomycin shows different patterns in Europe and in USA. In Europe, resistance to vancomycin has been detected not only in hospital samples, but also in community-acquired infections, as well as in asymptomatic carriers and farm animals. The emergence of such resistant strains has been associated with the use of avoparcin, a glycopeptide antibiotic that has been used as a growth promoter in farm animals [47]. The use of avoparcin may have provided a selective pressure for the emergence of VRE in poultry and pigs, which probably are a major reservoir of VRE for humans [50]. Some human isolates are indistinguishable from isolates derived from animal sources, which suggests that VRE from animals may pass to humans via the food chain. For this reason, avoparcin administration has been restricted in the European Union. In contrast, in USA, where avoparcin has never been licensed, vancomycin resistance has only been detected in hospitals. Emergence of such resistance is associated with frequent vancomycin administration, especially via the oral route, and with the use of high doses of the antibiotic [17].

The spread of VRE involves the clonal dissemination of a single resistant strain and the genetic transfer of resistance-encoding plasmids and/or transposons between different strains or even between different species. Since phenotypic detection of low vancomycin resistance levels by the usual sensitivity tests is difficult, molecular techniques such as Polymerase Chain Reaction (PCR), genetic fingerprinting, Pulsed Field Gel Electrophoresis-Restriction Fragment Length Polymorphisms (PFGE-RFLPs), etc. permit an improvement not only in diagnosis but also in the knowledge of the numbers and types of resistant strains at a given time in a given location. This knowledge is useful for the design of plans for prevention, care and control of the emergence of vancomycin resistance, and for the establishment of rules for the proper use of antibiotics.

Molecular methods to study glycopeptide resistant enterococci

Since some vancomycin resistance genes are transferable among different enterococcal species or even among different genera [54], the lack of prompt detection of enterococci may cause delays in attempting to eliminate VRE colonization and infection. Conventional identification methods, which are based

on culturing bacteria, require two to three days to provide results, and even then are not discriminatory enough. The development of rapid, sensitive PCR-based assays has improved the speed and accuracy of the diagnosis of enterococcal infections. PCR provides a means for culture-independent detection of enterococci in a variety of clinical specimens, and can yield results in just a few hours. Several PCR-based methods for the specific detection of enterococci have been reported [14, 19, 27]. One such method is based on targetting the *ddl* gene, permitting the specific detection of *E. faecalis* and *E. faecium* [19]. For the specific detection of *E. gallinarum* and *E. casseliflavus/E. flavescens*, a PCR assay amplifying different *vanC* genes has been developed [14, 19]. Recently, Ke et al. [27] have developed a rapid PCR assay for the specific detection of all enterococci of clinical importance at the genus level. By targeting the *tuf* gene, which encodes elongation factor EF-Tu, this method makes it possible to detect most enterococcal species to a highly sensitive degree, with acceptable specificity [27].

PCR can be used to discriminate between different kinds of genes encoding resistance to glycopeptides. In this instance, primers that specifically hybridize to different conserved DNA sequences in the different resistant genotypes are used to amplify particular gene fragments that allow different resistance types to be distinguished [19, 40, 51]. Recently, a Multiplex PCR assay that allows simultaneous detection of glycopeptide resistance genes (*vanA*, *vanB*, *vanC-1*) and identification at the species level, of clinically relevant enterococci (*E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*) has been developed [19]. Another method developed by Patel et al [40] is a PCR-RFLP assay which can be performed directly with isolated colonies of *Enterococcus* spp. to detect and discriminate *vanA*, *vanB*, *vanC-1* and *vanC-2/3* genes. The recent cloning and characterization of the *vanD* and *vanE* genes will permit the development of PCR assays for the specific detection of these new types of glycopeptide resistance [24, 41]. Subsequent DNA sequencing of the PCR fragments gives information on the variability of glycopeptide resistance genes within each type of resistance class.

Other molecular tests allow to obtain an accurate, precise identification of each isolate, as well as to estimate the genetic diversity within each resistance type. The determination of 16S ribosomal RNA sequences has become a common procedure [27, 33]. Fragments of the 16S rRNA can be retrieved from the bacteria by applying PCR with a set of primers known as 16S rRNA primers. The 16S rDNA fragment can be sequenced, and the sequence compared to all the isolates and to those in a large database. The sequence information is sufficient to classify a strain to a genus or species level. Alternatively, ribotyping or ARDRA (Amplified Ribosomal DNA Restriction Assay) can be performed. To carry out the ribotyping, a 16S rDNA fragment is labeled and hybridized against digested genomic DNA in order to obtain a hybridization pattern which, depending on the experimental conditions, may show specificity at subspecies,

species or genus levels [49]. ARDRA involves the digestion of the PCR fragment with restriction enzymes that cut in the variable or hypervariable regions of the 16S rRNA gene.

Additionally, other methods are available to perform rapid analyses that provide a tool for determining subtle differences among highly-related enterococci. Such molecular tools include REP-PCR (Repetitive Extragenic Palindrome PCR), RAPD (Random Amplified Polymorphic DNA) and RFLP [18, 33, 51]. REP-PCR is based on the fact that genomes of microorganisms contain repetitive sequences. In the genomes of different enterococci, the repetitive sequences are in different relative locations to each other. Distances between pairs of repetitive elements that are not more than a few kilobase-pairs apart can be determined by PCR, and the result is a number of DNA fragments differing in size. In this way, subtle genome differences between strains can be detected relatively quickly, although the method does not work when a particular strain contains too few or unknown repetitive elements. RAPD has been shown to be very efficient in distinguishing related enterococcal isolates [33]. This method is based on the amplification of genomic DNA sequences by using a single random primer that is not targeted towards any specific DNA sequence in the genome. Under low-stringency conditions, a number of different sites of annealing are present on the genome depending on the length, on the nucleotide sequence and on the G+C content of the primer. This technique performed on a given genomic DNA generates a pattern of amplification products.

RFLP analyses also attempt to generate banding patterns that reflect genetic differences between bacteria. RFLP analyses can be performed in several different variations: patterns generated by ARDRA (see this section above) and PFGE macrorestriction have been the most successful to date. PFGE macrorestriction patterns involve the comparison of total DNAs cleaved with a restriction enzyme that cuts infrequently, and separated by PFGE [30, 51]. PFGE has been demonstrated to be a suitable typing method for enterococci [51]. Furthermore, the capacity of PFGE to separate long DNA fragments, even chromosomal or high-molecular-weight extrachromosomal elements, combined with hybridization techniques, allows elucidation of the type of genetic element harboring a specific DNA sequence.

Finally, it should be taken into account that, in some studies, only the combination of different molecular techniques will yield the desired degree of discrimination.

Concluding remarks

The mechanism by which resistant enterococci evade the action of glycopeptides is based on the use of altered peptidoglycan precursors that have low affinity for the antibiotic. Genetic variations have led to the existence of at least five different glycopeptide resistance types, some of which are encoded in transferable genetic elements, i. e. transposons or plasmids (Table 1). Two of these five types, VanD and VanE, have been

described only recently, and we should be ready to face the emergence of new resistance types. Furthermore, the transferable nature of inducible resistance operons is a putative risk of the spread of glycopeptide resistance to more virulent bacteria. Although the transfer of vancomycin resistance genes has not been reported in a clinical setting, the transfer of *vanA* genes from *E. faecalis* to *S. aureus* has been demonstrated in vitro [37].

Since phenotypic typing methods are not discriminatory enough, to stop the spread of VRE, it is essential to use molecular techniques that allow a rapid, accurate identification of enterococci and their resistance type. Thus, fast, accurate molecular methods will be an essential diagnostic tool for microbiology laboratories.

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