Heterologous expression of glycopeptide resistance vanHAX gene clusters from soil bacteria in Enterococcus faecalis

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Objectives: The aim of the study was to determine whether glycopeptide resistance gene clusters from soil bacteria could be heterologously expressed in Enterococcus faecalis and adapt to the new host following exposure to vancomycin.

Methods: The vanHAX clusters from Paenibacillus thiaminolyticus PT-2B1, Paenibacillus apiarius PA-B2B and Amycolatopsis coloradensis DSM 44225 were separately cloned in an appropriately constructed shuttle vector containing the two-component regulatory system (vanRS) of Tn1546. The complete vanAPT operon (vanRSHAXY) from P. thiaminolyticus PT-2B1 was cloned in the same shuttle vector lacking enterococcal vanRS. All plasmid constructs were electroporated into E. faecalis JH2-2 and the MICs of vancomycin and teicoplanin were determined for each recombinant strain before and following exposure to sublethal concentrations of vancomycin.

Results: The vanHAX clusters from P. thiaminolyticus and P. apiarius conferred high-level vancomycin resistance (MIC ≥ 125 mg/L) in E. faecalis JH2-2. In contrast, cloning of the vanHAX cluster from A. coloradensis did not result in a significant increase of vancomycin resistance (MIC = 0.7 mg/L). Resistance to vancomycin was not observed after cloning the complete vanAPT operon from P. thiaminolyticus (MIC = 2 mg/L), but this recombinant rapidly adapted to high concentrations of vancomycin (MIC = 500 mg/L) following exposure to sub-lethal concentrations of this antibiotic.

Conclusion: The results showed that vanAPT in P. thiaminolyticus is a possible ancestor of vanA-mediated glycopeptide resistance in enterococci. Experimental evidence supported the hypothesis that enterococci did not acquire glycopeptide resistance directly from glycopeptide-producing organisms such as A. coloradensis.

Keywords: vanA, Amycolatopsis coloradensis, Paenibacillus

Introduction

Vancomycin and teicoplanin are glycopeptide antibiotics of critical importance in the treatment of severe nosocomial infections caused by staphylococci and enterococci. Resistance to this class of antibiotics was initially reported in enterococci in 1988 and has recently emerged in Staphylococcus aureus.²³ Six types of glycopeptide resistance operons have been described in enterococci (vanA to vanE and vanG).⁴⁻⁵ The vanA type is the most common in enterococci and the only one reported in S. aureus. This operon is associated with the transposable element Tn1546 and contains two regulatory genes (vanR and vanS), three resistance genes (vanH, vanA and vanX) and two accessory genes that are not required for glycopeptide resistance (vanY and vanZ). The minimal requirement for glycopeptide resistance is the expression of D-lactate dehydrogenase (vanH), D-Ala-D-Lac ligase (vanA) and DD-peptidase (vanX) genes, which results in the synthesis of peptidoglycan precursors with low affinity for glycopeptides and elimination of native (susceptible) precursors, therefore conferring high-level resistance to glycopeptides.⁴

Glycopeptide resistance operons also occur in Gram-positive bacilli. The vanF operon is associated with Paenibacillus popilliae, a biopesticide which was used in the United States for more than 50 years for suppression of Japanese beetle...
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populations. Recently, operons with up to 94% gene identity to \textit{Tn1546} have been described in \textit{Paenibacillus} spp. isolated from soil. Genetic elements resembling \textit{vanA} operons have also been reported in clinical isolates of \textit{Bacillus circulans}, \textit{Oerskovia turbata} and \textit{Arcanobacterium haemolyticum}. More distantly related \textit{vanHAX} gene clusters are known to occur in glycopeptide-producing actinomycetes. The \textit{vanHAX} clusters in producers have been regarded as the possible ancestors of glycopeptide resistance operons in enterococci; however, the lack of a two-component regulatory system and the low similarity with enterococcal \textit{vanHAX} clusters (54–64% predicted amino acid identity) suggest that glycopeptide resistance operons in enterococci were not directly acquired from glycopeptide producers. Other bacteria, most probably Gram-positive endospore-forming bacilli, may have acted as donors of glycopeptide resistance to enterococci.

In this study, the \textit{vanHAX} clusters from two soil \textit{Paenibacillus} strains and the glycopeptide producer \textit{Amycolatopsis coloradensis} were cloned into \textit{Enterococcus faecalis} to examine their ability to confer resistance to vancomycin and teicoplanin. The aim of the study was to determine whether glycopeptide resistance gene clusters of soil origin could be heterologously expressed in enterococci. Such information is essential for clarifying the possible role played by different soil bacteria in the acquisition of glycopeptide resistance by enterococci.

Materials and methods

Bacterial strains

The avoparcin-producing strain \textit{A. coloradensis} DSM 44225 was originally isolated from soil in Colorado. Glycopeptide-resistant \textit{Paenibacillus thiaminolyticus} PT-2B1 and \textit{Paenibacillus apiarius} PA-B2B were isolated from Danish soil. \textit{E. faecalis} JH2-2, a glycopeptide-susceptible strain commonly used for gene transfer experiments in \textit{Enterococcus}, was used as a recipient for studying heterologous expression of glycopeptide resistance. \textit{Enterococcus faecium} BM4147 was the first strain in which glycopeptide resistance of the \textit{vanA} type was described and was a positive control in the cloning experiments.

Sequencing of the \textit{vanHAX} cluster in \textit{A. coloradensis} DSM 44225

We sequenced the complete DNA region covering the \textit{vanHAX} cluster in \textit{A. coloradensis} DSM 44225, including 123 bp upstream of the start codon of \textit{vanH} and 281 bp downstream of the stop codon of \textit{vanX} (3113 bp). The D-Ala-D-Ala ligase from \textit{A. coloradensis} was amplified and sequenced using degenerate primers designed to detect \textit{vanA} and \textit{vanB} homologues. The flanking regions were sequenced by inverse PCR. Chromosomal DNA was purified using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim Germany), digested with EcoRV (New England Biolabs, Beverly, MA, USA), which did not cut within the amplified fragment, and purified using a GFX PCR DNA gel band purification kit (Amersham Biosciences, Buckinghamshire, UK). Subsequently, chromosomal DNA was circularized using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) and 2 \muL of the ligation mix was used for PCR amplification and DNA sequencing using specific primers designed to read outwards from the initial sequence. Based on these sequencing results, additional restriction digestions of the chromosomal DNA were carried out using \textit{SalI} and \textit{SacI}, and the circularization of purified restricted DNA was repeated as described above. Again, the circularized DNA was amplified with primers designed to read upstream and downstream of the previously sequenced DNA. These PCR products were sequenced and new primers were designed based on this new sequence, until the complete sequence of the gene cluster was obtained. Sequencing of the PCR products was carried out using an AmpliTag dye terminator kit and a 373A automatic sequencer (Applied Biosystems, Foster City, CA, USA). The Vector NTI suite, v8.0 (Invitrogen), was used to assemble sequencing fragments.

Construction of pHHA223

The \textit{vanHAX} genes of \textit{P. apiarius} PA-B2B, \textit{P. thiaminolyticus} PT-2B1 and \textit{A. coloradensis} DSM 44225 were cloned in the vector pHHA223. This vector was based on the promoter probe vector \textit{pAK80}, which carries an erythromycin resistance cassette for selection in both Gram-positive and Gram-negative bacteria. The pHHA223 vector was designed to contain the regulatory system from \textit{Tn1546} (\textit{vanRS}) as well as the corresponding promoters upstream of the \textit{vanK} and \textit{vanH} genes, respectively. It was constructed by digesting \textit{pAK80} with \textit{BglII} and \textit{SalI} to substitute the \textit{lacLM} genes with a small oligo linker. This linker was created using primers 842 and 843 (all primers are listed in Table 1), containing an internal \textit{SmaI} site and overhangs compatible with \textit{BglII} and \textit{SalI} digested DNA. The digested \textit{pAK80} and the linker were ligated to generate the plasmid pHHA214. pHHA214 was then digested with \textit{SalI} and \textit{SmaI}, and was ligated to a PCR-generated fragment containing the transcriptional terminator from \textit{rrnB} present on the pBAD myc-his/c plasmid (Invitrogen), which was amplified using primer 1534 carrying a \textit{SmaI} site, and R583 carrying a \textit{SalI} site, thus generating the plasmid pHHA222. The two-component regulatory system from \textit{Tn1546} (\textit{vanRS}), its promoter (\textit{P\textit{vanR}}) and the \textit{vanA} promoter situated downstream (\textit{P\textit{vanH}}) were amplified from \textit{E. faecium} BM4147 using primers 1306 containing a HinIII site and 1307 containing a BglII site. The PCR product was digested with HinIII and BglII and ligated in pHHA222 digested with the same two enzymes, resulting in the plasmid pHHA223. The integrity of pHHA223 was confirmed by plasmid isolation using a Qiagen Plasmid Midi kit (Qiagen Inc., Mississauga, ON, Canada) followed by sequencing using primers flanking the inserted DNA as well as primers matching the internal sequence. In all cases, the following PCR programme was used: 5 min at 95°C, 25 cycles including 10 s at 98°C, 15 s at 60°C and 2 min at 72°C, followed by 10 min of a final extension at 72°C.

Cloning of \textit{vanHAX} clusters in pHHA223

The three \textit{vanHAX} gene clusters of soil origin were cloned in pHHA223, generating the variants pHHA224 (\textit{P. apiarius}), pHHA227 (\textit{P. thiaminolyticus}) and pHHA228 (\textit{A. coloradensis}). In addition, the \textit{vanHAX} cluster from \textit{E. faecium} BM4147 was cloned in pHHA223 to form pHHA225.

All \textit{vanHAX} clusters were amplified using Phusion™ High-fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) with the primers listed in Table 1. Amplification was carried out using the following programme: 30 s at 98°C, 25 cycles with 10 s at 98°C, 15 s at 68°C and 2 min at 72°C, followed by 10 min at 72°C. Purified PCR products were digested either with \textit{BglII} plus \textit{SalI} or, for the \textit{vanHAX} cluster from \textit{A. coloradensis}, with BamHI plus \textit{SalI} and ligated into pHHA223. All constructs were transferred into electrocompetent \textit{E. coli} DH10B cells (Invitrogen) and subsequently transferred into electrocompetent \textit{E. faecalis} JH2-2 as described.
Previously, the sequence of the insert in each plasmid construct was confirmed.

**Cloning of vanAPT operon from P. thiaminolyticus in pHHA222**

The complete vanAPT operon (vanRSHAXY) from *P. thiaminolyticus* was cloned in pHHA222. Two primers (1542 and 1543) were designed to amplify a 7318 bp fragment covering the vanAPT operon in *P. thiaminolyticus* PT-2B1 (GenBank ID: DQ018710) and the 1151 bp region upstream of vanAPT containing a putative promoter. Both primers were designed to contain BgII sites (underlined) for cloning the vanAPT operon in the BgII site of pHHA222. The final construct was named pHHA226. The integrity of pHHA226 was confirmed by plasmid isolation as described earlier followed by sequencing using internal primers covering the complete gene cluster.

**MIC determination**

*E. faecalis* JH2-2 transformed with pHHA224, pHHA225, pHHA226, pHHA227 and pHHA228 was tested for susceptibility to vancomycin and teicoplanin. Susceptibility was assessed by the broth microdilution method according to the CLSI guidelines.²² The MICs of vancomycin were determined in cation-adjusted Mueller–Hinton broth using concentrations between 0.2 and 2000 mg/L. The MICs of teicoplanin were determined using custom-made panels covering a range from 2 to 32 mg/L (Trek Diagnostic Systems, Basingstoke, UK).

**Growth experiments**

Recombinant strains were grown on Mueller–Hinton agar (Oxoid, Basingstoke, UK) plates containing 20 mg/L erythromycin to maintain the transformed plasmids and incubated overnight at 37°C. Bacterial cultures were diluted in Mueller–Hinton II broth according to the CLSI recommendations for antibiotic susceptibility testing²² and 100 μL was inoculated into 100-well Bioscreen microplates containing an equal volume of Mueller–Hinton broth supplemented with 40 mg/L erythromycin and vancomycin at concentrations ranging from 0 to 128 mg/L. The Bioscreen microwell plate was inserted into Bioscreen C apparatus (Oy Growth Curves AB Ltd., Helsinki Finland) and analysed using the software Research Express (Transgalactic Ltd, Helsinki, Finland). Hardware settings were as follows: temperature 37°C, shaking before measurement (10 s, medium, 80 steps) and measuring at OD₅₉₀ every 15 min in 24 h. Each strain was tested in the same medium at least three times and the minimal doubling time (Tₘ₉₀) and the time until the maximal growth rate was reached (T₉₀ max) were calculated by averaging these data.

Adaptation of pHHA226 to JH2-2 was studied following exposure to sub-lethal concentrations of vancomycin. JH2-2 containing pHHA226 and pHHA222 (control) was exposed to gradually increased concentrations of vancomycin (0.5–6 mg/L). The two recombinant strains were grown overnight at 37°C in Mueller–Hinton II broth containing 20 mg/L of erythromycin and 0.5 mg/L of vancomycin. Aliquots of 125 μL of each of the two cultures were transferred into fresh tubes containing the same concentration of vancomycin. This was repeated until the strain carrying pHHA226 was able to grow in the presence of the higher concentration of vancomycin (6 mg/L). This adapted strain was then tested for susceptibility to vancomycin and teicoplanin, as described earlier.

**Accession number**

The complete sequence of the vanHAX cluster in *A. coloradensis* DSM 44225 was submitted to GenBank (accession no. DQ246438).

**Results and discussion**

The existence of a vanHAX-like gene cluster in the avoparcin-producing soil bacterium *A. coloradensis* has been reported previously.¹²,¹³ We determined the entire nucleotide sequence of the vanHAX gene cluster in *A. coloradensis*, revealing the
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<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>MIC of vancomycin (mg/L)</th>
<th>MIC of teicoplanin (mg/L)</th>
<th>Origin of the vanHAX genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH2-2 (negative control)</td>
<td>0.7</td>
<td>≤0.5</td>
<td>NA</td>
</tr>
<tr>
<td>BM4147 (positive control)</td>
<td>750</td>
<td>&gt;32</td>
<td>Tn1546</td>
</tr>
<tr>
<td>JH2-2 (pHHA222)</td>
<td>0.7</td>
<td>≤0.5</td>
<td>NA</td>
</tr>
<tr>
<td>JH2-2 (pHHA223)</td>
<td>0.7</td>
<td>≤0.5</td>
<td>NA</td>
</tr>
<tr>
<td>JH2-2 (pHHA224)</td>
<td>125</td>
<td>4</td>
<td><em>P. apiarius</em></td>
</tr>
<tr>
<td>JH2-2 (pHHA225)</td>
<td>500</td>
<td>4</td>
<td>Tn1546</td>
</tr>
<tr>
<td>JH2-2 (pHHA227)</td>
<td>250</td>
<td>4</td>
<td><em>P. thiaminolyticus</em></td>
</tr>
<tr>
<td>JH2-2 (pHHA228)</td>
<td>0.7</td>
<td>≤0.5</td>
<td><em>A. coloradensis</em></td>
</tr>
</tbody>
</table>

NA, not applicable.

The vanHAX gene clusters from *P. apiarius* (pHHA224), *A. coloradensis* (pHHA228), *P. thiaminolyticus* (pHHA227) and *E. faecium* BM4147 (pHHA225) were cloned into the vancomycin-responsive expression vector pHHA223. This strategy positioned the different vanHAX gene clusters downstream of the vanA promoter from Tn1546 under inducible control of the vanR4 and vanS4, thus excluding differential expression of their native promoters. *E. faecalis* JH2-2 transformants containing pHHA224, pHHA227 and pHHA225 (positive control) were fully resistant to vancomycin (Table 2). In contrast, the vanHAX cluster from *A. coloradensis* (pHHA228) did not result in any change in susceptibility to vancomycin or teicoplanin compared with the negative control (JH2-2 carrying pHHA223). Comparison of growth experiments using JH2-2 carrying either pHHA228 or pHHA223 showed that pHHA228 did not confer any growth advantage in the presence of vancomycin. Both recombinants exhibited growth at 0.6 mg/L with the same minimal doubling time ($T_{2_{\text{min}}}$) and full growth inhibition at 0.7 mg/L (data not shown). Introduction of the vanHAX cluster from *A. coloradensis* under the control of the vanA promoter and the vanR4 and vanS4 regulatory system into *E. faecalis* did not result in any growth advantage in the presence of vancomycin. As both the promoter and the regulatory system originated from Tn1546, this suggested that expression of the vanHAX genes from *A. coloradensis* in *E. faecalis* did not lead to functional proteins. An explanation for this could be insufficient translation caused by the significant difference in codon usage between high GC content actinomycetes and the low GC content *E. faecalis*; however, this was not examined further. As functional protein synthesis is a prerequisite for establishment and adaptation of the gene cluster in enterococci, it seems unlikely that glycopeptide resistance originated in enterococci from the acquisition of this gene cluster.

The levels of vancomycin resistance of *E. faecalis* JH2-2 transformants varied considerably depending on the cloned gene cluster, with the vanHAX cluster from *E. faecium* (pHHA225) conferring higher levels of resistance than those from *P. thiaminolyticus* (pHHA227) and *P. apiarius* (pHHA224). Heterologous expression of glycopeptide resistance in *E. faecalis* JH2-2 was correlated to the level of nucleotide identity of the cloned vanHAX cluster to Tn1546 since the vanHAX clusters in *P. thiaminolyticus* and *P. apiarius* are 92 and 87% identical to Tn1546, respectively. The observed differences in the vancomycin resistance phenotypes of the three recombinant strains were confirmed when minimal doubling time ($T_{2_{\text{min}}}$) was studied in the presence of vancomycin (Figure 1). Above 16 mg/L, JH2-2 carrying pHHA224 grew significantly slower than JH2-2 carrying pHHA225 or pHHA227. Furthermore, the time necessary to reach this minimal growth rate ($T_{\text{Vmax}}$) was significantly delayed in JH2-2 carrying pHHA224 (data not shown). Accordingly, the vanHAX cluster from *P. apiarius* seemed not to be functionally adapted to enterococci and could represent either an earlier step in the evolution of Tn1546 or a parallel evolution. Introduction of either pHHA224 or pHHA227 into *E. faecalis* JH2-2 conferred reduced susceptibility to teicoplanin (MIC ≤ 4 mg/L). This could be due to the lack of vanZ in the cloned gene clusters, as this gene may be required to confer full resistance to teicoplanin.

![Figure 1](https://academic.oup.com/jac/article-abstract/57/4/648/669450)
The genetic construct containing the entire vanA<sub>PT</sub> operon from \textit{P. thiaminolyticus} (pHHA226) resulted in only a small decrease in vancomycin susceptibility (MIC = 2 mg/L) and no change in teicoplanin susceptibility (MIC < 0.5 mg/L). However, acquisition of this operon by enterococci could result in a significant selective advantage in the presence of low concentrations of vancomycin. We tested whether exposure to sub-lethal concentrations of vancomycin could increase functional expression of the gene cluster in the heterologous host. Indeed, after 4 days of continued growth (approximately 27 generations), the strain was able to survive at higher concentrations of vancomycin (MIC = 500 mg/L) and teicoplanin (MIC = 8 mg/L). In contrast, JH2-2 containing pHHA222 was not able to adapt to higher concentrations of glycopeptides and maintained low MICs of vancomycin (0.7 mg/L) and teicoplanin (≤0.5 mg/L). Therefore, the complete vanHAX cluster from \textit{P. thiaminolyticus} conferred an initial growth advantage to \textit{E. faecalis} in the presence of sub-lethal concentrations of vancomycin as well as the ability to adapt to higher concentrations of the antibiotic within a short time. An explanation for the initial low level of resistance towards vancomycin of the cloned gene cluster in \textit{E. faecalis} could be that either the regulatory genes or the vanA promoter was not optimal for expression in this new host and therefore had to be adapted to the new environment. The nature of this adaptation still remains to be resolved. However, the requirement of relatively few generations indicates that only a few adaptive mutations are necessary in order to achieve high-level resistance to vancomycin.

It has been suggested that DNA contamination originating from the avoparcin-producing \textit{A. coloradensis} leads to the uptake of glycopeptide resistance genes by animal commensal bacteria.\textsuperscript{13} This DNA would then subsequently be taken up either through an intermediate bacterial host or directly by enterococci, thus ultimately leading to the emergence of glycopeptide-resistant enterococci. As an alternative hypothesis, Guardabassi et al.\textsuperscript{9} indicated that vanA operons nearly identical to those occurring in clinical enterococci might have existed in soil bacteria belonging to the genus \textit{Paenicbacillus} a long time before avoparcin was introduced as a feed additive in animal production. According to this hypothesis, glycopeptide resistance operons were acquired by enterococci from the bacteria belonging to the genus \textit{Paenicbacillus} or other soil bacilli where these genetic elements had previously emerged. This study corroborates this second hypothesis, indicating that vanA operons from paenibacilli could have been adapted in enterococci, conferring on them an ecological advantage in the presence of antibiotic selective pressure.

On the basis of current knowledge, the vanA operons from paenibacilli such as \textit{P. apiarius} and \textit{P. thiaminolyticus} described here can be considered the probable ancestors of enterococcal vanA operons. Nevertheless, transfer of vanA<sub>PT</sub> or vanA<sub>PT</sub> to enterococci has not been demonstrated \textit{in vitro}. Transfer might have occurred as a consequence of a rare recombination event and this could reflect the difficulties in reproducing such an event in the laboratory. It should be noted that the vanA<sub>PT</sub> operon is not associated with the transposase and the resolvase typical of Tn1546.\textsuperscript{9} Thus, it seems that Tn1546 has evolved from two components, the gene cluster conferring inducible glycopeptide resistance, which was probably acquired from Gram-positive bacilli, and the set of genes allowing transposition, the origin of which remain unknown. Interestingly, a small DNA fragment with 95\% identity to part of the Tn1546 transposase has recently been associated with the vanF gene cluster of \textit{P. popilliae}, indicating that both components could in fact have been present in paenibacilli simultaneously.\textsuperscript{23} Further research on the possible occurrence of this recombinase in glycopeptide-susceptible enterococci and in soil bacterial communities may provide useful information to understand the evolutionary process leading to the origin of Tn1546.

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Transparency declarations

None to declare.

References

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