The vanC-3 vancomycin resistance gene cluster of Enterococcus flavescens CCM 439

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Enterococcus flavescens CCM 439 is phenotypically similar to Enterococcus casseliflavus; it possesses intrinsic low-level resistance to vancomycin and has the VanC phenotype. The complete vanC-3 vancomycin resistance gene cluster was cloned and sequenced, and found to contain five open reading frames. These encoded five proteins that displayed a high degree of amino acid identity to the proteins of the vanC-2 cluster of E. casseliflavus. The serine racemases displayed the lowest degree of identity (97%), whereas the response regulators VanRC-2 and VanRC-3 were 100% identical. Long-PCR-RFLP analysis of the vanC-3 and vanC-2 gene clusters distinguished E. flavescens CCM 439 from E. casseliflavus ATCC 25788 due to the absence of a single EcoRV restriction endonuclease site from the E. flavescens gene cluster. However, the lack of nucleotide divergence between the sequences of the vanC-2 and vanC-3 clusters casts doubt on the validity of E. flavescens and E. casseliflavus being classed as distinct species.

Keywords: Enterococcus flavescens, vanC-3, vancomycin resistance

Introduction

The VanC vancomycin resistance phenotype is possessed by three species of enterococci, Enterococcus gallinarum, Enterococcus casseliflavus and Enterococcus flavescens. They have intrinsic, low-level resistance to the glycopeptide antibiotic vancomycin with MIC values ranging from 2 to 32 mg/L, but remain susceptible to the related antibiotic teicoplanin. Resistance to vancomycin is mediated by the production of cell wall precursors which terminate in D-alanyl-D-serine (D-Ala-D-Ser).1 In the absence of vancomycin, VanC strains that are inducible for resistance synthesize precursors that terminate in D-alanyl-D-alanine (D-Ala-D-Ala). The vanC-2 vancomycin resistance gene cluster of E. casseliflavus consists of five genes.2 The first three genes of the cluster, which are essential for resistance, encode a D-Ala: D-Ser ligase, VanC-2, followed by VanXYC-2, a protein possessing both D,D-dipeptidase and D,D-carboxypeptidase activities, and a serine racemase, VanTC-2. Expression of the resistance genes is controlled by a two-component regulatory system, which is present downstream of vanTC-2, consisting of a response regulator, VanRC-2 and a histidine kinase, VanSC-2. The proteins encoded within the vanC-2 gene cluster all display a high degree of amino acid identity to the equivalent proteins from the vanC cluster of E. gallinarum. The highest degree of amino acid identity, 91%, was observed between the response regulators and the lowest, 65%, between the serine racemases, VanTC-2 and VanT.2

E. flavescens is a yellow-pigmented species of Enterococcus that has only been identified recently.3 Like E. casseliflavus, which also possesses yellow pigmentation, E. flavescens is motile and possesses intrinsic low-level resistance to vancomycin; it is differentiated from E. casseliflavus only through its inability to produce acid from the fermentation of ribose.3 Since its first identification, some doubt has remained over the validity of describing E. flavescens as a distinct...
species. Only an internal portion of the vanC-3 D-Ala:D-Ser ligase gene from the vanC-3 cluster has been sequenced previously, and this 500 bp fragment displays 98.3% nucleotide identity to the vanC-2 gene from E. casseliflavus.4

This study describes the sequencing of the complete vanC-3 gene cluster of E. flavescens and comparison with the vanC-2 cluster of E. casseliflavus by sequence analysis and restriction fragment length polymorphisms (RFLPs).

Materials and methods

Bacteria

E. flavescens CCM 439 was originally derived from a clinical isolate and was obtained from the Central Public Health Laboratory, London NW9 5HT, UK.

Three strains of E. casseliflavus were used in the course of this study, ATCC 25788 (the type strain), NCTC 2321 and E93/640. E. casseliflavus E93/640 was also obtained from the Central Public Health Laboratory, and was derived from a clinical isolate. Bacteria were grown in Brain Heart Yeast extract (0.5% w/v) medium (Difco Bacto) at 37°C with gentle shaking and maintained on BHY agar.

Plasmid construction and cloning of the vanC-3 gene cluster by PCR

A PCR approach was used to clone and sequence the genes of the vanC-3 vancomycin resistance gene cluster. Primers were designed using sequence information from the vanC-2 gene cluster of E. casseliflavus.

A 1.6 kb PCR product containing the vanC-3 and vanXYC3 genes was obtained with primers C1 (5′-CTAAGAGCTCTCGGAAAAGCGGAAGGAAG) and S2 (5′-GTAATCTAGACTACTTTGAACTAGAGGT). The PCR product was digested with XbaI (Roche Molecular Biochemicals, Mannheim, Germany) and SacI (Roche), purified and ligated with pUC18 digested with the same enzymes to give plasmids pUCF1 and pUCF2, respectively. Plasmid pUCF3, containing vanRC3 and vanSC3, was constructed by cloning the 1.7 kb PCR product obtained using primers R1 (5′-CTCAGAGCTCGATCTAGATGTTGGAG) and S2 (5′-GTAAGGATCTTAAAGCGGTTGGTTACGA) into pUC18 after both the product and vector had been digested with SacI and BamHI (Roche). Plasmids were transformed into Escherichia coli XL1-Blue.

The intergenic region between vanTC3 and vanRC3 was amplified by PCR using Pwo polymerase (Roche) and primers T3 (5′-TTGCACCTGATCTTGAGG) and R4 (5′-GTAA-TCTAGAGCCAAAGCGGTCTGGTCCA), located at the 3′ and 5′ ends of vanTC3 and vanRC3, respectively, and was sequenced directly. The sequences of vanC-3 and vanXYC3 of vanTC3 and of vanRC3 and vanSC3 were obtained by sequencing the inserts of plasmids pUCF1, pUCF2 and pUCF3, respectively. Sequencing was carried out by the dye terminator method using fluorescent cycle sequencing with dye-labelled terminators (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer) on a 373A automated DNA sequencer. Sequence analysis was carried out using the programs of the Genetics Computer Group (GCG) v.9 (Madison, WI, USA).

The complete nucleotide sequence of the vanC-3 gene cluster has been deposited in GenBank under accession number AY033764.

RFLP analysis of long-PCR (L-PCR) amplicons

Genomic DNA was extracted from E. flavescens and E. casseliflavus and 100 ng amounts were used as templates for L-PCR.5 The complete vanC-3 and vanC-2 gene clusters were amplified using primers C1 and S2 and an Expand Long Template PCR System (Roche). Amplification was carried out using the following protocol: (i) 94°C for 2 min; (ii) 10 cycles of 94°C for 20 s, 55°C for 30 s and 68°C for 10 min; (iii) 20 cycles of 94°C for 20 s, 65°C for 30 s and 68°C for 10 min (with the elongation time increased by 20 s/cycle); and (iv) 68°C for 10 min. L-PCR products were digested with 10 U EcoRV and the resulting fragments were separated by electrophoresis through a 0.7% agarose gel containing 0.5 mg/L ethidium bromide. The gel was run in TBE electrophoresis buffer (0.09 mM Tris–HCl, pH 8.0, 0.09 M boric acid, 2 mM EDTA).

Results and discussion

Analysis of the vanC-3 vancomycin resistance gene cluster of E. flavescens CCM 439

The vanC-3 vancomycin resistance gene cluster of E. flavescens contained five open reading frames. The genes encoding the vancomycin resistance proteins in the vanC-3, vanC and vanC-2 clusters had the same organization. The proteins encoded by the vanC-3 gene cluster displayed extensive amino acid identity to those encoded by the vanC-2 cluster (Table 1). The lowest degree of amino acid identity, 97%, was observed between VanTC3 and VanTC2, and the highest, 100%, between VanRC3 and VanRC2. The 99 bp intergenic region between vanTC3 and vanRC3 (98 bp in E. casseliflavus) displayed 98.0% nucleotide identity to the intergenic region between vanTC2 and vanRC2.

Resistance to vancomycin in E. flavescens CCM 449 was inducible, but, in common with E. casseliflavus, it required...
several hours of incubation in growth medium containing a low concentration of vancomycin (2 mg/L) before growth resumed (data not shown), indicating that the operon was tightly regulated. The vanR<sub>C-2</sub> and vanS<sub>C-3</sub> genes were exactly the same length as vanR<sub>C-3</sub> and vanS<sub>C-3</sub> from E. casseliflavus and were separated by a region of 62 nucleotides, which displayed 100% nucleotide identity over 62 nucleotides to the same region in vanR<sub>C-2</sub>–vanS<sub>C-2</sub>, although the region from E. casseliflavus contained an additional six nucleotides. The high degree of nucleotide and amino acid identity between the vanC-3 and vanC-2 gene clusters and the proteins they encode demonstrates that there has been little sequence divergence. This lack of nucleotide divergence is not limited to the vancomycin resistance genes. Sequencing of internal portions of the D-Ala:D-Ala ligase and sodA superoxide dismutase genes from E. casseliflavus and E. flavescens revealed that they were 98.5% and 99.5% identical, respectively.2,4 The minimal degree of inter-species sequence divergence was much lower than expected for housekeeping genes from different species and also relative to estimates of sequence diversity between the genomes based on DNA–DNA hybridization experiments.3

The genes of the vanC-3 cluster were considered different from those of the vanC-2 cluster because the host organism was distinct from E. casseliflavus. However, the highest degree of sequence divergence observed between the two gene clusters was only 3.6%, between vanXY<sub>C-3</sub> and vanXY<sub>C-2</sub>. Alleles of other vancomycin resistance genes display greater sequence divergence than the vanC-3 and vanC-2 clusters; vanB3 displays 5% nucleotide divergence from vanB, and vanD4 exhibits 17% sequence divergence from vanD.7,8

L-PCR-RFLP analysis of the vanC-2 and vanC-3 gene clusters of E. casseliflavus and E. flavescens

To evaluate the closeness of the relationship between E. casseliflavus and E. flavescens, the vanC-2 gene clusters of three strains of E. casseliflavus and the vanC-3 gene cluster were amplified by L-PCR to produce fragments of 5.8 kb in each instance. With EcoRV, the three strains of E. casseliflavus all gave rise to the same RFLP (Figure 1). The sizes of the restriction fragments corresponded to the three target sites within the cluster predicted by sequence analysis. The RFLP profile of the vanC-3 L-PCR product of E. flavescens contained only two bands, indicating the absence of a single EcoRV site within the cluster (Figure 1). This led to the production of a restriction fragment of 4 kb instead of one of 1.7 kb and another of 2.3 kb as seen with E. casseliflavus strains.

Previously, it was shown that DNA from strains of E. flavescens, digested with restriction endonucleases, hybridized to probes based on E. casseliflavus genes, although their hybridization profile was distinct from those of E. casseliflavus isolates.4 These results support the L-PCR-RFLP analysis of the vanC-2 and vanC-3 gene clusters. Other techniques, such as PCR, PFGE and 16S rRNA sequence analysis, were unable to differentiate between E. casseliflavus and E. flavescens.9-11 RFLP analysis can detect single nucleotide changes that affect the presence or absence of restriction sites, and, as shown here, can differentiate between some strains of the two species. However, further investigation is required to determine whether this difference is found consistently in additional strains of both E. flavescens and E. casseliflavus.

### Table 1. Percentage identity between the deduced amino acid sequences of the proteins of the vanC-2 and vanC-3 clusters of E. casseliflavus and E. flavescens, respectively

<table>
<thead>
<tr>
<th>% Amino acid identity</th>
<th>VanC-2</th>
<th>VanXY&lt;sub&gt;C-3&lt;/sub&gt;</th>
<th>VanT&lt;sub&gt;C-3&lt;/sub&gt;</th>
<th>VanR&lt;sub&gt;C-3&lt;/sub&gt;</th>
<th>VanS&lt;sub&gt;C-3&lt;/sub&gt;</th>
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<tr>
<td>VanC-2</td>
<td>98.7</td>
<td>98.4</td>
<td>97.0</td>
<td>100</td>
<td>99.0</td>
</tr>
<tr>
<td>VanXY&lt;sub&gt;C-2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VanT&lt;sub&gt;C-2&lt;/sub&gt;</td>
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<tr>
<td>VanR&lt;sub&gt;C-2&lt;/sub&gt;</td>
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<td>VanS&lt;sub&gt;C-2&lt;/sub&gt;</td>
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![Figure 1](https://academic.oup.com/jac/article-abstract/51/3/703/897253/25788, lane 3; E. flavescens CCM 439, lane 4; E. casseliflavus NCTC 2321, lane 5. Sizes of the restriction fragments (kb) are shown to the left-hand side. Digested L-PCR products with EcoRV site within the cluster predicted by sequence analysis. The RFLP profile of the vanC-3 L-PCR product of E. flavescens contained only two bands, indicating the absence of a single EcoRV site within the cluster (Figure 1). This led to the production of a restriction fragment of 4 kb instead of one of 1.7 kb and another of 2.3 kb as seen with E. casseliflavus strains.)
It therefore appears that the allocation of *E. flavescens* as a species distinct from *E. casseliflavus* is tenuous. Although a single nucleotide change leading to the absence or presence of a restriction site will affect the number of fragments obtained by L-PCR-RFLP analysis, it seems unlikely that this would be sufficient to differentiate *E. flavescens* from the many strains of *E. casseliflavus* that appear to show variation from the type strain. *E. flavescens* is differentiated from *E. casseliflavus* on its inability to acidify ribose, but this technique is not always sufficient to identify isolates of the two species correctly. It may therefore be more appropriate to describe *E. flavescens* as a phenotypic variant or subspecies of *E. casseliflavus*.

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References