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Molecular Characterization of the *vanE* Gene Cluster in Vancomycin-Resistant *Enterococcus faecalis* N00-410 Isolated in Canada

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The *vanE* operon was characterized from *Enterococcus faecalis* N00-410 (MIC of vancomycin = 24 μg/ml). The organization of the *vanE* operon was identical to that of the *vanC1* operon from *Enterococcus gallinarum*, with protein identities ranging from 46 to 63%. An open reading frame located downstream of the *vanE* operon showed significant homology to a number of integrase genes, all of which are located downstream of the chromosomal GMP synthase gene *guaA*.

In enterococci, normal peptidoglycan precursors have D-Ala–D-Ala termini that strongly bind vancomycin, whereas in vancomycin-resistant enterococci, alternate biosynthetic pathways lead to precursors with termini that bind vancomycin poorly, thus conferring resistance (4, 18). The *vanA* (3), *vanB* (9, 15), and *vanD* (5, 6, 14) genes code for D-Ala–D-Lac ligases and are responsible for the acquired intermediate- to high-level resistance found mainly in *Enterococcus faecalis* and *Enterococcus faecium*. Intrinsic low-level vancomycin resistance is conferred by *vanC1*, *vanC2*, and *vanC3*, which code for D-Ala–D-Ser ligases found on the chromosomes of *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens*, respectively (7, 13). The acquired, nontransferable VanE D-Ala–D-Ser ligase found in *E. faecalis* also confers a low-level resistance phenotype (8). The function of the *E. faecalis* WCH9 putative vancomycin resistance gene *vanG* is unknown (11). The identification of the first *vanE*-containing *E. faecalis* isolated in Canada has recently been reported (17). In this report, we describe the characterization of the *vanE* resistance locus and the genes flanking this region.

E. faecalis N00-410 (MIC of vancomycin = 24 μg/ml) (17) and *E. faecium* ATCC 19434 were grown at 35°C in brain heart infusion broth or cation-adjusted Mueller-Hinton broth. Induction studies were performed as previously described (5). Transfer experiments were attempted by liquid mating with selection on phenol red agar plates (Difco) containing 1% L-arabinose and 5 μg of vancomycin/ml (11). Antimicrobial susceptibilities were determined by using Etest strips (AB Biodisk) or agar dilution according to NCCLS guidelines (12) for high levels of streptomycin and gentamicin. Genomic DNA was extracted from enterococci as previously described (5). An N00-410 DNA library (~10-kb *Sau*3A fragments) in ZAP Express (Stratagene) was screened with a *vanE* PCR product generated with primers VANE1 and VANE2 (8). Labeling and detection of the probe were performed per the manufacturer's instructions (Amersham Pharmacia Biotech). The sequence was obtained by primer walking and by using the EZ::TN<TET-1> insertion kit (Epicentre Technologies). Inverse PCR was carried out with primers vanRE-1 (5′-TCTCG GCTTTTCATGCATC-3′) and vanSE-DN1 (5′-GAATGAAA TTAATCATATTCG-3′) and with *Eco*RV-cut and -religated N00-410 DNA. Primers Eint-DN1 (5′-ATTCAAGGGATATT TTCAATAGC-3′) and guaDN-1 (5′-TTGCACATGTAAAC CGTATCG-3′) were used to amplify a 0.9-kb fragment overlapping the inverse PCR product. Homology searches were conducted with BLAST (National Center for Biotechnology Information website [http://www.ncbi.nlm.nih]) (1).

E. faecalis N00-410 (MIC of vancomycin = 24 μg/ml) was isolated from an infected ankle wound of a patient who had no travel history outside of Manitoba for 20 years, had no extensive contact with out-of-province visitors, and had never received vancomycin (17). The strain was susceptible to teicoplanin (0.5 μg/ml), ampicillin (0.75 μg/ml), tetracycline (0.75 μg/ml), chloramphenicol (3 μg/ml), and high levels of streptomycin and gentamicin. This is only the second *vanE*-containing vancomycin-resistant *Enterococcus* strain isolated, the first being *E. faecalis* BM4405, which was isolated from a patient in Chicago who had received vancomycin (8).

A total of 10,749 bp was characterized by sequence analysis (GenBank accession no. AF430807) of two *vanE*-positive clones, pE52A (6.9-kb insert) and pE51A (5.4-kb insert), a 2.1-kb inverse PCR fragment, and part of a 0.93-kb PCR product (Fig. 1). Five open reading frames (ORFs) constituting the *vanE* operon were found: *vanE* (D-Ala–D-Ser ligase), $vanXY_E$ (D,D-dipeptidase/D,D-carboxypeptidase), $vanT_E$ (serine racemase), $vanR_E$ (response regulator), and $vanS_E$ (sensor kinase) (Fig. 1). A comparison with the gene from the *vanE* type strain BM4405 (8) showed 4.1% nucleotide differences (97% amino acid identity), and thus, the genes may be considered variants. The overall gene order and protein similarity indicate that the *vanE* operon is the functional equivalent of the *vanC1* operon from *E. gallinarum* (2), a finding which is not unexpected, as the *vanE* type strain BM4405 was found to synthesize peptidoglycan precursors terminating in D-serine residues (8). As in the *vanC1* operon, some of the *vanE* operon genes overlap one

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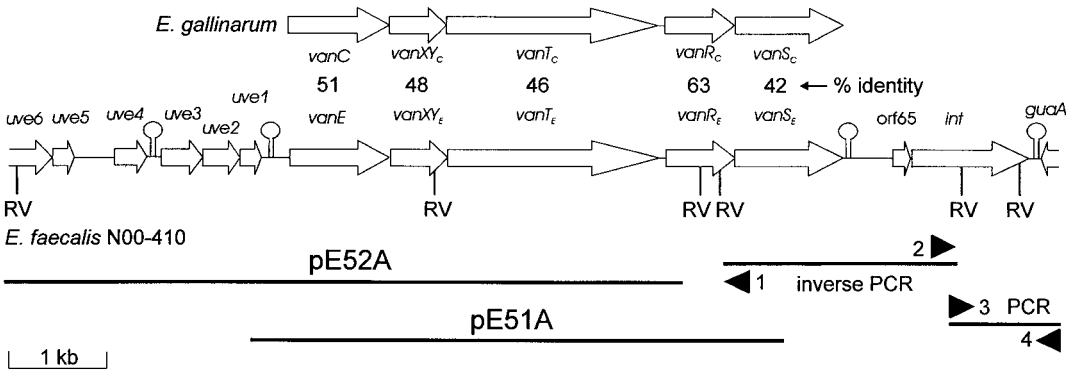

FIG. 1. Genetic organization of the *vanE* locus from *E. faecalis* N00-410 showing regions cloned into plasmids and regions isolated by inverse PCR and PCR. The direction of transcription is indicated by arrows, and putative stem-loop structures are indicated. Primers used in inverse PCR and PCR are indicated by black arrowheads: primer 1 is vanRE-1, primer 2 is vanSE-DN1, primer 3 is Eint-DN1, and primer 4 is gua-DN1. The organization of the *vanC1* locus from *E. gallinarum* is shown at the top. Percent identities of the corresponding *vanC1* and *vanE* operon proteins are shown. RV indicates *Eco*RV sites.

another: $vanXY_E$ overlaps *vanE*, $vanT_E$ overlaps $vanXY_E$, and $vanR_E$ overlaps $vanS_E$. The percent G+C content of the *vanE* operon genes is between 30.7 and 35.7%, which is slightly lower than the 37.5% for *E. faecalis* V583 (http://www.tigr.org). Beginning 31 bp downstream of the $vanS_E$ stop codon is a region of dyad symmetry ($\Delta G = -12.6$ kcal/mol) followed by a T-rich region which may play a role in *vanE* operon transcription termination. Growth studies with N00-410 showed that vancomycin resistance was inducible (data not shown), indicating that expression of the *vanE* operon may involve not only translational coupling but an active response regulator-sensor kinase system.

Six ORFs were detected upstream of *vanE* (*uve1* to *uve6*) (Fig. 1). The *uve6* product exhibited 36% identity to the hypothetical Spy1691 protein of *Streptococcus pyogenes* (GenBank accession no. AE06599). The product of *uve1* was 37% identical over its full length to the sigma factor SpoIIIG of *Bacillus thuringiensis* (accession no. I40582), and the product of *uve2* was 26% identical over its full length to the sigma factor SpoIIG of *Bacillus subtilis* (accession no. M57606). In both cases, however, the enterococcal protein was about half the length of the *Bacillus* protein. The products of three other ORFs, *uve3*, *uve4*, and *uve5*, had no significant homology to extant proteins. The percent G+C content for the genes in this region was between 27.5 and 32.2%. Two regions of dyad symmetry were detected here: one downstream of *uve4* ($\Delta G = -14.1$ kcal/mol) and one downstream of *uve1* ($\Delta G = -17.5$ kcal/mol) (Fig. 1).

Three ORFs were found downstream of the *vanE* operon: *orf65*, *int410*, and the 3′ end of *guaA*, encoding GMP synthase (Fig. 1). The Int410 protein had 69% identity to the integrase of Tn*5801* (Int5801, Orf SAV0392) from *Staphylococcus aureus* Mu50 (10) and the Int459 integrase of the *tet*(M) element from *Clostridium perfringens* CW459 (16). The Orf65 protein was 80% identical to the product of the SAV0393 gene, *orf66* (66 amino acids), found just upstream of the *int5801* gene. Furthermore, we identified a gene identical to *orf66* in exactly the same location upstream of *int439*, although it was not anno-

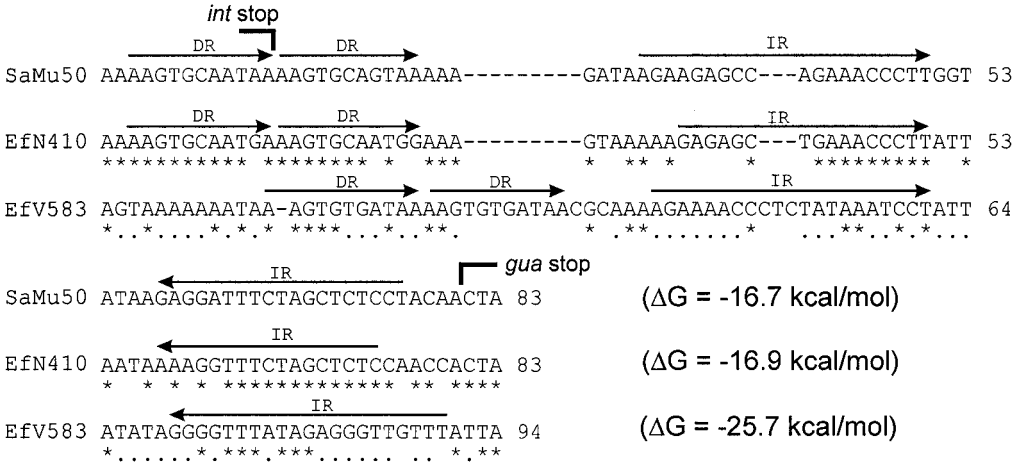

FIG. 2. Alignments of the *int-guaA* intergenic regions from *S. aureus* Mu50 (SaMu50), *E. faecalis* N00-410 (EfN410), and *E. faecalis* V583 (EfV583). The sequence from the equivalent region in *C. perfringens* CW459 is identical to that of *S. aureus* Mu50. Asterisks indicate an identical nucleotide in all three sequences, and periods indicate an identical nucleotide in two out of three sequences. The ΔG values of the inverted repeats are indicated after each sequence. DR, direct repeat; IR, inverted repeat.

tated in the GenBank entry for the *C. perfringens tet*(M) element (accession no. AF329848). A comparison of the complete *C. perfringens tet*(M) element with Tn*5801* revealed that they share >97% sequence identity (data not shown) and that in both organisms, the elements are inserted downstream of *guaA*. Using *int410* to query the *E. faecalis* V583 genome (http://www.tigr.org), we identified an integrase gene (*int583*) downstream of *guaA* whose product had 53% identity to the Int410 protein. Furthermore, overlapping *int583* by 8 bp was *orf70* (70 amino acids), whose product exhibited 52 and 47% identity to Orf65 and Orf66, respectively. The location, size, and basic pI values of the proteins of *orf65*, *orf66*, and *orf70* indicate that they may be excisionase proteins. Thus, it appears that the transposon-like elements described above belong to a group with integrative functions recognizing sequences downstream of *guaA*. Analysis of the *int-guaA* intergenic regions allowed for the identification of some common features (Fig. 2). In *S. aureus* and *C. perfringens*, the sequences are identical and the same length as the N00-410 region (67 bp), with which they share 67% identity. The *E. faecalis* V583 region (78 bp) exhibits 58% identity to the N00-410 region and 49% identity to the *S. aureus* and *C. perfringens* regions. Inverted repeats in each region may be involved in transcription termination of the convergently orientated *int* and *guaA* genes. Direct repeats of 11 bp in *S. aureus*, *C. perfringens*, and N00-410 involve a duplication of the end of the *int* genes and in *E. faecalis* V583 are found just downstream of the *int* gene. A direct repeat consensus sequence, AAGTGYRRTRR (Y = pyrimidine, R = purine), can be postulated and may be involved in the integration of these elements, as may the inverted repeats. Since *guaA* is a housekeeping gene, we assume it to be outside of the transposon-like elements.

The transfer of vancomycin resistance to *E. faecium* ATCC 19434 was unsuccessful. Transfer to an *E. faecalis* strain was not carried out due to the lack of a suitable recipient strain. Fines et al. (8) were unable to demonstrate the transfer of vancomycin resistance from *E. faecalis* BM4405 to *E. faecalis* JH2-2. Whether Tn*5801* is transferable is unknown, while in *C. perfringens* CM459, the *tet*(M) gene appears to be nontransferable (16).

We have characterized the *vanE* locus from an *E. faecalis* strain isolated in Canada. The gene order and protein similarity indicate a close relationship to the *vanC1* operon from *E. gallinarum*. Downstream is an integrase gene, which may have been involved in the initial acquisition of the *vanE* operon. The N00-410 *vanE*-containing element, the *C. perfringens tet*(M) element, Tn*5801* from *S. aureus* Mu50, and a transposon-like element from *E. faecalis* V583 belong to a family of elements that can integrate downstream of *guaA* genes.

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