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Transferable *vanB2* Tn*5382*-Containing Elements in Fecal Streptococcal Strains from Veal Calves

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Three vancomycin-resistant veal calf fecal streptococci, identified as *Streptococcus gallolyticus* ($n = 2$) and *Streptococcus lutetiensis*, were shown to harbor *vanB2* Tn*5382*-like elements earlier described in enterococci. One *S. gallolyticus* strain had a 1,495-bp IS*256*-related element inserted in $vanS_B$. The *vanB2* Tn*5382* element present in the plasmid-free *S. lutetiensis* strain was transferable to *Enterococcus faecium* BM4105-RF, *Enterococcus faecalis* JH2-2, and its recombination-deficient derivative, UV202. The transfer frequencies were comparable between recipient strains (from 1×10^{-7} to 7×10^{-6}). All transconjugants acquired a *vanB*-containing chromosomal insert of approximately 100 kb, apparently by site-specific integration. Secondary transconjugants were not observed in intraspecies retransfer experiments. These observations are consistent with a conjugative, selftransmissible, integrative element that might be involved in the interspecies spread of *vanB2* resistance determinants. Two JH2-2-derived transconjugants had also gained additional copies of large *vanB*-containing chromosomal fragments, a process that involves unexplained mechanisms that seems to require functional host cell-dependent recombination mechanisms.

The *vanB* gene cluster is a common cause of transferable high-level vancomycin resistance in human clinical enterococci worldwide (3, 5, 6, 19, 31–33, 40). Its origin remains unknown. The host range and mechanisms for intercellular spread are only partially understood. *Enterococcus faecium* and *Enterococcus faecalis* are the main enterococcal hosts, but *vanB* has also been described in *Enterococcus gallinarum* (16, 35) and *Enterococcus durans* (18). Nonenterococcal reservoirs have also been reported, such as three fecal veal calf strains of *Streptococcus gallolyticus* (25) and one human fecal *Streptococcus bovis* isolate (27). Recently, the *vanB2* gene cluster was identified in a human fecal *Eggerthella lenta*-related strain and in a nonidentified human fecal *Clostridium* species (37).

The *vanB* operons described so far seem to have a conserved order of genes ($vanR_B$, $vanS_B$, $vanY_B$, *vanW*, $vanH_B$, *vanB*, and $vanX_B$). Due to sequence diversity, they can be divided in at least three distinct subtypes, *vanB1* to *B3*, indicating different origins (7, 8, 11, 24).

Intra- and interspecies transfer of the *vanB* operon has been linked to the movement of large (90 to 250 kb) chromosomal fragments or conjugative plasmids (3, 5, 6, 31–33, 40). Recently, the 34-kb *vanB2*-containing Tn*1549*, which is similar to the Tn*916*-Tn*1545* family of conjugative transposons, was completely sequenced (11). Tn*1549* appears to be similar to the earlier described and partially sequenced Tn*5382* (3). These putative conjugative transposons are thus designated Tn*5382*-like. Several studies suggest that the *vanB2* operon is the most prevalent *vanB* subtype and that it is universally present as an integral part of Tn*5382*-like elements (5, 11, 23). Although molecular evidence for precise chromosomal or plasmid insertions of a *vanB2* Tn*5382*-like element in enterocooci has been presented (3, 6, 11), direct evidence for conjugative transposition of Tn*5382*-like elements has not been given (6). Rather, transfer of Tn*5382*-like elements takes place as an integral part of variably sized chromosomal elements or conjugative plasmids (3, 5, 6). In the present study, we describe the genetic characterization and transferability of *vanB2* Tn*5382*-containing elements in three fecal veal calf streptococci phenotypically identified as *S. gallolyticus* (25).

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *vanB* clusters of the *vanB*-positive *Streptococcus* strains 5-F9, 4-C11, and 4-G10 (Table 1), isolated from mixed fecal samples from veal calf herds in The Netherlands (25), were examined. *E. faecium* BM4105-RF, *E. faecium* BM4105-Str (30), *E. faecalis* JH2-2 (17), *E. faecalis* BM4110, and the recombination-deficient derivative of JH2-2, *E. faecalis* UV202 (41), were used as recipient strains in the conjugation experiments.

Species identification by *sodA* sequence. DNA sequence determinations were done by direct sequencing of PCR amplicons (7). The *sodA* degenerate primers *d1* and *d2* were used to amplify $sodA_{int}$, an internal fragment of approximately 480 bp in streptococci, representing 85% of the *sodA* gene (28).

PCR amplification and nucleotide sequencing. Amplification of DNA, direct sequencing of PCR amplicons, and restriction analysis of *vanB* long PCR amplicons by restriction fragment length polymorphism (RFLP) were conducted as described previously (7). A 1,132-bp $vanR_BS_B$ region of strain 4-C11 was amplified with primers 5′-GTTTGATGCAGAGGCAGACGACT-3′ and 5′-CCTCC AACAGAACGCTTACAG-3′.

Computer analysis. Editing, initial analysis of sequences, and alignments were performed by using the Sequence Navigator software package (Applied Biosystems, Foster City, Calif.). Nucleotide sequences were compared to those in the GenBank, EMBL, DDBJ, and PDB databases, and protein sequences were compared to nonredundant GenBank CDS translations and sequences in PDB, SwissProt, SPupdate, and PIR by using the BLASTN, BLASTX, and BLASTP local alignment search tools (1). Possible open reading frames (ORFs) were

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TABLE 1. Properties of *vanB* streptococci in this study

Strain	PFGE pattern	Species, as determined by *sodA* sequencing	Vancomycin resistance genotype[a]	*vanB* localization	Transfer frequency[b]	
					E. faecium BM4105-RF[c]	*E. faecalis* JH2-2/ isogenic UV202[c]
5-F9	I	*S. lutetiensis*	*vanB2*	Chromosomal	1×10^{-7}	$2 \times 10^{-6}/7 \times 10^{-6}$
4-C11	II	*S. gallolyticus*	*vanB2 vanA*	Chromosomal	ND[d]	ND/NT[e]
4-G10	III	*S. gallolyticus*	*vanB2 vanA*	Chromosomal	ND	ND/NT

[a] *vanA* genotype determined by Mevius et al. (25).
[b] Number of transconjugants per donor cell.
[c] Recipient strains.
[d] ND, not detected.
[e] NT, not tested.

found by using the ORF finder located at the National Center for Biotechnology Information website.

Conjugation experiments. Filter matings were done with a donor/recipient ratio of 19:1 (5). Various dilutions of resuspended bacteria were spread on brain heart infusion agar containing 8 μg of vancomycin/ml, 20 μg of rifampin/ml, and 10 μg of fusidic acid/ml. Transfer frequencies were expressed as the number of transconjugants per donor cell.

PFGE. The *vanB* strains and transconjugants were typed by *Sma*I digestion and pulsed-field gel electrophoresis (PFGE) of genomic DNA (5) with the run increased to 30 h. A chromosomal *vanB* location was assessed by sequential *vanB*, 16S rDNA, and 23S rDNA hybridizations of PFGE-separated I-*Ceu*I (New England Biolabs, Beverly, Mass.)-digested DNA. Agarose plugs were prepared as described previously (5) with 1 mg of proteinase K/ml and digested with the intron-encoded endonuclease I-*Ceu*I, which recognizes a 23-bp sequence specific for 23S rRNA genes (21). Digested DNA was electrophoresed in a 1.2% agarose gel by using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, Calif.). The pulse time was increased from 60 to 90 s over 22 h at 200 V.

DNA isolation and digestion with restriction enzymes. Total DNA was isolated by using guanidinium isothiocyanate by a protocol slightly modified from that described by Bickley and Owen (2). Streptococci were grown overnight in brain heart infusion broth, and cells from 1 ml of culture were harvested for lysis. Cold 3 M sodium acetate (60 μl) was added subsequent to cell lysis to salt out proteins. Plasmid DNA was isolated as described by Werner et al. (38). Cleavage of DNA with restriction endonucleases was performed as recommended by the manufacturer (New England Biolabs).

DNA transfer and hybridization. Colony blotting and Southern transfer of digested genomic DNA were performed as previously described (5). Probes were labeled by using a PCR digoxigenin (DIG) probe synthesis kit, and detection was performed by using a DIG luminescent detection kit (Roche Diagnostics, Basel, Switzerland). Total DNAs from the following bacteria were used as a template for probe synthesis. The 23S rDNA probe (669 bp with primers 5′-CGCATGT ACAGGATAGGTAGG-3′ and 5′-AGGTGGGCTTCACACTTAGAT-3′) was from *E. faecium* ATCC19434; the 16S rDNA probe (5) was from *E. faecalis* DS16C2 (10); the Tn*5382* probe was from *E. faecium* C68 (3); and the *vanB* probe (7) was from *E. faecalis* V583 (9).

Nucleotide sequence accession numbers. Novel nucleotide accession numbers described in the text are AY035703 to AY035715.

RESULTS AND DISCUSSION

Species identification of 5-F9, 4-C11, and 4-G10 by *sodA* sequence determination. The three strains have previously been identified as *S. gallolyticus* by their growth characteristics, biochemical activities, and whole-cell protein analysis (25). The results of Poyart et al. (28) indicate that the superoxid dismutase (*sodA*) gene has a higher divergence and thus might be a more discriminative target sequence than the 16S rDNA for differentiating closely related streptococcal species. Sequence determination revealed that 4-C11 and 4-G10 had identical $sodA_{int}$ sequences (GenBank accession no. AY035714 and AY035715) that were indistinguishable from those of *S. gallolyticus* NEM1203 and NEM1204 (GenBank accession no. AJ297196 and AJ297197, respectively). The $sodA_{int}$ region in 5-F9 (GenBank accession no. AY035713) showed only 81% identity to the 4-C11 and 4-G10 sequences but 100% identity to the sequences of NEM760 and NEM1603 (GenBank accession no. AJ297188 and AJ297205, respectively), recently classified as *Streptococcus lutetiensis* (29).

Molecular characterization of the *vanB* operons. 5-F9, 4-C11, and 4-G10 were found to be genomically diverse by PFGE (Table 1). The $vanS_B$ to $vanY_B$ intergenic regions (GenBank accession no. AY035706 to AY035708) and the *vanB2* ligase gene sequences (GenBank accession no. AY035703 to AY035705) were identical to *vanB2* operon sequences (7, 12). The RFLP patterns of *vanB* long PCR amplicons from strains 4-G10 and 5-F9 (Fig. 1, lanes 3 and 5) were also identical to the RFLP-2 pattern of *vanB2* clusters found in enterococci (7). The RFLP-2 pattern gives two bands of approximately 1 kb, one in the $vanR_BS_B$ region and one in the $vanY_B$ region. Strain 4-C11 showed a divergent RFLP-2 pattern (RFLP-2#) in which the 1-kb *Bsp*HI fragment covering the $vanR_BS_B$ region in RFLP-2 has been replaced by restriction fragments of approximately 1,350, 600, and 550 bp (Fig. 1, lane 4), indicating the presence of an insertion in this region. The RFLP-2# pattern

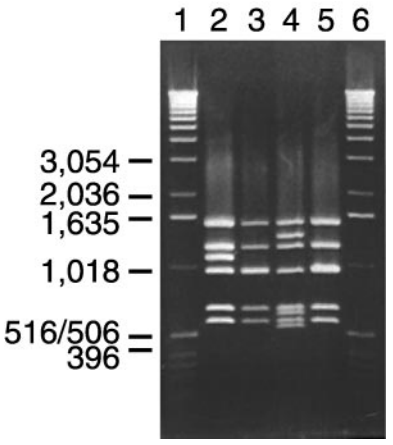

FIG. 1. Restriction fragment analysis of *vanB* long PCR amplicons. *Bsp*HI/*Dra*I-digested *vanB* long PCR amplicons were analyzed by agarose gel electrophoresis. Lanes: 1 and 6, 1-kb ladder (Invitrogen Corporation, Carlsbad, Calif.); 2, *vanB1* strain V583 with RFLP-1; 3, *vanB2* strain 4-G10 with RFLP-2; 4, *vanB2* strain 4-C11 with a 1,504-bp insertion in $vanS_{B2}$ leading to replacement of the 969-bp fragment with restriction fragments of 1,327, 614, and 532 bp (RFLP-2#); 5, *vanB2* isolate 5-F9 with RFLP-2. Molecular sizes shown to the left of the gel (in base pairs) refer to the 1-kb ladder.

```
Inverted right end:   ...GCAAACAGTGTTTGTGTCAAGTTATTCTCGGAAAGTTTTTCAAAAGTCCTAAATCCATTTCTT...
                                 ||| |||| || |  | ||||||||     || | | ||  | ||   |
Left end:          ...AACACTGTTTGCGTTAGTGTAAAATAGTACTCGGAAAAACGTTTACATGTTTTCAAAATAAAAAAA...
```

FIG. 2. Alignment of the terminal portions of the insertion in the $vanS_B$ gene of strain 4-C11. The left end following a 12-bp sequence of $vanS_B$ (bold) is aligned to the inverted version of the right end. Vertical lines indicate identical nucleotides. A putative imperfect inverted repeat of 25 bp is underlined. The 12-bp sequence of $vanS_B$ (bold) immediately in front of the left end is identical to 12 bp at the right end (bold) of the insertion when this sequence is not inverted.

was accounted for by DNA sequencing, which identified a 1,504-bp insertion starting 103 bp into $vanS_{B2}$ (GenBank accession number AY035712). Analysis of the inserted DNA revealed a single 1,353-bp ORF oriented in the same direction as the *vanB* cluster. This ORF encoded a putative 450-amino-acid protein with 29% identity (amino acid 29 to 160) to 139 amino acids found in putative transposases of IS*Rm3* elements from *Rhizobium meliloti* (36, 39). IS*Rm3* belongs to the IS*256* family of insertion sequences (ISs), and the $vanS_{B2}$ insertion shares several characteristics with this IS family (22). Briefly, the insert comprises a potential DDE motif together with a K/R residue, 12 bp at the terminus, which were identical to the $vanS_{B2}$ sequence at the region of insertion and an imperfect IR of 25 bp located at the terminal ends of the insert, leaving 9 bp external to the repeat, which might be a duplication of the target sequence (Fig. 2) (GenBank accession number AY035712). IS elements have been described earlier in *vanB2* Tn*5382*-like elements (5, 20). However, these IS elements did not interrupt genes involved in expression of the resistance phenotype. Because of the insertion, $vanS_{B2}$ is likely not expressed in the *vanB* operon of strain 4-C11. However, this strain also carries a *vanA* cluster (25), and thus the phenotypical consequences of a nonfunctional $VanS_{B2}$ or a lack of $VanS_{B2}$ are not revealed.

Presence of Tn*5382*-like elements. Southern hybridization of total DNAs showed homology of the Tn*5382* probe to an approximately 2.5-kb *Dde*I restriction fragment in 5-F9 and a 2-kb *Dde*I restriction fragment in both 4-C11 and 4-G10, consistent with the presence of a single Tn*5382*-like copy (data not shown). DNA sequencing of the $vanX_B$ Tn*5382* joint region revealed that the *vanB2* clusters are integral parts of Tn*5382*-like elements in *S. gallolyticus* and *S. lutetiensis*. The DNA sequences (GenBank accession no. AY035709 to AY035711) were identical to Tn*5382*-like elements described in enterococci (5). Thus, the DNA sequence and RFLP analyses show that highly similar *vanB2* operons with linkage to Tn*5382*-like elements are present in enterococci (3, 5, 7, 11) and in streptococci, suggesting a common origin for these operons.

S. gallolyticus strains have been isolated from diverse habitats, including feces of many animals, as well as from animals with bovine mastitis, human clinical sources, and the sheep rumen (26). *S. lutetiensis* (earlier classified as *Streptococcus infantarius* subsp. *coli*) has been isolated from various human specimens, including human feces (34). Thus, these species are able to survive in the gastrointestinal tract and thereby have the opportunity to exchange genetic material with other gastrointestinal commensals. The *vanB2* operon has recently been found in human fecal anaerobic bacteria, but possible linkage to Tn*5382*-like elements was not determined (37). The G + C content of different parts of Tn*5382*-like elements are 48 (*vanB* cluster and right extremity, which are implicated in the excision-integration process) to 55 mol% (left extremity, which is probably implicated in conjugative transfer) (GenBank accession no. AF192329). Enterococci (34 to 42 mol%), streptococci (34 to 46 mol%), *Clostridium innocuum* (41 to 45 mol%), and *Eggerthella* (30 to 40 mol%) genomes have lower G + C contents, suggesting a different origin of *vanB2* Tn*5382*-like elements. Nevertheless, detection of *vanB2* Tn*5382*-like elements in two different *S. gallolyticus* strains, in one *S. lutetiensis* strain, and in genomically unrelated enterococci in both Europe (5, 11, 24) and the United States (3, 5, 14) suggests that these are widespread and successful mobile elements.

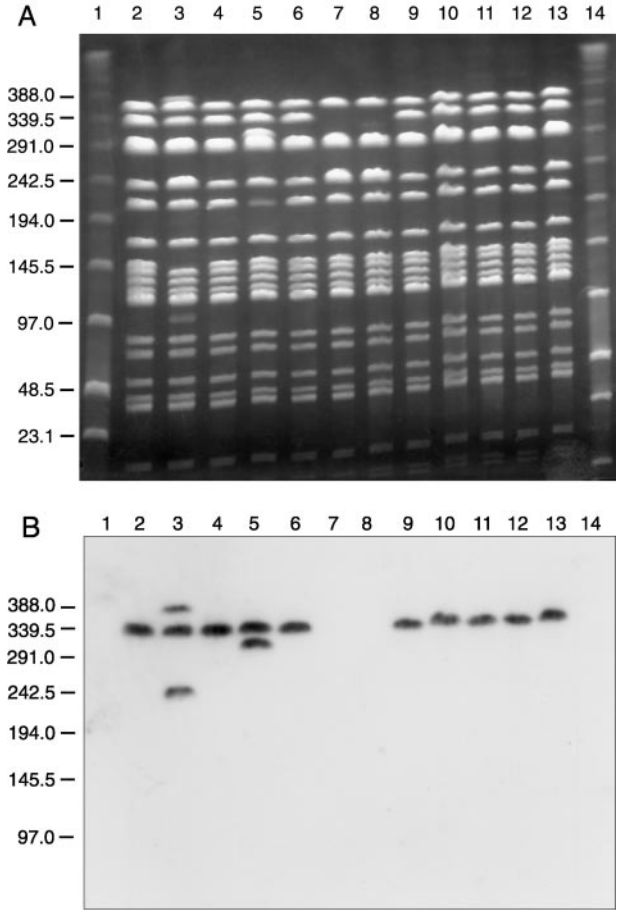

FIG. 3. PFGE of *Sma*I-digested total DNA of the recipients and transconjugants from matings using 5-F9 as donor and JH2-2 and UV202 as recipients (A) and corresponding Southern hybridization with a *vanB* probe (B). Lanes: 1 and 14, low-range PFG markers (New England Biolabs); 2 to 6, transconjugants obtained after mating with recipient JH2-2; 7, recipient JH2-2; 8, recipient UV202; 9 to 13, transconjugants obtained with UV202 as recipient. JH2-2 and UV202 are isogenic strains and thus have indistinguishable PFGE patterns.

Localization of the *vanB*-containing elements. The strains were characterized with regard to plasmid or chromosomal localization of the *vanB* gene cluster by Southern hybridization of plasmid DNA and I-*Ceu*I PFGE fragments. Hybridization with the *vanB* probe, and not with the 16S rDNA probe, to plasmid DNA indicates a plasmid localization of the *vanB* operon, whereas cohybridization of the *vanB* and either 16S rDNA or 23S rDNA probes to I-*Ceu*I PFGE fragments was interpreted as a chromosomal location of the *vanB* cluster. A chromosomal location of the *vanB* operon was shown in all three strains (data not shown).

Mobility of the *vanB* gene clusters. Filter mating experiments were performed between the *vanB*-containing streptococci as donor strains and plasmid-free enterococcal recipients. Five vancomycin-resistant transconjugants from each mating pair were analyzed for the presence of *vanB* by PFGE and subsequent hybridization. All transconjugants tested harbored *vanB* and showed PFGE patterns related to that of the recipient.

Filter mating experiments using 4-C11 and 4-G10 as donors gave no detectable transconjugants (transfer rates, $<1 \times 10^{-9}$). Successful mating was shown with the 5-F9 strain, consistent with the findings of Mevius et al. (25). Transfer frequencies of about 7×10^{-6} transconjugants per donor were observed with the recombination-deficient recipient *E. faecalis* UV202. The transfer rates were somewhat lower with *E. faecalis* JH2-2 (2×10^{-6} transconjugants per donor) or *E. faecium* BM4105-RF (1×10^{-7} transconjugants per donor) as recipients. *Sma*I PFGE analysis and subsequent *vanB* hybridization of the transconjugants (Fig. 3A, lanes 2 to 6 and 9 to 13) derived from the *E. faecalis* recipients JH2-2 and UV202 (Fig. 3A, lanes 7 and 8, respectively) or the *E. faecium* recipient BM4105-RF (data not shown) revealed a reproducible insertion event of an approximately 100-kb *vanB*-containing element which was confirmed by *Sfi*I/*Not*I PFGE analysis of the JH202- and UV202-derived transconjugants (data not shown). The JH2-2- and UV202-derived transconjugants (Fig. 3A and B, lanes 2 to 6 and 9 to 13, respectively) showed the replacement of an approximately 240-kb fragment (the double band becomes a single band) by a 340-kb *vanB*-containing fragment. BM4105-RF-derived transconjugants (data not shown) showed the replacement of a 290-kb fragment by an approximately 390-kb *vanB*-containing fragment.

A 100-kb *vanB*-containing insertion event in the corresponding *Sma*I fragment, comparable to that demonstrated in this study, has previously been shown in BM4110- and BM4105-RF derived transconjugants obtained by using the *vanB2 S. bovis* NEM760 as donor (27). BM4110 and JH2-2 have the same PFGE patterns, as they are isogenic strains derived from JH2. Thus, it seems that streptococci might harbor a *vanB2*-containing transferable 100-kb element that integrates in a site-specific manner in enterococcal recipients. The arrival of the 100-kb *vanB* Tn*5382*-containing element in the recombination-deficient recipient strain UV202 as well as its ancestor JH2-2 at comparable transfer frequencies indicates that it does not require a functional host cell recombination system for integration. Thus, the element shares many properties with conjugal self-transmissible, integrating elements (15). Further studies are in progress to characterize this element.

Retransfer experiments using 5-F9-derived transconjugants as donors and BM4105-Str and BM4110 as recipients showed no detectable secondary transconjugants ($<1 \times 10^{-9}$). However, Poyart et al. (27) were able to show successful retransfer of their 100-kb *vanB*-containing element from *S. bovis* between enterococcal strains in the presence of the plasmid pIP964. The plasmid contents of 5-F9 and selected transconjugants were therefore analyzed to examine if plasmids could be involved in the observed transfer events. Plasmid DNA was not detected in repeated experiments (data not shown), indicating plasmid-independent chromosomal transfer. Hence, enterococci may not be able to express the transfer functions of the streptococcal 100-kb element, or the factors required for transfer could be retained in the donor after conjugation.

Two of 11 transconjugants obtained by JH2-2 matings revealed the presence of additional *vanB*-containing fragments (Fig. 3, lanes 3 and 5). These results have been reproduced and confirmed by hybridization of *Sfi*I/*Not*I PFGE fragments (data not shown). The integration of additional large *vanB*-containing fragments seems to involve a host cell-encoded recombination mechanism, since this phenomenon was not observed in the 11 UV202-derived transconjugants tested.

The potential spread of vancomycin resistance determinants to other pathogenic bacteria is underlined by the expanded species distribution of the *vanB2* Tn*5382*-containing elements, as seen in this study, by the broad host range demonstrated by the Tn*916*-Tn*1545* family of conjugative transposons in general, as well as by the recent description of *vanA* gene clusters in clinical *Staphylococcus aureus* strains (4, 13). Further studies are needed to clarify the reservoirs for transferable vancomycin resistance determinants and address mechanisms for transposition and conjugative mobilization in order to prevent and control their spread.

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