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Transfer of Vancomycin Resistance Transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus* spp. in the Gut of Gnotobiotic Mice

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The vancomycin resistance *vanB2* gene cluster is disseminated worldwide and has been found in phylogenetically remote bacterial genera. The *vanB2* operon is part of conjugative transposons Tn*1549*/Tn*5382*, but conjugative transposition of these elements has not been demonstrated. We have obtained transfer of a Tn*1549*-like element (referred to herein as "Tn*1549*-like") from *Clostridium symbiosum* MLG101 to *Enterococcus faecium* 64/3 and *Enterococcus faecalis* JH2-2 in the digestive tract of gnotobiotic mice and to *E. faecium* 64/3 in vitro. Retransfer of Tn*1549*-like from an *E. faecium* transconjugant also containing Tn*916* to *E. faecium* BM77 was obtained in vitro, albeit at a very low frequency. Transfer efficiency was found to be both donor and recipient dependent. Pulsed-field gel electrophoresis analysis of total SmaI-digested DNA of 48 transconjugants indicated in 27 instances the acquisition of ca. 34 kb of DNA. Two transconjugants harbored two copies of the transposon. Sequencing of the flanking regions of Tn*1549*-like in 48 transconjugants revealed 29 integration events in 26 loci in the *E. faecium* genome, and two hot spots for insertion were identified. Integration of the transposon was associated with the acquisition of 5 ($n = 18$) or 6 ($n = 7$) bp of donor DNA or with 5-bp duplications of target DNA in the remaining transconjugants. These data demonstrate functionality of the Tn*1549*-like element and attest that the transfer of the *vanB* operon between enterococci and human commensal anaerobes occurs in the intestinal environment.

Emergence of vancomycin resistance in enterococci was reported in 1986, approximately 30 years after the introduction of this antibiotic into clinical practice (25). More recently, vancomycin resistance was detected in strains of *Staphylococcus aureus*, *Oerskovia turbata*, *Arcanobacterium haemolyticum*, *Streptococcus bovis*, *Streptococcus gallolyticus*, *Streptococcus lutetiensis*, *Bacillus circulans*, *Paenibacillus*, and *Rhodococcus*, as well as in anaerobic bacteria belonging to the *Clostridium* genus and *Eggerthella lenta* (6, 15, 26, 29, 30, 32, 35, 36, 42). As a consequence, glycopeptide resistance is considered as a global threat to public health, and control of its dissemination constitutes a crucial challenge. Acquired resistance to glycopeptides in enterococci is due to production of modified peptidoglycan precursors ending in D-alanine-D-lactate (D-Ala-D-Lac) (VanA, -B, and -D) or D-alanine-D-serine (D-Ala-D-Ser) (VanC, -E, and -G), to which glycopeptides exhibit low binding affinities, combined with the elimination of high-affinity D-Ala-D-Ala-ending precursors synthesized by the host Ddl ligase (4). Expression of the resistance gene clusters is controlled by two-component regulatory systems that are composed of VanR-type response regulators acting as transcriptional activators and VanS-type histidine kinases that are associated with the membrane (2). The regulatory and resistance genes are transcribed from distinct promoters that are coordinately regulated (1).

Among the glycopeptide resistance determinants, VanA and VanB are the two most commonly encountered in clinical settings (12). VanA-type resistance is mediated in enterococci (3) and, more recently, in *Staphylococcus aureus* by transposon Tn*1546* or closely related elements (13). Two major subtypes of *vanB* operons, *vanB1* and *vanB2*, have been described so far (9, 16, 33). Clusters related to *vanB1* are generally carried by large (90- to 250-kb) elements that are transferable by conjugation from chromosome to chromosome (37). The more common *vanB2* operon is generally associated with Tn*1549*- and Tn*5382*-like transposons that are closely related (here referred to, for the sake of simplicity, as "Tn*1549*-like") (9, 21). Tn*1549* is entirely sequenced (accession number AF192329), whereas Tn*5382* is partly sequenced (accession numbers AF063010 and AF063900). Minor base differences of the *vanB* operon were detected in the related elements found in anaerobes, which are structurally similar to Tn*1549* (7). These genetic elements possess features of conjugative transposons of the Tn*916* family and are capable of excision to form a circular intermediate. Tn*1549* and Tn*5382* have not been shown to promote conjugative transposition but can be transferred passively as an integral part of variable-size chromosomal fragments or of plasmids (15, 17). The *vanB2* gene has been detected in anaerobic bacteria in Australia (42) and, more recently, in Canada (18). The linkage of *vanB2* with Tn*1549*-like elements in members of various genera including *Enterococcus*, *Streptococcus*, *Clostridium*, *Eggerthella*, and *Ruminococcus* (6, 16, 17) suggests that the spread of this resistance determinant is due to transposition. The chromosomal sequence of *Enterococcus faecalis* V583 indicated that more than a quarter of the genome probably consists of mobile foreign DNA and that the *vanB* operon is part of a mobile element that contains 53 genes, including Tn*1549* (34). The aim of this work was to test inter-

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. lenta sp.		
MLG043	Vm (*vanB2*)	42
Clostridium sp.		
MLG055	Vm (*vanB2*)	42
C. symbiosum spp.		
MLG101	Vm (*vanB2*)	42
MLG101-1	Vm (*vanB2*) spontaneous derivative harboring a second copy of Tn*1549*-like	This study
E. faecalis spp.		
BM4110	Str, JH2 spontaneous mutant	10
JH2-2	Fus, Rif, JH2 spontaneous mutant	24
JH2-2::Tn*916*	Fus, Rif, Tet	20
T1	JH2-2::Tn*1549*-like, Fus, Rif, Vm	This study
T1::Tn*916*	Fus, Rif, Vm, Tet	This study
T1(pCM100)	Fus, Rif, Vm, Sp, Cm, *xis-int*$_{\text{Tn}\textit{1549}\text{-like}}$	This study
T1::Tn*916*(pCM100)	Fus, Rif, Vm, Sp, Cm, Tet, *xis-int*$_{\text{Tn}\textit{1549}\text{-like}}$	This study
E. faecium spp.		
BM77	Str spontaneous mutant	Wild-type strain
BM4105	Fus, Rif	10
BM4105S	Str	10
64/3	Fus, Rif,	44
T2-28	64/3::Tn*1549*-like, Fus, Rif, Vm	This study
T2::Tn*916*	Fus, Rif, Vm, Tet	This study
T2(pCM100)	Fus, Rif, Vm, Sp, Cm, *xis-int*$_{\text{Tn}\textit{1549}\text{-like}}$	This study
T2::Tn*916*(pCM100)	Fus, Rif, Vm, Tet, *xis-int*$_{\text{Tn}\textit{1549}\text{-like}}$	This study
T29	BM77::Tn*1549*-like, Fus, Rif, Vm	This study
Plasmids		
pAT79	P2, Cm, Sp	2
pCM100	pAT79Ω*xis-int*$_{\text{Tn}\textit{1549}\text{-like}}$	This study

[a] Abbreviations: Cm, chloramphenicol resistance; Fus, fusidic acid resistance; Rif, rifampin resistance; Sp, spectinomycin resistance; Str, streptomycin resistance; Tet, tetracycline resistance; Vm, vancomycin resistance; *xis*$_{\text{Tn}\textit{1549-like}}$, excisionase of Tn*1549*-like; *int*$_{\text{Tn}\textit{1549}\text{-like}}$, integrase of Tn*1549*-like; P2, promoter of the *aphA-3* gene from enterococcal plasmid pJH1 (GenBank accession number V01547).

generic transfer of vancomycin resistance between *Clostridium* and *Enterococcus* species and to characterize the mechanism involved in this process.

MATERIALS AND METHODS

Bacterial strains and plasmids. The origins and properties of bacterial strains and plasmids are listed in Table 1. *Eggerthella lenta* and *Clostridium* sp. were grown under anaerobic conditions at 37°C on prereduced brain heart infusion broth (Difco Laboratories, Detroit, MI) and agar supplemented with 5% horse blood (Bio-Rad, Marnes-la-Coquette, France). The MICs of vancomycin were determined by the Etest procedure (AB Biodisk, Solna, Sweden) or by twofold serial dilution in agar.

Conjugation experiments. *Eggerthella lenta* MLG043, *Clostridium* sp. strain MLG055, and *Clostridium symbiosum* MLG101-1 carrying a *vanB2* Tn*1549*-like transposon were used as donors. *Enterococcus faecalis* JH2-2 and BM4110 and *Enterococcus faecium* strains 64/3, BM77, and BM4105 were used as recipients. Transconjugants were used as donors in retransfer experiments. Filter matings were carried out on sterile filter membranes as described previously (14). Antibiotics were used alone or in combination at the following concentrations to counterselect donor strains: vancomycin, 8 μg/ml; streptomycin, 1,000 μg/ml; spectinomycin, 80 μg/ml; and rifampin, 100 μg/ml. Mating experiments were carried out by filter mating in the absence or presence of a subinhibitory level (0.2 μg/ml) of vancomycin.

In vivo transfer. Groups of five germfree consanguineous C3H mice supplied by INRA (Jouy-en-Josas, France) were inoculated intragastrically with a challenge of 10^8 CFU of donors. After massive and prolonged colonization of the gut by the bacteria, the animals were inoculated with 10^8 CFU of the recipient enterococci. Mice were maintained in a positive-pressure incubator to prevent any bacterial contamination. After control of the persistence of the association of *Enterococcus* spp. with the putative anaerobic donor, vancomycin was added to the drinking water at concentrations increasing from 15 to 120 μg/ml throughout the experiment (Fig. 1). Fecal samples were plated on brain heart infusion agar containing 8 μg/ml of vancomycin and incubated at 37°C under aerobic conditions. Attempts to increase retransfer efficiency included introduction in two transconjugants of either Tn*916* by conjugative transposition or of a shuttle plasmid by electrotransformation to overexpress Int$_{\text{Tn}\textit{1549}\text{-like}}$ and Xis$_{\text{Tn}\textit{1549}\text{-like}}$, as well as both Tn*916* and pCM100. Transconjugants were tested for resistance to vancomycin (MIC > 8 μg/ml), and the presence of *vanB2* was screened for by PCR with primers VBa and VBb.

PCR amplification. Primers used for amplification or sequencing are listed in Table 2. PCR was performed with a Ready-To-Go kit (Amersham Biosciences, Orsay, France) in a GeneAmp 2400 PCR system (Perkin-Elmer Cetus, Norwalk, Conn.). PCR elongation times and temperatures were adjusted according to the expected size of the amplicon and melting temperature of the primers, as recommended by the manufacturer. Circular intermediates of Tn*1549*-like were screened by amplification and sequencing of the 250-bp PCR product overlapping the joint region using the VB2 and VBR2 primers. In the absence of an amplification product, nested PCR was carried out using internal primers VB1 and VBR3.

Characterization of Tn*1549*-like targets. Thermal asymmetric interlaced PCR (TAIL-PCR) and inverse PCR (IPCR) were used to determine the 5′- and 3′-flanking regions of Tn*1549*-like in the three anaerobic donors and in 48 randomly selected transconjugants (27). TAIL-PCR was performed using an Expand Long Template PCR system kit (Roche, Mannheim, Germany) for strains MLG043, MLG055, and MLG101-1 and an *E. faecium* 64/3 transconju-

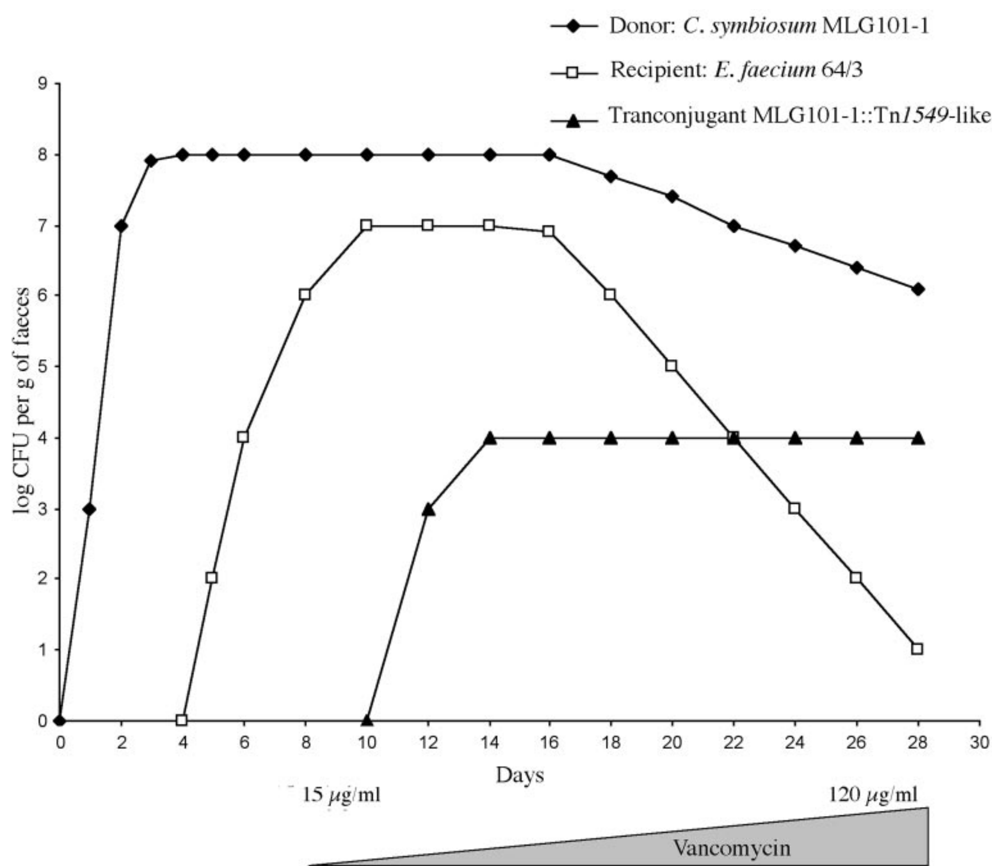

FIG. 1. Bacterial counts in fecal samples from gnotoxenic mice receiving vancomycin in drinking water. Vancomycin was added step-wise in incrementally increasing amounts (15 μg/ml on day 8 to 120 μg/ml on day 24).

gant obtained in vivo. Primers AD1, AD2, AD3, and AD4 were used with primers VBR1, VBR2, VBR3, and VBR4, respectively, to examine flanking sequences at the right extremity of Tn*1549*-like, and VB0, VB1, VB2, and VB3 were used for the left end. IPCR was performed as follows. Total DNA of transconjugants was digested by (i) DraI or HinfI and (ii) AluI, DdeI, or Sau3AI, self-ligated, and used as the template for IPCR with AL/VBR2 and AR/VB2, respectively. PCR products were purified with a High Pure PCR product purification kit (Roche) before sequencing. Total DNA from the strains was prepared with a blood genomics kit (Amersham).

Nucleotide sequencing. Both DNA strands were sequenced with synthetic oligonucleotides by use of an ABI PRISM 310 automated sequencing apparatus (Perkin-Elmer Applied Biosystems). Determination was carried out by direct

TABLE 2. Oligodeoxynucleotide primers

Primer[a]	Sequence (5′–3′)	Position in Tn*1549* (start, end)[b]	Source or reference
AF (+)	GCTATGGCAGTTTTCCGTGTG	483, 504	15
AR (−)	AACGCTTCTTCATGGCTCTTG	−33441, −33421	15
VB0 (+)	TGCCGGAAAAGCCCGGAAACACG	33720, 33742	This study
VB1 (+)	GAGGGGGAAATGGTGAGAGGT	33662, 33682	This study
VB2 (+)	AGAAATGGAACGGCTGGCAGC	33601, 33621	This study
VB3 (+)	TTCCAATATCACCATGACGCTG	33541, 33562	This study
VBa (−)	GCTGCGGAGCTTTGAATATC	−29217, −29198	This study
VBb (+)	CGTGTGCTGCAGGATACTAC	28768, 28789	This study
VBR1 (−)	TGCATCAGCCGTTCAAACGCC	−298, −278	This study
VBR2 (−)	CGCGTTTACGGTGTCGTATTC	−178, −158	This study
VBR3 (−)	TGCGGCTCAATCCGAAAGTAG	−100, −80	This study
VBR4 (−)	TGCGAAATGCCCGTATTTCCGG	−48, −27	This study
xisF (+)	TGAGAGCTCAGGAGGCTGCATTATGAACCATG[c]	32139, 32157	This study
intR (−)	ATGTCTAGAGTCAGGCTGCCAGCCGTTCC[d]	−33626, −33607	This study

[a] +, sense primer; −, antisense primer.
[b] Nucleotide numbering according to numbering of GenBank accession no. AF192329.
[c] SacI restriction site underlined.
[d] XbaI restriction site underlined.

TABLE 3. Experiments for transfer of vancomycin resistance from anaerobes to *Enterococcus* spp. in gnotobiotic mice

Recipient	Result[a] for donor		
	E. lenta MLG043	*C. innocuum* MLG055	*C. symbiosum* MLG101-1
E. faecalis JH2-2	0/2	0/2	1/2
E. faecium 64/3	0/2	0/2	2/2
E. faecium BM4105	0/2	0/2	0/2

[a] Expressed as number of experiments leading to transconjugants/total number of experiments.

TABLE 4. Conjugative transfer of Tn*1549*-like by filter mating

Donor[a]	Recipient	Transfer frequency[b]
E. lenta MLG043	*E. faecium* 64/3	$<10^{-9}$
Clostridium sp. strain MLG055	*E. faecium* BM4105	
	E. faecalis JH2-2	
C. symbiosum MLG101-1	*E. faecium* 64/3	10^{-9} to $<10^{-7}$
C. symbiosum MLG101-1	*E. faecium* BM4105	$<10^{-9}$
	E. faecalis JH2-2	
T1, T1(pCM100), T1::Tn*916*, T1::Tn*916*(pCM100)	*E. faecium* BM77	$<10^{-9}$
T2, T2(pCM100), T2::Tn*916*(pCM100)	*E. faecalis* BM4110	
T2::Tn*916*	*E. faecium* BM77	ca. 10^{-9}
T2::Tn*916*	*E. faecalis* BM4110	$<10^{-9}$

[a] Abbreviations: T1, *E. faecalis* JH2-2::Tn*1549*-like; T2, *E. faecium* 64/3::Tn*1549*-like.
[b] Transconjugants per recipient.

sequencing of PCR products or, when necessary, after cloning with TopoTA or TopoXL kits (Invitrogen, Groningen, The Netherlands). Plasmid DNA was purified with a QIAprep Spin Miniprep kit (QIAGEN, Inc., Chatsworth, Calif.).

Analysis of transconjugants by pulsed-field gel electrophoresis (PFGE) and Southern hybridization. Total DNA from recipients and transconjugants embedded in agarose plugs was digested overnight at 27°C with 25 U of SmaI, and fragments were separated on a 0.8% agarose gel by using a CHEF-DRIII apparatus (Bio-Rad) under the following conditions: total migration, 24 h; initial pulse, 60 s; final pulse, 120 s; voltage, 6 V/cm; included angle, 120°; and temperature, 14°C. Resulting fragments were transferred to a Hybond N+ nylon membrane (Amersham) and hybridized to a probe specific for the xis_{Tn1549} and int_{Tn1549} genes corresponding to a 1,500-bp PCR product obtained from MLG101-1 using the xisF and intR primers. Total DNA from anaerobes and from three transconjugants (T1, T10, and T11) was digested with NdeI. Restricted DNA was electrophoresed on a 0.8% TAE (40 mM Tris-acetate, 1 mM EDTA)-agarose gel, transferred to a nylon membrane, and hybridized under stringent conditions with the *xis-int* probe (40). Hybridization signals were detected by chemiluminescence using CDP-*star* reagent (Amersham Biosciences) according to the manufacturer's recommendations.

Cloning and overexpression of the *xis* and *int* genes. A 1,487-bp SacI-XbaI PCR fragment obtained with primers XisF and IntR and containing the xis_{Tn1549} and int_{Tn1549} genes was cloned into shuttle vector pAT79 and resequenced. In this plasmid, designated pCM100, the *xis* and *int* genes were placed under the control of transcription and translation signals of gram-positive bacteria. pCM100 was then introduced into *E. faecium* 64/3::Tn*1549*-like and *E. faecalis* JH2-2::Tn*1549*-like by electrotransformation with selection on 80 μg/ml of spectinomycin.

Nucleotide sequence accession numbers. The target sequences for Tn*1549*-like in *E. faecalis* JH2-2, *E. faecium* 64/3, and *E. faecium* BM77 have been deposited in the GenBank data library under accession no. DQ119765 to DQ119820.

RESULTS

In vivo transfer of vancomycin resistance. Experiments were carried out in the digestive tract of gnotobiotic mice to mimic the natural conditions that prevail in vivo for vancomycin resistance transfer to take place. Irrespective of the donor (*C. symbiosum* MLG101-1, *Clostridium* sp. strain MLG055, or *E. lenta* MLG043) and of the recipient (*E. faecium* 64/3 or BM4105 or *E. faecalis* JH2-2) used, feces from challenged mice contained high numbers (10^9 to 10^{11} CFU/g of feces) of both donor and recipient (Fig. 1). A high level of colonization by the donor and recipient strains combined with the presence of vancomycin in drinking water created conditions helpful for the selection of transconjugants. Two transconjugants, T2 and T3, were detected using MLG101-1 as a donor and *E. faecium* 64/3 as a recipient in two independent experiments. A third transconjugant, T1, was obtained following transfer from MLG101-1 to *E. faecalis* JH2-2 in one out of two experiments (Table 3). Transfer of vancomycin resistance from MLG101-1 to *E. faecium* BM4105 or from MLG043 or MLG055 to any of the three recipients used was not obtained. All three transconjugants had MICs of vancomycin higher than 8 μg/ml and were shown to contain the *vanB* gene by PCR. Transfer of vancomycin resistance from T2 to BM77, BM4105, and BM4110 was not obtained in three independent experiments. The level of circular intermediate could be a limiting step in transfer of Tn*1549*-like. In order to circumvent this possibility, the *xis* and *int* genes of Tn*1549* were cloned into a shuttle vector generating pCM100 to overproduce the circular intermediate. Attempts to retransfer vancomycin resistance from T2 after the introduction of Tn*916* and/or plasmid pCM100 were unsuccessful.

In vitro transfer of vancomycin resistance. Based on six independent experiments, transfer of vancomycin resistance from MLG101-1 to *E. faecium* 64/3 was estimated to occur at frequencies of between 10^{-8} and 10^{-9} transconjugants per recipient (Table 4). Forty-eight transconjugants resistant to vancomycin and containing *vanB* were selected for further analysis. Transfer was not detected from MLG101-1 to *E. faecium* BM4105 or *E. faecalis* JH2-2 or from MLG043 or MLG055 to any of the recipients. When using T2 as a donor (containing Tn*916* and pCM100 or not), a single transconjugant (T29), transferred from T2::Tn*916* to *E. faecium* BM77, could be detected. Subinhibitory concentrations of vancomycin did not significantly influence transfer frequencies.

Characterization of the junction fragments of Tn*1549*-like in transconjugants. The junction fragments of Tn*1549*-like in MLG101-1, MLG043, and MLG055, in the three transconjugants obtained in vivo, and in the randomly selected 45 clones obtained in vitro were characterized by using TAIL-PCR or IPCR. For all transconjugants obtained from in vivo, in vitro, and retransfer experiments, a total of 29 unique integration events were observed from a total of 48 transconjugants studied (Fig. 2). In order to rule out mismatches during amplification, the nucleotide sequences of the target sites were confirmed by sequencing the conventional PCR products obtained with new primers from *E. faecium* 64/3 DNA. Quasi-identity with sequences available from a partial genome sequencing of *E. faecium* (GenBank accession no. AAAK00000000) was detected for 20 transconjugants, and homology with sequences in GenBank is summarized in Table 5. The flanking junctions of

	Insertion target		Tn*1549*-like Left end Right end		
Aa.	TATAACACATAAGAGTTGTATAACCGGCCGCTATATAGCCAAACTTTT	AATGCT	AAAATTTTAG---ATATAATTTT	GTATA	AAAATATAGTTTGTTATGATATTGACCAACGCTCCTGTAAATGTTGCA
Ab.	ACAGAGATTAAGGCGTACAATGATTTCTTGCCATTACTCCGCTTGTCT	ATTTT	AAAATTTTAG---ATATAATTTT	ATCCAA	AAAATATGGGTATTTGATAGTTCATCAGCCAGTACGTCGACTCTTTTG
T1a.	GAGCGTAGCAAAGAAAATAACGCCAGCTGACGTTCATTTTTACAGACG		AAAATTTTAG---ATATAATTTT	ATTTT	AAAGACATTGAAAGGATGTGGTGGCCTAGGACCAACATCATTGCTTCA
T1b.	TTTCGCAAAACTTCTGGTGATTTAACATTCAAATCAAAAGCAACTTTT	ATTTT	AAAATTTTAG---ATATAATTTT		AAAACAAAACTATCAGCAATTTCTAAGTTCTTTGGAAATTCGAGATTT
T2.	GAATCACCGTACATAAATATTACTCGTTAAGGGGAATTGAGAAATTTT	GTATA	AAAATTTTAG---ATATAATTTT		GTATGAAAATTTAAGAATTAGTTAGTATGCGCAAGTCAAAAATATGAA
T3.	GACACCCACGTTTCTCATGTTGTCACCAAAACGAGCCACTTTTATTCC		AAAATTTTAG---ATATAATTTT	ATTTT	AAAACTTTCATTATAGGCTACTGCAACATCCATCCAATCAGCAATCTC
T4.	AGAAATCTAGATTCTGATGTAATAGACCTTTATTCTAATAAAGCTTTT	ATTGA	AAAATTTTAG---ATATAATTTT		ATTGAAAAAGGTTTGAGGAATACGTTGTCTCCTGCAAACGTAAAGAGC
T5.	TCTTGTATAGCTTCTGTCTTAGAGAAGCAAGGAGCTATATTTTGTGCT		AAAATTTTAG---ATATAATTTT	AATGCT	ACAATCTTAAAAAGGTAGAGAGAAGACCAAATGGAAGACAGAGGAGCC
T6.	AGAAATCAAGGAGCAGATACGCCAGTTGTTTAATTTGAATATTATTTT	GTATA	AAAATTTTAG---ATATAATTTT		AAAATTAAAATTTTTATTCTGTTCTAAACATTTTTGGTTGTTTAGAAC
T7.	TTAAAACGCTTTCTGAAAGTGGCGGATTTTTTGCCTTTTTAAAATTTT	AATGCT	AAAATTTTAG---ATATAATTTT		ATTCTAAAATTTACTCTATTTGTGGGTGTTTTGTGCTTTTTCTCTTCC
T8.	TTAAAACGCTTTCTGAAAGTGGCGGATTTTTTGCCTTTTTAAAATTTT	GTATA	AAAATTTTAG---ATATAATTTT		ATTCTAAAATTTACTCTATTTGTGGGTGTTTTGTGCTTTTTCTCTTCC
T9.	TTAAAACGCTTTCTGAAAGTGGCGGATTTTTTGCCTTTTTAAAATTTT	ATTCT	AAAATTTTAG---ATATAATTTT		ATTCTAAAATTTACTCTATTTGTGGGTGTTTTGTGCTTTTTCTCTTCC
T10.	AGGAATCATTCAGCCTGTGATACGCAGTCAGGCAAATTATTTTATCCA	ATCCAA	AAAATTTTAG---ATATAATTTT		AAAAAATATTCGCTACGAGATCGTTGGTGAGGATCATACTGTTTTGAC
T11a.	TTTTCCTTAGTGAGTATCAAAAAGATAGTGATATTGAATATAAATTTT	AATGCT	AAAATTTTAG---ATATAATTTT		ATTTGGAAAATGAAGCACTACTCAATGGTACTTTCGAAGTACTAGGTT
T11b.	TAGGATTGTCTCTAAAGCTTAGTTTCTTTTTCTTTATATTTTCATAAT		AAAATTTTAG---ATATAATTTT	AATGCT	GAAAAGTGTATAGGATTTTTTAATTGGTACTTTTCGACAGTAATATGT
T12.	AGAAATCAAGGAGCAGATACGCCAGTTGTTTAATTTGAATATTATTTT	ATTTT	AAAATTTTAG---ATATAATTTT		AAAATTAAAATTTTTATTCTGTTCTAAACATTTTTGGTTGTTTAGAAC
T13.	AAAAGAGTATCAGAACAAAAATAATAACACGCATCTTGTAAAACTTTT	GTTCA	AAAATTTTAG---ATATAATTTT		GTTCAAAAAGTTCGTTTTTCCCTATTCCCTCCTCTTTTTTCAGGTTTT
T14.	GAAGTATTGTCAAGAATGGAAAGAGGCGATGTATTGACCCATTGTTTT	GTATA	AAAATTTTAG---ATATAATTTT		AATGGAAAACCAAACGGTATTTTAGATCCAAAGACAGATCAAATTAAA
T15.	TATAGTCTTATTTGTTGACTTTAGTCTACTTTGAGACTGTGAACTTTT	GTATA	AAAATTTTAG---ATATAATTTT		TATGAAAAACTTTAGGAAAAATCATTGTTTTCAGATGAAAATATTTGA
T16.	TGTCACACCAAGCATGAAACAATTAGAAGAAAAAACAGCAAAATTTTT	AGATG	AAAATTTTAG---ATATAATTTT		AGATGAAAACCAATTGACTTATGATACGATCGAAACATTTTCCACACC
T17.	TAAAACAATTGAAGACAGAGTTAGAAAAGTATGCTTACTTTTTATTTT	ATTTT	AAAATTTTAG---ATATAATTTT		ACTTTTAAAATCTTACTGTATCAACTTATCGCAATTGCAGAAAATTGA
T18.	TTTTTCTAACAAATAATCTAATAAAACTACTTTTTTGAAAGATGTTTT	AATGCT	AAAATTTTAG---ATATAATTTT		AGTTAAAAAAGTTTGCATCATTTCACCTCAATAAGTTACTTTTGTTTA
T19.	AAGCTTCAGCGATAGCTGTCATTTACTCCCTGTTGATTGCTATGTTTT	GTATA	AAAATTTTAG---ATATAATTTT		ACTATAAAACGGTAAAACTAAAAGATATACCTAATATGTTGTTTCAAT
T20.	ACCAGCGTGCTAATTTCTCACCCTCATCTTTCCCTGAACTTTTGTTAT	GTATA	AAAATTTTAG---ATATAATTTT		AAAAAACTAGCAGCTTTTCCATTCTTTACCTCCTATTTACTCTTCAAA
T21.	AGCATTTTTTTCAATAGTTTTCCACAGAAACCGAAGAAAAAAATTTTT	TAATT	AAAATTTTAG---ATATAATTTT		TAATTACAATTATCCACACAATCGACAAAAAGAAAAAAGAGAAAAACA
T22.	TAAGAATCGTTCCCATGAATTTTGCTCTGATAGCAATGTTTTGTTTAT		AAAATTTTAG---ATATAATTTT	ATTTT	AAAAATACGTTCTATTGTAAACATGACACTGTGAAGTGCCATTTATAC
T23.	TGGAAATGGCACATCTAGCGAAAGAAAAAAATGTTTGGGTTGTTTCCC		AAAATTTTAG---ATATAATTTT	ATTTT	AAAACATCGACGGTTTGCACGCAAAGGCTAAAAGCCCGCAACTCGTCA
T24.	GGTATAAAAGATCATGAATCGATCTTCTATACCGCTTGTTTTTACTAT		AAAATTTTAG---ATATAATTTT	GTATA	AAAATGATTGATTTAGCCCGCGGGCTCTAAACGATCAATATAAGGACT
T25.	AACTTATATTATAGTCTCTTTTGGTAGGGAACCATCTTATTTTTAATG		AAAATTTTAG---ATATAATTTT	GTATA	AAAATCAAGCATTTTAGCTATTGAAGAATGTTGATTTTATAGGGATTC
T26.	GAAAGCATTGGATTTTGAAACAGCCGCTAATGTGCGAGATATGATTTT	GTATA	AAAATTTTAG---ATATAATTTT		AGAATTAAAAGCTTCAAAGTAGGAGGACAACATGGCTAACGATAAAAT
T27.	ATTGGATTTTGAAACAGCCGCTAATGTGCGAGATATGATTTTAGAATT		AAAATTTTAG---ATATAATTTT	GTATA	AAAAGCTTCAAAGTAGGAGGACAACATGGCTAACGATAAAATTACGAT
T28.	GATAATTCTTCTATAAAAACACTATCGGGTTCAAAGTAGTTGTAATTA		AAAATTTTAG---ATATAATTTT	AATGCT	AAAACTTTCCACATAAAGTAGATAGCCGGAAGAGTCAAAATAGCTAA
T29.	CCGCGGACACGTCTTTCTTTATAATTTCCTCCTTGAATATCATAACTT		AAAATTTTAG---ATATAATTTT	GTATA	TTCGTTACAAAGAACGAGGCATTTTTTTTGGGAACAGGTTGCATTTGC

FIG. 2. Target DNA sequences at the integration sites of Tn*1549*-like in *C. symbiosum* MLG101-1, *E. faecalis* JH2-2, *E. faecium* 64/3, and *E. faecium* BM77. Insertion target rows: A, *C. symbiosum* MLG101-1; T1, *E. faecalis* JH2-2; T2 to T28, *E. faecium* 64/3; T29, *E. faecium* BM77. Coupling sequences are colored, each color corresponds to a specific sequence, and target duplication is indicated by yellow boxes.

Tn*1549*-like in the 29 distinct transconjugants showed acquisition of five (18 transconjugants) or six (7 transconjugants) base pairs originally flanking the transposon in MLG101-1. In the remaining transconjugants, surprisingly, 5-bp duplications of the target site were detected. Except for two hot spots for insertion common to three (T7 to T9) and two (T6 and T12) independent events and easily recognized at the coupling sequence level, 24 different integration sites were recovered for *E. faecium*, whereas two integration sites were detected in the *E. faecalis* JH2-2 transconjugant T1 (Fig. 2). These data showed that Tn*1549* can transpose at multiple sites in the *E. faecium* genome, although only two hot spots for insertion were identified.

DNA analysis of transconjugants by PFGE. Total DNA from *E. faecium* 64/3 and BM77, *E. faecalis* JH2-2, and transconjugants T1 to T29 was analyzed by PFGE after digestion by SmaI. The acquisition of a ca. 34-kb fragment corresponding to Tn*1549* was detected in 27 out of the 29 transcon-

TABLE 5. Insertion targets of Tn*1549*-like in *E. faecium* strains 64/3 and BM77

Transconjugant	*E. faecium* strain DO[a]	Target
E. faecium 64/3		
T2	Contig 636 (19731)	Hypothetical protein
T3	Contig 646 (32512)	G2160: L-arabinose isomerase
T5	Contig 655 (25838)	Noncoding sequence
T6 and T12	Contig 605 (10224)	Noncoding sequence
T7, T8, and T9	Contig 626 (20593)	Noncoding sequence
T10	Contig 651 (27393)	COG0642: signal transduction histidine kinase
T11	Contig 596 (8946)	COG4698: uncharacterized protein conserved in bacteria
T13	Contig 578 (8952)	Noncoding sequence
T14	Contig 635 (19630)	COG3964: predicted amidohydrolase
T15	Contig 632 (3516)	Noncoding sequence
T16	Contig 618 (10216)	COG0751: glycyl-tRNA synthetase, beta subunit
T17	Contig 627 (18298)	COG3279: response regulator of the LytR/AlgR family
T19	Contig 633 (17428)	COG1593: TRAP-type C4-dicarboxylate transport system, large permease component
T20	Contig 621 (11540)	COG1597: sphingosine kinase and enzymes related to eukaryotic diacylglycerol kinase
T24	Contig 619 (13913)	Noncoding sequence
T25	Contig 611 (3757)	Noncoding sequence
T26 and T27[b]	Contig 559 (T26, 6622; T27, 6628)	COG0556: helicase subunit of the DNA excision repair complex
T28	Contig 655 (59076)	Hypothetical protein
E. faecium BM77		
T29	Contig 603 (17495)	Hypothetical protein

[a] Identity with *E. faecium* strain DO (GenBank accession no. AAAK00000000) was 98% to 100% in all sequences from *E. faecium* 64/3 and 88% with contig 603 from *E. faecium* BM77. The position of the insertion in *E. faecium* DO is shown in parentheses.
[b] Insertions differ by a 6-bp shift.

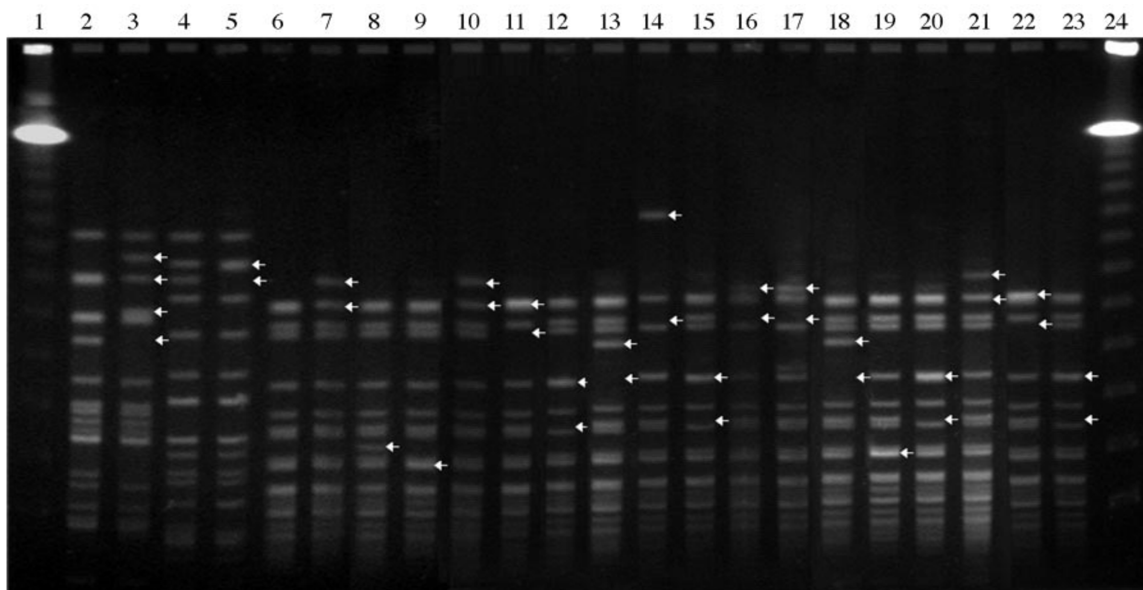

FIG. 3. SmaI PFGE profiles of *Enterococcus* recipient strains and of the transconjugants. Lanes: 1, 48.5- to 970-kb lambda ladder (Bio-Rad); 2, *E. faecalis* JH2-2; 3, T1 (JH2-2::Tn*1549*-like); 4, *E. faecium* BM77; 5, T29 (BM77::Tn*1549*-like); 6, *E. faecium* 64/3; 7, T2 (64/3::Tn*1549*-like); 8, T10; 9, T7; 10, T4; 11, T5; 12, T6; 13, T25; 14, T11; 15, T12; 16, T13; 17, T14; 18, T21; 19, T17; 20, T19; 21, T20; 22, T24; 23, T15; 24, lambda ladder. Arrows indicate the fragments that varied in size by acquisition of the ca. 34-kb Tn*1549*-like element. In some cases, comigrating fragments were observed. For T1 and T11, two copies of Tn*1549*-like were detected.

jugants (Fig. 3). The restriction profile of T1, compared to that of *E. faecalis* JH2-2, suggested the integration of two copies of Tn*1549*-like. By contrast, acquisition of a fragment larger than 68 kb was detected in T11, suggesting cotransfer of additional genetic material to the two copies of the transposon. Acquisition of additional material was also detected in T16 (data not shown).

Copy number of Tn*1549*-like in donors and transconjugants. Total DNA of MLG101-1, MLG043, MLG055, T1, T10, and T11 was analyzed after digestion by NdeI and hybridized with a probe specific for the int_{Tn1549} and xis_{Tn1549} genes. Since NdeI does not cut in these genes, the presence of two hybridizing fragments in MLG101-1, T1 (data not shown), and T11 indicated that these strains harbored two copies of Tn*1549*-like; by contrast, a single copy of the transposon was detected in T10 (data not shown). MLG101 was previously reported as harboring a single copy of the transposon (7), indicating that duplication in MLG101-1 had spontaneously occurred during subculturing.

Detection of circular intermediates. The presence of circular intermediates of Tn*1549* was detected in MLG101-1, MLG043, and MLG055 and in all 29 transconjugants by PCR or nested PCR using primers designed to direct polymerization outward from the ends of the transposon. The circularized end products from MLG101-1, MLG043, MLG055, T1, T2, T3, and T11 and from T1(pCM100) and T2(pCM100) overproducing Int_{Tn1549} and Xis_{Tn1549}, which led as expected to an increased amount of the circular intermediate (data not shown), were sequenced. Analysis of these products showed that the coupling sequences which joined the ends of the circularized transposon consisted of 5 or 6 bp. These data are in agreement with that for the base pairs acquired at the target level during insertion (Fig. 2).

DISCUSSION

The presence of Tn*1549*-like elements in many distinct strains of various genera, including enterococci and anaerobic bacteria, suggests that this element is a functional conjugative transposon. Until now, however, transfer of Tn*1549* was associated with the movement of plasmids or of large elements of various sizes (9, 17, 21, 37, 39). To study the intergeneric transfer of vancomycin resistance, we took into account the failure of previous in vitro experiments to demonstrate the active movement of Tn*1549*-like elements (9, 15, 21) and the fact that the intestinal ecosystem is the most probable habitat for meetings between gram-positive anaerobic bacteria and *Enterococcus* species. The digestive tract of humans and animals, in which very dense and diverse microbial populations live in intimate contact, sometimes as part of biofilms, constitutes an extremely favorable ecosystem for gene transfer (5, 19, 31). We have performed experiments in the digestive tract of gnotoxenic mice to mimic the natural conditions that prevail in vivo for vancomycin resistance transfer to take place. Intergeneric transfer was achieved both in vivo and in vitro into *E. faecium* 64/3. A single *E. faecalis* transconjugant was obtained in vivo, whereas in vitro experiments led to higher numbers of events in *E. faecium* 64/3. It is unclear whether the Tn*1549*-like elements included in this study differ from Tn*1549* and Tn*5382* with respect to transfer ability. Our results indicate that the nature of the strains involved in conjugation was the main factor influencing the occurrence of transfer, with MLG101-1 being the most efficient donor and *E. faecium* 64/3 being the best recipient. This suggests that host factors may be involved in the transfer process.

Interestingly, MLG101-1 harbored two copies of Tn*1549*-like elements. It has been proposed for Tn*916* that multiple copies of the transposon can increase the transfer frequency

(38). Although two copies of Tn*1549* were also detected in T1 and T11, all attempts to transfer resistance in vivo and in vitro from these transconjugants were unsuccessful. We recently performed conjugation between MLG101 and *E. faecium* 64/3 in an in vitro experiment and showed that transfer also occurred. This indicated that a second copy of Tn*1549*-like was not essential for conjugative transposition. To account for transfer of Tn*1549*-like elements from *C. symbiosum* to *Enterococcus* spp. we hypothesize that conjugative transposition could be more easily detected after intergeneric transfer, since plasmid replication or homologous recombination via chromosomal fragments should be strongly reduced, two features which could impair the detection of the conjugative transposition of Tn*1549* in enterococci. Furthermore, the detection of transconjugants is also made difficult because *vanB* can confer a low level of resistance to vancomycin and requires induction for phenotypic expression (23).

In order to explain the weak transfer efficiency of Tn*1549*-like elements, we have carried out in vitro experiments and showed that vancomycin did not increase transfer frequency. In contrast, tetracycline induces transfer of Tn*916* by the formation of a large transcript through the circular intermediate (11). In addition, overproduction of Int and Xis in T1 and T2 did not influence transfer or the rate thereof. A similar feature has been reported for Tn*916* excision, which is necessary but not sufficient for the occurrence of conjugal transfer (28). Because of the failure to retransfer vancomycin resistance from the transconjugants, we introduced Tn*916*, which is capable of mobilizing plasmids or other transposons (20), into T1 and T2 and into their derivatives overexpressing Int and Xis. Despite numerous in vivo and in vitro attempts, we obtained only a single transconjugant from T2::Tn*916*, which indicates that retransfer from *E. faecium* 64/3 was possible but did not give evidence for a role of Tn*916* in mobilization of Tn*1549*-like.

The fact that in our experiments MLG101-1 was the only efficient donor for conjugative transposition could also be due to a polar effect of the genomic environment. The location and orientation of transfer genes in Tn*1549* are in part similar to those of Tn*916*, and transcription of these genes may have resulted following transposon insertion near an active promoter. Examination of the sequence in MLG101-1 for a putative promoter sequence upstream from the genes involved in transfer of Tn*1549* did not show consensus promoter motifs. Thus, the reason why MLG101-1 could act as a donor whereas MLG055 and MLG043 did not remains unexplained.

Conjugative transposition of Tn*916* has been studied extensively (38). Transposition of Tn*916* proceeds by excision and formation of a circular intermediate in which the ends of the transposon are separated by six nucleotides, resulting from staggered cleavages by Int (43). The excised transposon contains a heteroduplex consisting of the 6-bp sequences termed coupling sequences. Insertion is the reverse of excision, since staggered cuts occur at the junction of the circular molecule and the new target site. This produces an integrated transposon flanked by heteroduplex coupling sequences which are resolved by DNA replication. Analysis of the junction fragments flanking the end of Tn*1549*-like in 29 different transconjugants indicated that 24 coupling sequences were inherited from the donor. Both GTATA and AATGCT sequences originated from flanking sequences of the first copy of the transposon in MLG101, whereas ATTTT and ATCCAA were attributed to the second copy (Fig. 2). This resulted in asymmetric acquisition of either five or six nucleotides from the donor, depending on the level of DNA cutting. In addition, five events corresponded to 5-bp nucleotide duplications of the target site, similar to what was seen for duplications produced by other kinds of transposons, although the mechanism is completely different. Such a feature has been reported for Tn*916* and was attributed to mismatch repair following integration of the transposon (41). This could have also been the result of two successive transposition events, in which the transposon brings a coupling sequence from the intermediate integration site, which is identical to that of the final target. However, the fact that five independent transposition events led to exact 5-bp duplication does not favor this hypothesis. Moreover, we might have predicted that random cutting upon insertion would have generated distinct 5-bp flanking regions, which were not observed. Interestingly, three different events, either with acquisition of five or six nucleotides or with duplication of five base pairs, were observed in T7, T8, and T9 at the same locus. Two other events with acquisition of five nucleotides in T6 and T12 were detected at another locus. These two loci are likely to be two hot spots for Tn*1549*-like insertion (Fig. 2).

The acquisition of fragments larger than 68 kb by T11 and T16 is not clearly understood and is probably due to chromosomal rearrangement during DNA replication, since analysis of the flanking regions of Tn*1549* gave evidence of a transposition process.

Surprisingly, T1 and T11 contained two copies of Tn*1549*-like elements, as did MLG101-1. Since conjugative transposons do not transpose in a replicative manner, the presence of multiple copies could be explained either by intracellular transposition between daughter chromosomes after passage of the replication fork (38), by cotransfer, or by independent acquisition of the two copies by horizontal transfer.

The presence of *vanB2* on a functional conjugative transposon accounts for its spread in both anaerobes and enterococci. These elements have a broad host range that includes numerous genera of gram-positive bacteria. It has been recently reported that mobile elements designated as integrative and conjugative elements encode integrative and putative transfer functions related to those of conjugative transposons (8). Such structures have been found in the genomes of various bacteria with low G+C content, including *Bacillus subtilis*, *Butyrivibrio fibrisolvens*, *Clostridium difficile*, *E. faecalis*, *Listeria monocytogenes*, *S. aureus*, *Streptococcus mutans*, and *Streptococcus thermophilus*. A similar structure or a conjugative transposon has probably recruited the *vanB* operon from a glycopeptide producer to form a Tn*1549*-like element.

Recently, operons closely related to *vanA* and *vanB* have been reported for *Paenibacillus thiaminolyticus* and *Paenibacillus apiarius* (22). Anaerobic bacteria could have played a role as an intermediate for the transfer of *vanB*-mediated glycopeptide resistance from glycopeptide producers to enterococci. Anaerobes which constitute the majority of the bacteria in the digestive tract and enterococci largely present are also very common in soil. The high density and the promiscuity of these bacteria in the digestive tract, associated with the presence of glycopeptide, create favorable conditions for the occurrence of transfer. Our data provide further support for the hypothesis

that *vanB*-containing, naturally occurring anaerobes may be associated with the emergence of new strains of vancomycin-resistant enterococci under appropriate clinical selective conditions (6, 42). Due to their broad host range, conjugative transposons are exceedingly important in bacterial evolution, and the fact that Tn*1549* is a genuine conjugative transposon highlights the transfer potential of VanB-type glycopeptide resistance in pathogenic species. This fact argues in favor of detecting the carriage of the *vanB* gene directly from the feces by PCR in order to control the spread of this resistance determinant.

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