ICESlu\textsubscript{van}, a 94-Kilobase Mosaic Integrative Conjugative Element Conferring Interspecies Transfer of VanB-Type Glycopeptide Resistance, a Novel Bacitracin Resistance Locus, and a Toxin-Antitoxin Stabilization System

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A 94-kb integrative conjugative element (ICE\textsubscript{Slu\textsubscript{van}}) transferable to \textit{Enterococcus faecium} and \textit{Enterococcus faecalis} from an animal isolate of \textit{Streptococcus lutetiensis} consists of a mosaic of genetic fragments from different Gram-positive bacteria. A variant of ICE\textsubscript{Slu\textsubscript{van}} was confirmed in \textit{S. lutetieniensis} from a patient. A complete Tn5382/Tn1549 with a vanB2 operon is integrated into a streptococcal ICE\textsubscript{de3396} -like region harboring a putative bacteriophage exclusion system, a putative agglutinin receptor precursor, and key components of a type IV secretion system. Moreover, ICE\textsubscript{Slu\textsubscript{van}} encodes a putative MobC family mobilization protein and a relaxase and, thus, in total has all genetic components essential for conjugative transfer. A 9-kb element within Tn5382/Tn1549 encodes, among others, putative proteins similar to the TnpX site-specific recombinase in \textit{Faecalibacterium} and VanZ in \textit{Paenibacillus}, which may contribute to the detected low-level teicoplanin resistance. Furthermore, ICE\textsubscript{Slu\textsubscript{van}} encodes a novel bacitracin resistance locus that is associated with reduced susceptibility to bacitracin when transferred to \textit{E. faecium}. The expression of a streptococcal pezAT toxin-antitoxin-encoding operon of ICE\textsubscript{Slu\textsubscript{van}} in \textit{S. lutetieniensis}, \textit{E. faecium}, and \textit{E. faecalis} was confirmed by reverse transcription (RT)-PCR, indicating an active toxin-antitoxin system which may contribute to stabilizing ICE\textsubscript{Slu\textsubscript{van}} within new hosts. Junction PCR and DNA sequencing confirmed that ICE\textsubscript{Slu\textsubscript{van}} excised to form a circular intermediate in \textit{S. lutetieniensis}, \textit{E. faecalis}, and \textit{E. faecium}. Transfer between \textit{E. faecalis} cells was observed in the presence of helper plasmid pIP964. Sequence analysis of the original \textit{S. lutetieniensis} donor and enterococcal transconjugants showed that ICE\textsubscript{Slu\textsubscript{van}} integrates in a site-specific manner into the C-terminal end of the chromosomal tRNA methyltransferase gene \textit{rumA}.

Horizontal gene transfer is a key factor in bacterial evolution, and mobile genetic elements (MGEs) play an important role in the dissemination and persistence of antimicrobial resistance in enterococci. Genome sequence analysis and comparative genome hybridizations of seven \textit{Enterococcus faecium} isolates from various sources have revealed large differences in genome size, mostly due to the variable presence of mobile genetic elements. The \textit{E. faecium} pan-genome is considered to be unrestricted in size (1), implying that excess genes, such as those involved in environmental persistence, colonization, and virulence, can easily be incorporated into the \textit{E. faecium} gene pool. Up to 38% of the \textit{E. faecium} genome may be noncore, and differences in gene content indicate that gain and loss of genes as important in the evolution of \textit{E. faecium} (1, 2, 3). These findings are consistent with the presence of more than 25% mobile or foreign DNA in \textit{Enterococcus faecalis} V583 (3, 4), suggesting extensive genome plasticity.

The first high-level vancomycin-resistant enterococci (VRE) were described in 1988 (5, 6). VRE have since become an increasing nosocomial problem both in Europe and the United States (7, 8). The origin of vancomycin resistance determinants is not known, although soil bacteria seem to represent a rich and assorted reservoir of genes closely related to \textit{vanA} (9). Moreover, analyses of genomes from invasive \textit{vanB}-positive \textit{E. faecium} isolates and \textit{vanB}-positive anaerobic gut commensals demonstrate that \textit{vanB} resistance in enterococci commonly arises through gene transfer from members of other bacterial genera in the human gastrointestinal tract (10). The \textit{vanB} cluster is mainly found in \textit{E. faecium} and \textit{E. faecalis}, though it has also been described in isolates of \textit{vanB}-resistant \textit{Enterococcus gallinarum} (11, 12, 13, 14), \textit{Enterococcus hirae} (15), \textit{Enterococcus durans} (16), \textit{Enterococcus casseliflavus} (17), \textit{Staphylococcus} (18), \textit{Streptococcus} (19, 20, 21, 22), \textit{Eggerthella}, \textit{Clostridium}, \textit{Ruminococcus} (23, 24, 25, 26, 27), and \textit{Atopobium} (27).

The \textit{vanB} gene cluster has a conserved gene order and can be divided into three genetic subtypes, \textit{vanB1}, \textit{vanB2}, and \textit{vanB3} (28, 29, 30, 31, 32). As an integral part of the conjugative transposon Tn5382/Tn1549, \textit{vanB2} is the most-widespread subtype in clinically important enterococci (30, 33, 34, 35, 36, 37, 38). Tn5382/Tn1549 is able to support transfer of the \textit{vanB2} operon from \textit{Clostridium perfringens} to \textit{Enterobacter cloacae} and \textit{Salmonella typhimurium} (27, 28).
and *E. faecalis* self-encoded transfer functions consistent with an integrative and strain that contains no visible plasmids into enterococci suggests appeared to integrate in a site-specific manner (20, 42). Transfer containing \(\text{vanB}_2\) was unable to transfer between enterococci in the intestinal environment (39), but the element was unable to contribute to the detected low-level resistance to teicoplanin, a novel bacteracian locus which seems to contribute to bacitracin resistance in *S. lutetensis* and *E. faecium* and a streptococcal toxin-antitoxin (TA) \(\text{pezAT}\) locus, as well as all essential components for conjugative transfer. ICESluvan excises to form a circular intermediate in *S. lutetensis*, *E. faecalis*, and *E. faecium* and integrates in a site-specific manner. However, transfer between enterococci required a helper plasmid. The expression of the \(\text{pezAT}\) operon indicates a functional toxin-antitoxin system.

### MATERIALS AND METHODS

**Bacterial strains and construction of BAC clones.** The bacterial strains used in this study and their relevant characteristics are given in Table 1. Briefly, genomic DNA from the *E. faecium* transconjugant MM5-F9a was used to generate a bacterial artificial chromosome (BAC) library with an average insert size of 90 kb (MWG Biotech AG). MM5-F9a was obtained by transfer of the ICESluvan element from *S. lutetensis* 5-F9, isolated from

###TABLE 1 Bacterial strains used in this study and their relevant characteristics

| Strain   | Species           | Properties                  | MIC \(\text{mg liter}^{-1}\) | Reference(s) |
|----------|-------------------|-----------------------------|-----------------------------|--------------|
|          |                   |                             | Vancomycin | Teicoplanin | Bacitracin |           |
| 5-F9     | *S. lutetensis*   | Van'- veal calf strain with \(\text{vanB}_2\) Tn5382 chromosomally located | \(\geq 256\) | 3 | \(\geq 256\) | 19, 20 |
| NEM760   | *S. lutetensis*   | Van'- human patient strain with \(\text{vanB}_2\) chromosomally located | \(\geq 256\) | 4 | \(\geq 256\) | 22, 42 |
| 4-C11    | *S. gallolyticus* | Van'- veal calf strain with nontransferable \(\text{vanB}_2\) Tn5382 and \(\text{vanA}\) | \(\geq 256\) | 32 | 24 | 19, 20 |
| 4-G10    | *S. gallolyticus* | Van'- veal calf strain with nontransferable \(\text{vanB}_2\) Tn5382 and \(\text{vanA}\) | \(\geq 256\) | 64 | \(\geq 256\) | 19, 20 |
| C68      | *E. faecium*      | \(\text{vanB}_2\) Tn5382 type strain | this study |
| BM4105-RF| *E. faecium*      | \(\text{Rif}^R\) \(\text{Fus}^R\) plasmid-free recipient | 1.5 | 0.5 | 48 | 46 |
| MM5-F9a  | *E. faecium*      | Transconjugant resulting from mating of 5-F9 and BM4105-RF | 32 | 3 | \(\geq 256\) | 20 |
| OG1-RF   | *E. faecalis*     | \(\text{Rif}^R\) \(\text{Fus}^R\) recipient derived from OG1 | 4 | 0.25 | 24 | 73 |
| OG5-F9a  | *E. faecalis*     | Transconjugant resulting from mating of 5-F9 and OG1-RF | \(\geq 256\) | 3 | 32 | this study |
| JH2-2    | *E. faecalis*     | \(\text{Rif}^R\) \(\text{Fus}^R\) plasmid-free recipient derived from JH2 | 3 | 1 | 16 | 74 |
| JHS-F9a  | *E. faecalis*     | Transconjugant resulting from mating of 5-F9 and JH2-2 | \(\geq 256\) | 3 | 8 | 20 |
| UV202    | *E. faecalis*     | \(\text{Rif}^R\) \(\text{Fus}^R\) plasmid-free recipient, recombination-deficient derivative of JH2-2 | 1.5 | 0.5 | 128 | 75 |
| UV5-F9d  | *E. faecalis*     | Transconjugant resulting from mating of 5-F9 and UV202 | \(\geq 256\) | 3 | 192 | 20 |
| BM4110   | *E. faecalis*     | \(\text{Str}^R\) plasmid-free recipient strain derived from JH2 | 1.5 | 0.25 | 96 | 76 |
| BM4110 pIP964 | *E. faecalis* | \(\text{Str}^R\) recipient strain derived from JH2 containing plasmid pIP964 (pCF10 rep agg; \(\text{Tra}^R\), 65 kb) | 1.5 | 0.38 | \(\geq 256\) | 76 |
| ICESluvan BM4110 pIP964 | *E. faecalis* | 1st-generation transconjugant resulting from mating of donor 5-F9 and BM4110 pIP964 | \(\geq 256\) | 3 | \(\geq 256\) | this study |
| ICESluvan pIP964 JH2-2 | *E. faecalis* | 2nd-generation transconjugant resulting from mating of ICESluvan BM4110 pIP964 and JH2-2 | \(\geq 256\) | 3 | \(\geq 256\) | this study |
| ICESluvan pIP964 UV202 | *E. faecalis* | 2nd-generation transconjugant resulting from mating of ICESluvan BM4110 pIP964 and UV202 | \(\geq 256\) | 3 | 192 | this study |

\(\text{vanA}\), vancomycin resistant; \(\text{Rif}^R\), rifampin resistant; \(\text{Fus}^R\), fusidic acid resistant; \(\text{Str}^R\), streptomycin resistant.

tridium to enterococci in the intestinal environment (39), but the transposon is more often transferred as a part of larger chromosomal elements or plasmids (30, 35, 37, 40, 41).

An approximately 100-kb transferable chromosomal element containing \(\text{vanB}_2\) has been described in a patient isolate of *Streptococcus lutetensis* previously designated *Streptococcus bovis* biotype II with \(\text{vanB}_3\) (22, 42) and in the plasmid-free *S. lutetensis* strain 5-F9 from animal feces (20). The \(\text{vanB}_2\) cluster of the 5-F9 strain has previously been shown to be an integral part of a Tn5382/Tn1549 element (20) and was transferred to *E. faecium* and *E. faecalis* at relatively high frequencies (10\(^{-7}\) to 10\(^{-5}\)). In contrast, the element was unable to transfer between *E. faecium* and *E. faecalis* strains (20, 42), which suggests a coresident transfer system in *Streptococcus* (42). Retransfer of the ~100-kb element from the patient isolate between *E. faecalis* strains, including to a recombination-deficient recipient, was obtained in the presence of a conjugative helper plasmid (42), and the ~100-kb element appeared to integrate in a site-specific manner (20, 42). Transfer of this ~100-kb chromosomal element from the *S. lutetensis* strain that contains no visible plasmids into enterococci suggests self-encoded transfer functions consistent with an integrative and conjugative element (ICE) (20). The transfer mechanism has not been resolved.
the face of a real calf in the Netherlands, to recipient E. faecium BM4105-R by filter mating (20). The vanB2-containing strains S. lutentesis NEM760, Streptococcus galolyticus 4-C11, and S. galolyticus 4-G10 were included to search for ICESlvan in streptococci. E. faecalis transconjugants OG5-F9a, JH5-F9a, and UV5-F9d and laboratory strains OG1-1R, JH2-2, BM4110, and UV202 were used for various studies of ICESlvan as indicated below. Total DNA from E. faecium C68 (40) was used as the template for probe synthesis.

Selection and sequencing of BAC clones. Sequential dot blot and Southern hybridizations were used to identify BAC clones positive for both vanB2 and Tn5382/Tn1549. PCR-based probes were made (PCR digoxigenin [DIG] probe synthesis kit; Boehringer Mannheim) for the probes (nDNA was isolated from BAC clones that scored positive for one or both probes (n = 24). Clones were grown overnight in yeast extract-tryptone (YT) medium containing 12.5 μg ml−1 chloramphenicol, plasmid replication was induced (Epicentre induction solution: Epicentre), and BAC DNA was purified using the E.Z.N.A BAC/PAC kit (Omega Bio-Tek). NotI (New England BioLabs)-digested plasmid DNA was separated on a 1.2% agarose gel, 5 to 15 s, 6 V/cm, 16 h, at 15°C on a CHEF-DR III (Bio-Rad) and analyzed by Southern hybridization. Five clones positive for both probes and with an average insert size of 90 kb were selected for sequencing.

Libraries of the BAC inserts of each selected clone were constructed with the use of multiplex identifiers (MIDs) and sequenced to an average depth of 91.5-times coverage using 454 GS-FLX technology. The BAC inserts were de novo assembled individually with Newbler (Roche).

End sequencing of the BAC inserts provided additional geographical information for contig ordering. End sequencing and extension of contigs were set up using extracted BAC DNA and BigDye 3.1 for cycle sequencing and an ABI prism 377 genetic analyzer (Applied Biosystems). The cycle sequencing program was as follows: initial denaturation at 96°C for 5 min followed by 99 cycles of 96°C for 30 s, 52°C for 10 s, and 60°C for 4 min (primers available on request).

DNA extraction and sequencing of transconjugants. Genomic DNA from transconjugants was obtained using a bacterial DNA kit (E.Z.N.A.) with the following modifications: lysis was performed at 30°C for 40 min using 1.5 mg lysozyme and 100 U mutanolysin in a total volume of 220 μl. Direct genomic sequencing of the transconjugants was performed as for BAC end sequencing.

Gap closure and annotation. Abacas (http://abacas.sourceforge.net/index.html) was used to map contigs from the de novo assembly against Streptococcus suis BM407 (FM252032) and Tn1549 (AE192239). The sequence was annotated using Artemis software (43). Homology comparisons to nonredundant protein databases were performed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and FASTA (http://www.ebi.ac.uk/Tools/fasta33/index.html) software. Protein motifs were identified using Pfam (http://pfam.sanger.ac.uk/search) and Prosite (http://au.expasy.org/prosite/). Transmembrane domains were identified with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), and signal sequences were identified with SignalP version 3.0 (44). Comparison of the large mobile elements was facilitated using the Artemis Comparison Tool (ACT) (45), which enabled the visualization of BLASTN and TBLASTX comparisons between these sequences.

Verification of sequence assembly by PCRs. To verify the sequence assembly, over 40 PCRs with an average product size of 2.5 kb were performed in ICESlvan (see Table S1 in the supplemental material), using genomic DNA of transconjugants E. faecalis MM5-F9a and OG5-F9a and E. faecalis JH5-F9a as templates. Some of these primers were used to confirm the presence of ICESlvan regions (see Table S1) in streptococci (S. lutentesis 5-F9 and NEM760 and S. galolyticus 4-C11 and 4-G10) and to sequence the bacitracin locus in the E. faecalis transconjugants OG5-F9a and JH5-F9a.

RNA extraction for expression analyses. The expression of the pezAT operon was analyzed by semi-quantitative reverse transcription (RT)-PCR. Total RNA was extracted from stationary-phase cultures using an RNeasy minikit (Qiagen) with an extended lysis step of 1 h with 10 mg lysyoxine and 10 μg mutanolysin in a total volume of 200 μl. DNA contamination was removed by DNase I treatment, and RNA quality and quantity examined by gel analyses and spectrophotometric measurements prior to cDNA synthesis. Reverse transcription of 100 ng total RNA was performed using SuperScript II (Invitrogen). For each sample, a control reaction (without reverse transcriptase) with no enzyme was included. PCR was performed using primer sets specific for each of the genes (pezAF [5'-TGACCCCCGATGGTTTTC-3'] and pezAR [5'-TGCGCGAATTTTAATGAGAC-3']) and pezTF [5'-TTTTTTCTCTGAAAAATCCCTG-3'] and pezTR [5'-GACCGGCGAGCATTGAC-3']). In addition, PCR using primers pezTF and pezAR was performed. Amplicons were visualized on an agarose gel and confirmed by sequencing using BigDye 3.1 technology (Applied Biosystems).

Verification of ICESlvan circularization. Circularization of the ICESlvan element was examined by PCR using primers located on the very end of the element (see Fig. 2), as follows: 5'-AAGGGGAAATGACCAGTTACA-3' (nucleotides [nt] 93779 to 93798), 5'-CTGGCTGGAATATTAATGAAATGCCG-3' (nt 93385 to 93402), and 5'-TCCCAAACAGACATTCCAGAATC-3' (nt 130 to 151). PCR products were confirmed by sequencing.

Transfer of ICESlvan. Transfer of the ICESlvan element to E. faecalis strains BM4110 and JH2-2 with plPT964 and retransfer from these first-generation transconjugants to recipients (E. faecalis strains JH2-2, UV202, and BM4110) without this helper plasmid were performed by filter mating as previously described (33), with some modifications. Briefly, donor and recipient strains were grown to an A600 of 0.6 and mixed in a ratio of 1:1 in 2 ml of culture. The suspension was pelleted, resuspended in 150 μl brain heart infusion (BHI), and used for filter mating before application on selective agar plates using 8 mg liter−1 vancomycin, 1000 mg liter−1 streptomycin, 20 mg liter−1 rifampin, and 10 mg liter−1 fusidic acid. A control experiment (E. faecalis JH5-F9a × BM4110) with recipients containing no helper plasmid was also carried out. The presence of the vanB gene of ICESlvan was confirmed by PCR (29), and ICESlvan insertion in the transconjugant genome was confirmed by visual inspection of the Smal pulsed-field gel electrophoresis (PFGE) pattern. The presence of the pheromone-responsive helper plasmid plPT964 (46) was determined by PCRs that detect the pCF10 replication initiation gene repCF10 as described by Jensen et al. (47), and the presence of the pheromone plasmid’s conserved aggregation substance (agg) was detected using primers 5'-GC TCGTGGTGAATCGTCCTTCTCC-3' and 5'-CCTTCTACCTAATTGCCG CTAC-3'.

PFGE analyses. Total DNA was digested with SmaI (New England BioLabs) and analyzed by PFGE as previously described (48), with some modifications. Briefly, 5 μl lysozyme (100 mg ml−1) and 2 μl mutanolysin (10 μg ml−1) were added to the agarose plugs before molding, as well as to the lysis buffer.

Antimicrobial susceptibility testing. Susceptibility testing was performed using MIC test strips for vancomycin, teicoplanin (bioMérieux), and bacitracin (Liofilchem) on S. lutentesis 5-F9, E. faecalis JH5-F9a, JH2-2, OG5-F9a, and OG1-1R, and E. faecium MM5-F9a and BM4105-R according to the manufacturers’ recommendations.

Nucleotide sequence accession number. The whole sequence of ICESlvan is available in GenBank under the accession number HE963029.

RESULTS AND DISCUSSION

Gap closure and ICESlvan mosaic structure. The five selected BAC clones were sequenced to an average depth of 91.5-times coverage and assembled individually into an average of 28 contigs (>500 bp). Mapping of contigs against reference sequences and
end sequencing of BAC inserts provided scaffold information for the gap closure that resulted in one large, 94-kb contig (GenBank accession number HE963029) containing 86 open reading frames (ORFs) (Fig. 1; see also Table S2 in the supplemental material). Nine smaller contigs of >1 kb with BLAST similarities to unculturables organisms and transposases were discarded from further analysis. Extensive control PCRs (see Table S1) verified the assembly for *E. faecium* transconjugant MM5-F9a and confirmed the presence of the ICESlvan element in *E. faecalis* transconjugants OG5-F9a and JH5-F9a (Table 1). Some primer combinations used for MM5-F9a did not give PCR products with OG5-F9a and JH5-F9a. However, the presence of these regions was confirmed by altering the combination of the primers (denoted by plus signs in Table S1). Some of the PCRs worked at one time point and not at another, and when we sequenced the bacitracin locus of the transconjugant MM5-F9a and confirmed the ORFs (ORF4 to -13, ORF14, ORF69, and ORF75 to -86) showed similarities (Fig. 1; see also Table S2 in the supplemental material) (30). Five more ORFs were identified in the Tn5382/Tn1549 element inserted in ICESlvan compared to those from previous reports (see Table S2). Identical sequences were also present in Tn5382/Tn1549 but have not been annotated previously. In addition, a 9-kb contig located within ORF30 in Tn5382/Tn1549 was flanked by directly repetitive sequences with 291 of 292 nucleotides identical. Annotation of the 9-kb contig revealed a *TrpX* site-specific recombinase (ORF31), a pseudotransposase of the IS30 family (ORF33), a putative VanZ family protein (ORF34), a putative mobilization protein (ORF36), and a DNA primase (ORF37), as well as hypothetical proteins (ORF32, -35, and -38) with 40 to 96% amino acid identity to putative proteins in *Faecalibacterium*, *Lactobacillus*, and *Paenibacillus* species, respectively (see Table S2).

The mosaic pattern of the ICESlvan element, which consists of genes from several different streptococcal species, *Faecalibacterium*, enterococci, and possibly other intestinal bacteria (see Table S2 in the supplemental material), indicate that mobilization and recombination events have been key factors in the assembly of this element. A mosaic pattern was also observed for ICESde3396, which is a montage of genes derived from group A, B, and G Streptococcus organisms, in addition to genes acquired from non-streptococcal Gram-positive bacteria, such as *Listeria innocua* and *E. faecalis* (49).

**ICESlvan variant confirmed in an*S. lutetiansis* isolate from a patient in France.** During examination of mixed fecal samples from 556 veal calf herds in the Netherlands, four vancomycin-resistant Streptococcus isolates were found. In addition to *S. lutetiansis* S-F9, *S. galloyticus* isolates 4-C11 and 4-G10 were positive for the *vanB* gene and found to have a *vanB2* cluster integrated in *S. lutetiansis* S-F9 (20). Furthermore, *S. lutetiansis* NEM760, isolated from a stool swab of a patient in France, showed transfer of
an approximately 100-kb element containing \textit{vanB2} (22, 42) that inserted into the same Smal PFGE fragment as ICE\textit{Slu}van in both \textit{E. faecium} BM4105-\textit{RF} and \textit{E. faecalis} JH2-derived recipients (20, 22, 42). Since 4-C11, 4-G10, and NEM760 are likely candidates to contain ICE\textit{Slu}van or variants of this element, we have examined these isolates by using primers selected to confirm the ICE\textit{Slu}van sequence assembly (see Table S1 in the supplemental material). The circa-100-kb element found in NEM760 was indeed very similar to ICE\textit{Slu}van, with some differences in the right end of the element, while 4-C11 and 4-G10 showed the presence of Tn5382/Tn1549 and adjacent regions but not the left or right end of ICE\textit{Slu}van, which is not surprising since transfer of \textit{vanB} was not achieved for these isolates (19, 20). The presence of highly similar variants of ICE\textit{Slu}van capable of interspecies transfer in two \textit{S. lutetiensis} isolates from completely different environments implies that ICE\textit{Slu}van is a successful element that may contribute to resistance in enterococci.

\textbf{ICE}\textit{Slu}van type IV secretion system components. A putative transfer system (ORF11 to -16 and ORF79 to -80) (see Table S2 in the supplemental material) was identified in the ICE\textit{Slu}van element, in addition to those encoded by Tn5382/Tn1549 (ORF21 to -23, ORF26, and ORF43 and -44) (see Table S2) (30). ORF11 to -15 were 94 to 99\% identical to ICE\textit{Sde}s3396_59 to ICE\textit{Sde}s3396_55 (49). The ORFs display the same genetic order in the ICE\textit{Slu}van element and belong to a type IV secretion system. Three factors have been recognized as important for DNA transfer by type IV secretion system in Gram-positive bacteria. These are murein hydrolase (50), which is involved in controlled local degradation of the peptidoglycan, making space for the formation of a mating channel, the VirB4 ATPase, which provides energy for the translocation, and the VirD4 coupling protein, which links the DNA transfer intermediate to the mating channel (50, 51). In the ICE\textit{Slu}van element, we identified two putative virB4 genes (ORF15 and ORF26) encoding ATPases, a putative virB6 gene (ORF13) encoding a putative membrane protein that has previously been shown to interact with several other Vir proteins mediating DNA substrate transfer through the cytoplasmic membrane channel (50), and two putative virD4 genes (ORF11 and ORF21) encoding coupling proteins of the TraG/TraD family. ORF11 is truncated by a frameshift after codon 465, but ORF21 encodes a putative coupling protein which belongs to the same coupling-protein family. Moreover, ICE\textit{Slu}van ORF16 encodes a putative \textit{N}-acetylglucosaminyl\textit{\&}-alanine amidase that may aid in local degradation of the peptidoglycan by hydrolyzing the amide bond between the \textit{N}-acetylglucosaminic acid side chain and \textit{\&}-alanine of the short peptide (52). Genes encoding putative mobilization proteins of the MobC family (ORF79 and ORF44) and relaxases (ORF80 and ORF43) were also identified in ICE\textit{Slu}van. Taken together, this indicates that the ICE\textit{Slu}van element contains most genes necessary for mobilization and conjugative transfer. Furthermore, ICE\textit{Slu}van is transferable by conjugation from \textit{S. lutetiensis} organisms with no visible plasmids, strongly suggesting that its transfer system is intact (20).

\textbf{ICE}\textit{Slu}van circularization, transfer, and integration. A putative site-specific serine recombinase (ORF86) (see Table S2 in the supplemental material) that can be involved in the genomic exit and integration of mobile genetic elements was found at the right flank of the ICE\textit{Slu}van. Although it lacks 1 or 20 amino acids in the C-terminal end compared to the sequences of its closest homologues, SsuIDRAFT_2393, identified in \textit{S. suis} (98\% amino acid identity), and SP70585_1107, identified in \textit{S. pneumoniae} (95\% amino acid identity), we hypothesize that the putative site-specific recombinase functions as normal in a transfer situation. This hypothesis was supported by circularization of the ICE\textit{Slu}van element both in the \textit{S. lutetiensis} donor 5-F9 and the \textit{Enterococcus} transconjugants, as shown by PCR and confirmed by sequence analyses (Fig. 2). Furthermore, we have transferred ICE\textit{Slu}van from \textit{S. lutetiensis} 5-F9 to both \textit{E. faecium} and \textit{E. faecalis} (20). Retransfer was only possible when ICE\textit{Slu}van was transferred into recipients that contain the helper plasmid pIP964 (Tra\textit{\&}), which is restricted to \textit{E. faecalis}. We did not test broad-host-range plasmids to achieve retransfer between \textit{E. faecium} strains. Retransfer from \textit{E. faecalis} BM4110 ICE\textit{Slu}van pIP964 to \textit{E. faecalis} JH2-2 or UV202 (Fig. 3, lanes 4 to 6) and from JH2-2 ICE\textit{Slu}van pIP964 to BM4110 (data not shown) was confirmed by growth on selective plates, ICE\textit{Slu}van- and pIP964-specific PCRs, Smal PFGE analysis (Fig. 3), and hybridization with \textit{vanB} probe (data not shown) showing transconjugant patterns with an approximately 100-kb enlargement of one of the two 240-kb fragments compared to the recipient patterns (Fig. 3, lanes 4 to 6). However, transconjugant BM4110 ICE\textit{Slu}van pIP964 had gained an extra copy of ICE\textit{Slu}van, as shown by the presence of a fragment around 220 kb in Fig. 3, lane 4, which hybridized with \textit{vanB} (data not shown). The presence of more than one copy of ICE\textit{Slu}van has been shown before in other JH-derived transconjugants (20). Both Smal PFGE patterns and DNA sequencing show that the ICE\textit{Slu}van element is integrated in a site-specific manner into the recipient chromosome, although the \textit{E. faecium} and \textit{E. faecalis} integration sites within the C-terminal end of \textit{tRNA methyltransferase} gene \textit{rumA} showed some sequence differences (Fig. 4). The same integration site was identified within each species for all transconjugants tested (data not shown). The ICE\textit{Slu}van integration site in \textit{S. lutetiensis} is also within the \textit{rumA} gene. However, the right flank of ICE\textit{Slu}van in \textit{S. lutetiensis} showed an extended 5.7-kb region identical to part of the \textit{E. faecalis} G1-01247 \textit{vanG} operon which was not transferred together with ICE\textit{Slu}van into the enterococci (see Fig. S1 in the supplemental material). Interestingly, ICE\textit{Slu}van ORF1 is a truncated homolog identical to the 13-amino-acid C-terminal part of \textit{S. pneumoniae} \textit{rumA} (see Table S2). This C-terminal part is in the same translational frame as the N-terminal part of the enterococcal \textit{rumA}, and together, they form a recombined full-length \textit{rumA} gene that is likely to be functional.

Since ICE\textit{Slu}van was shown to transfer from \textit{Streptococcus} to \textit{Enterococcus} but retransfer between enterococci required a helper plasmid, we hypothesize that a host factor is necessary for transfer. This has been shown for SX7 elements in \textit{Vibrio cholerae}, which require the host factors IHF and Fis for excision, recombination, and conjugation (53). Host factors may be chromosomally encoded and not on the MGE itself, leading to the inability of the MGE to conjugate in their absence. Transfer of the \textit{E. faecalis} V583 pathogenicity island (PAI), containing the features of an ICE, by an ICE-independent mechanism has been reported. Characterization of V583 PAI transconjugants showed cotransfer of selectable markers representing virtually all regions of the chromosome, including a vancomycin resistance transposon, capsule genes, and alleles which are used for multilocus sequence typing. PAI transfer was dependent upon helper plasmid function in the donors (54).

\textbf{Putative bacteriophage exclusion system.} The ICE\textit{Slu}van ORF17 located upstream and ORF60 to -64 located downstream from Tn5382/Tn1549 are 81 to 97\% identical to ICE\textit{Sde}s3396_55.
ORF60 and ORF17 are homologous to ICE\text{Sde}3396_48 and ICE\text{Sde}3396_54, encoding the abortive infection proteins \text{AbiGI} and \text{AbiGII}, respectively. The \text{Lactococcus lactis} \text{abiG} genes confer complete resistance to \text{H9278}712 phages (936 phage species) and partial resistance to \text{H9278}c2 phages (2 species) (55). Most \text{Abi} systems are plasmid encoded and are widespread in bacteria (56). The \text{abi} genes are also known to be involved in increased stress tolerance of the host. These genes are commonly found in \text{Streptococcus} (57). Transposon \text{Tn5382/Tn1549} was inserted in the C-terminal end after codon 251 of \text{abiGII} in ICE\text{Slu}van (Fig. 1), thereby disrupting this gene. Since this leads to an \text{AbiGII} protein lacking 30 amino acids compared to its closest homologue, it is not known whether these \text{abi} genes encode a functional system.

\textbf{Putative virulence determinants.} The ICE\text{Slu}van ORF61 was 95\% identical at the nucleotide level to ICE\text{Sde}3396_52, which encodes a putative agglutinin receptor precursor protein that harbors an LPXTG motif known to be common among several virulence factors in enterococci (58). Human salivary agglutinin has previously been shown to interact with streptococci in a calcium-dependent reaction for oral bacterial aggregation (59, 60). The ICE\text{Slu}van putative agglutinin receptor precursor is located next to a putative Ca\textsuperscript{2+} binding protein (ORF62). Studies have shown that transformation of nonaggregating \text{E. faecalis} with the streptococcal surface antigen SSP-5 confers an aggregation-positive phenotype in the presence of saliva agglutinin (61).

\textbf{Resistance to glycopeptides and bacitracin.} The ICE\text{Slu}van element encodes vancomycin resistance mediated by a \text{vanB2} cluster (ORF48 to -54) located in \text{Tn5382/Tn1549}. \text{S. lutetiensis} \text{5-F9} and the transconjugants, as well as \text{S. lutetiensis} \text{NEM760} and \text{S. gallolyticus} \text{4-C11} and \text{4-G10}, accordingly expressed resistance to vancomycin, whereas the recipient strains \text{E. faecium} \text{BM4105-RF} and \text{E. faecalis} \text{OG1-RF}, \text{JH2-2}, \text{UV202}, \text{BM4110}, and \text{BM4110 pIP964} were susceptible to vancomycin according to EUCAST clinical breakpoints for enterococci (MIC, \(\leq 4\) mg liter\textsuperscript{-1}) (Table 1). In addition, ORF34 on the 9-kb element inserted into \text{Tn5382/Tn1549} encoded a protein with 40\% amino acid similarity to a putative \text{VanZ} family protein from \text{Paenibacillus} (see Table S2 in the supplemental material).
CAST clinical breakpoints for enterococci) for *S. lutentensis* NEM760 and 5-F9 and the 5-F9 transconjugants, which was at least 3-fold higher than the MICs of the corresponding susceptible recipient strains (BM4105-RF and UV202 MIC, 0.5 mg liter\(^{-1}\); JH2-2 MIC, 1 mg liter\(^{-1}\); BM4110 plIP964 MIC, 0.38 mg liter\(^{-1}\); and OG1-RF MIC, 0.25 mg liter\(^{-1}\)) (Table 1). The finding of this vanZ gene illustrates the dynamics and the potential of the *Streptococcus* and *Enterococcus* genomes to acquire and collect genes which may increase their ability for environmental adaptation.

The streptococcal ICESluVan encodes resistance to cadmium and arsenic (49). These resistance genes were not identified in ICESluVan. Rather, ORF81 to -84 encode an ABC transporter (ATPase and permease) and a putative two-component system (histidine kinase and response regulator) resembling proteins BceA, -B, -R, and -S (previously designated MbrABCD), which are involved in bacitracin resistance in *S. mutans* through active efflux of bacitracin (GenBank accession number AB078507) (62, 63). ORF81 encodes 249 amino acids with 48% identity to the original *S. mutans* BceA (250 amino acids) and 82% identity to a putative BceA of *S. mitis* (GenBank accession number EFN94736) (245 amino acids), ORF82 encodes 672 amino acids with 29% identity to the original *S. mutans* BceB (667 amino acids) and 67% identity to a putative ABC transporter permease of *S. mitis* (GenBank accession number EFN94737) (671 amino acids), ORF83 encodes 524 amino acids with >20% identity to BceS (249 amino acids) and 65% identity to a putative membrane protein with a histidine kinase domain of *S. mitis* (GenBank accession number EFN94738) (524 amino acids), and ORF84 encodes 198 amino acids with 24% identity to BceR (223 amino acids) and 75% identity to a putative response regulator of *S. mitis* (GenBank accession number EFN94739) (198 amino acids). Compared to the bceABRS locus of *S. mutans*, ICESluVan holds a different gene order of the putative response regulator and histidine kinase. The putative bacitracin locus of ICESluVan shows about 20% amino acid identity to components encoded by a bcrABRS locus conferring high-level bacitracin resistance in *E. faecalis*, which show yet another synteny with the regulator gene upstream from the ATPase, permease, and kinase genes (64). Phenotypic testing indeed shows at

![FIG 3 PFGE of SmaI-digested total DNA of recipient, donor, and transconjugants from filter matings. Lanes 1 and 9, low-range PFGE marker; lane 2, ICESluVan donor S. lutentensis 5-F9; lane 3, recipient BM4110 containing plIP964; lane 4, 1st-generation transconjugant/2nd-generation donor BM4110 ICESluVan plIP964; lane 5, 2nd-generation transconjugant JH2-2 ICESluVan plIP964; lane 6, 2nd-generation transconjugant UV202 ICESluVan plIP964; lane 7, recipient JH2-2; lane 8, recipient UV202. Vancomycin, streptomycin, and rifampin resistance are indicated by plus signs opposite Vanr, Str\(^r\), and Rif\(^r\) Fus\(^r\), respectively. The presence of ICESluVan was verified by vanB PCR and visual inspection of the SmaI PFGE pattern, where ICESluVan insertion in a 240-kb band results in replacement of one of the 240-kb double bands with a 340-kb band, as shown previously for JH2-derived recipients (20). The presence of the pheromone-responsive plasmid pIP964 was verified by \(\text{rep}_{\text{IP964}}\) and \(\text{agg}\) PCRs.](http://jb.asm.org/)

![FIG 4 (A) Operon into which ICESluVan has been integrated in *E. faecium* MM5-F9a compared to the corresponding region in the fully sequenced *E. faecalis* OG1-RF (accession number CP002621). Dark gray shading indicates regions of similarity between compared CDSs. The tRNA (uracil-5)-methyltransferase \(\text{rnmA}\) disrupted by the insertion is colored light gray, and the insertion is indicated with a dotted line. (B) Sequence comparison of the insertion regions of ICESluVan in *E. faecium* MM5-F9a and *E. faecalis* OG5-F9a and the corresponding regions in their recipient strains, *E. faecium* BM4105-RF and *E. faecalis* OG1-RF, respectively. Vertical lines indicate identical nucleotides. Left- and right-end sequences of ICESluVan are shown in uppercase. Amino acid residues of CDSs that have been disrupted upon insertion of ICESluVan are marked (capital and boldface), together with their respective codons. #, stop codon.](http://jb.asm.org/)
least a 5-fold-increased MIC for bacitracin associated with transfer of ICESlu van from \textit{S. lutetiensis} S-F9 to \textit{E. faecium}. The \textit{E. faecium} transconjugant MM5-F9a expressed a bacitracin MIC of $\geq 256$ mg liter$^{-1}$, while the MIC of the corresponding \textit{E. faecium} recipient BM4105-RF was 48 mg liter$^{-1}$, implying functionality of the putative bacitracin locus of ICESlu van as a novel bacitracin resistance locus. On the other hand, the \textit{E. faecalis} transconjugants and their corresponding recipients showed similar low or high MICs to bacitracin, except for the second-generation transconjugant JH2-2 ICESlu plP964, which after transfer from the high-bacitracin-MIC background in \textit{E. faecalis} BM4110 plP964 showed at least a 16-fold-increased MIC compared to that of JH2-2 (Table 1). Undecaprenyl pyrophosphate phosphatase has recently been shown to account for the low-level resistance to bacitracin (MICs of 32 to 48 mg liter$^{-1}$) in both laboratory (JH2-2) and clinical (V583) strains of \textit{E. faecalis} (65), while variants of the transferable \textit{bcRABD} locus may be responsible for the high-level bacitracin resistance (MIC $\geq 128$ mg liter$^{-1}$) in enterococci (64, 66). Alternative primer combinations had to be used to confirm the bacitracin locus region of ICESlu van (see Q38 in Table S1 in the supplemental material) in \textit{E. faecalis} JH5-F9a and OG5-F9a compared to that in \textit{E. faecium} MM5-F9a, indicating some sequence differences. However, sequencing \textit{E. faecalis} JH5-F9a and OG5-F9a revealed identical nucleotides of the bacitracin locus (nt 85669 to 91794) compared to the sequence of MM5-F9a (GenBank accession number HE963029). Thus, host-related factors of \textit{E. faecalis} and \textit{E. faecium} may be a possible explanation for the lack of increased bacitracin MICs when ICESlu van was introduced directly into \textit{E. faecalis} strains with low-level resistance to bacitracin.

**ICESlu van expresses a pezAT TA system.** ICESlu van ORF75 and ORF76 are 93 and 89% identical at the nucleotide level to the pezAT plasmid maintenance system previously described in \textit{S. pneumoniae} (67), \textit{S. agalactiae} (57, 67), and \textit{S. suis} (57). The pezAT gene cassette consists of two genes, encoding an epsilon antitoxin and a zeta toxin, respectively. Toxin-antitoxin systems are traditionally known as plasmid addiction systems that ensure stable maintenance of plasmids in a bacterial population (68). However, during recent years, these gene loci have been found on MGEs and shown to promote the maintenance of ICE elements in \textit{V. cholerae} (69, 70). Experimental evidence also indicated that TA systems are involved in the stress response, enabling the cell to survive hostile growth conditions (71, 72). Analyses of the DNA sequence upstream from the pezAT locus showed the presence of a putative $-10$ promoter sequence (TATAAT) 34 bp upstream from an indicated start codon and a $-35$ promoter sequence (GT GCGTT) 19 bp from the putative Pribnow box. In addition, a putative ribosome-binding site (AGGAG) was shown 12 bp upstream from the putative start codon. All except the $-35$ sequence were identical to the \textit{S. pneumoniae} sequences (67). RT-PCR analyses revealed the expression of both pezAT genes (data not shown) in all of the donor and transconjugant strains tested. Figure 5 shows the PCR products of both genes. Moreover, both genes were detected in a single transcript (Fig. 5), confirming that pezA and pezT constitute an operon, as in \textit{S. pneumoniae} (67). PCR products were not detected in the absence of reverse transcriptase or in the recipient strain (BM4105-RF). All sequences were identical to the sequence found in our \textit{E. faecium} transconjugant. These observations suggest that this is a functional toxin-antitoxin system that may contribute to the stabilization of ICESlu van after integration into new hosts.

In conclusion, our report describes the complete genetic structure of a 94-kb element, named ICESlu van, originally detected in \textit{S. lutetiensis} from veal calf feces in the Netherlands. A variant of ICESlu van is confirmed for an \textit{S. lutetiensis} isolate from a patient in France. ICESlu van encodes glycopeptide and bacitracin resistance locus, a putative bacteriophage exclusion system, a putative virulence gene, and components necessary for conjugative transfer, as well as a toxin-antitoxin stabilization system. This element is a novel ICE, since it forms a circular intermediate, is self-transferrable from streptococci to enterococci, and integrates into the chromosome in a site-specific manner.

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