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Dissemination of an *Enterococcus* Inc18-Like *vanA* Plasmid Associated with Vancomycin-Resistant *Staphylococcus aureus*[▽]

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Of the 9 vancomycin-resistant *Staphylococcus aureus* (VRSA) cases reported to date in the literature, 7 occurred in Michigan. In 5 of the 7 Michigan VRSA cases, an Inc18-like *vanA* plasmid was identified in the VRSA isolate and/or an associated vancomycin-resistant *Enterococcus* (VRE) isolate from the same patient. This plasmid may play a critical role in the emergence of VRSA. We studied the geographical distribution of the plasmid by testing 1,641 VRE isolates from three separate collections by PCR for plasmid-specific genes *traA*, *repR*, and *vanA*. Isolates from one collection (phase 2) were recovered from surveillance cultures collected in 17 hospitals in 13 states. All VRE isolates from 2 Michigan institutions ($n = 386$) and between 60 and 70 VRE isolates ($n = 883$) from the other hospitals were tested. Fifteen VRE isolates (3.9%) from Michigan were positive for an Inc18-like *vanA* plasmid (9 *E. faecalis* [12.5%], 3 *E. faecium* [1.0%], 2 *E. avium*, and 1 *E. raffinosus*). Six VRE isolates (0.6%) from outside Michigan were positive (3 *E. faecalis* [2.7%] and 3 *E. faecium* [0.4%]). Of all *E. faecalis* isolates tested, 6.0% were positive for the plasmid, compared to 0.6% for *E. faecium* and 3.0% for other spp. Fourteen of the 15 plasmid-positive isolates from Michigan had the same Tn*1546* insertion site location as the VRSA-associated Inc18-like plasmid, whereas 5 of 6 plasmid-positive isolates from outside Michigan differed in this characteristic. Most plasmid-positive *E. faecalis* isolates demonstrated diverse patterns by PFGE, with the exception of three pairs with indistinguishable patterns, suggesting that the plasmid is mobile in nature. Although VRE isolates with the VRSA-associated Inc18-like *vanA* plasmid were more common in Michigan, they remain rare. Periodic surveillance of VRE isolates for the plasmid may be useful in predicting the occurrence of VRSA.

Currently, vancomycin-resistant *Staphylococcus aureus* (VRSA) infections are rare. Thus far, nine cases have been documented in the United States (9, 20), and two have been reported in other countries, including Iran (1) and India (22, 27). Despite this rarity, 7 of the 9 U.S. VRSA cases occurred in the metropolitan Detroit, MI, area. All U.S. VRSA isolates demonstrated either unique pulsed-field gel electrophoresis (PFGE) patterns or unique plasmid restriction patterns (20, 33). This suggests that each VRSA isolate, including the 7 from Michigan, acquired resistance independently and was not the result of transmission of a common VRSA strain between patients.

VRSA isolates are thought to occur by *in vivo* transfer of a *vanA* plasmid from an *Enterococcus* isolate to an *S. aureus* isolate. For most of the VRSA cases, a vancomycin-resistant *Enterococcus* (VRE) isolate was either coisolated from the same body site as the VRSA isolate or was found to colonize the patient at another body site, such as the nares or rectum (Table 1 shows a summary of VRE isolates from VRSA patients). There is limited evidence that these VRE isolates are the *vanA* donors. In previous studies (29, 30, 33), we found that certain VRSA isolates carried *vanA* on a transposon, and in some cases, a plasmid was identified that was identical to the *vanA* plasmid or transposon in a VRE isolate from the same patient. In VRSA cases 3, 4, and 5, the VRE and VRSA isolates shared the same *vanA* plasmid. For all other VRSA cases, the VRSA isolates carried the same Tn*1546*-like element as their associated VRE isolate (10, 33). Hence, it was proposed that the VRSA phenotype occurred by conjugative transfer of a *vanA* plasmid from VRE to *S. aureus* (29, 33).

Inc18 incompatibility plasmids are a family of broad-host-range conjugative plasmids that occur naturally in *Enterococcus* and *Streptococcus* spp. Plasmids pIP501 and pAMβ1 are two well-characterized examples in this group (15). These plasmids carry multiple antimicrobial-resistance genes, including genes that confer resistance to macrolides, lincosamides, and the streptogramin B (MLS) group, and they can be transferred to a wide variety of bacteria, including streptococci (26), lactococci (17), staphylococci (23), and enterococci (14). Specifically, Inc18-like *vanA* plasmids were identified in VRE iso-

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TABLE 1. Inc18-like *vanA* plasmid characteristics of VRSA and VRSA-associated VRE isolates from nine VRSA cases in the United States[c]

Case	State	Species	Site of isolation	Inc18-like *vanA* plasmid	Tn*1546* arrangement[a]	Tn*1546* insertion site[b]	Reference or source
1	MI	VRSA	Foot wound	No	Wild-type sequence	−	29, 33
		E. faecalis	Foot wound	Yes	Wild-type sequence	+	10
2	PA	VRSA	Foot	No	Insertions and deletions	−	4
		E. faecium	Suspected contamination	NA	NA		
3	NY	VRSA	Urine	No	Insertions and deletions	−	30
		E. faecium	Rectum	No	Insertions and deletions	−	Our unpublished data
4	MI	VRSA	Toe wound	Yes	Wild-type sequence	+	33
		E. faecalis	Rectal swab	Yes	Wild-type sequence	+	33
5	MI	VRSA	Abdominal wound	Yes	Wild-type sequence	+	33
		E. faecalis	Abdominal wound	Yes	Wild-type sequence	+	33
6	MI	VRSA	Wound	No	Wild-type sequence	−	33
		E. faecalis/*E. avium*	Rectal	Yes	Wild-type sequence	+	33
7	MI	VRSA	Left elbow	Yes	NA	−	33
		NA	NA	NA	NA	−	
8	MI	VRSA	Foot	No	NA	−	Our unpublished data
		NA	NA	NA	NA	−	
9	MI	VRSA	Foot	No	NA	−	Our unpublished data
		E. faecalis	NA	NA	NA	NA	

[a] The wild-type arrangement of Tn*1546* was considered to be the prototype (2).
[b] The Tn*1546* insertion site test result was defined as positive (+) if the junction sites were the same as those of plasmids pWZ909, pWZ7140, or pWZ1668; if not, the test result was defined as negative (−).
[c] NA, not available.

lates from 4 Michigan VRSA patients (cases 1, 4, 5, and 6) and in VRSA isolates from 3 patients (cases 4, 5, and 7) (10, 33). In all VRSA cases where an *Enterococcus* sp. with an Inc18-like *vanA* plasmid was found, the isolate was *E. faecalis* but each isolate demonstrated a different PFGE pattern, indicating that there was not a single enterococcus *vanA* donor in Michigan. These results suggest that Inc18-like *vanA* plasmids may be more likely than other *vanA* plasmids to transfer from an *Enterococcus* sp. to *S. aureus*. If VRE isolates with Inc18-like *vanA* plasmids are more common in Michigan than in other geographic areas, this may at least partially explain why VRSA isolates have occurred primarily in Michigan. In this study, we examined health care-associated VRE isolates from institutions in various geographical locations within the United States for the presence of an Inc18-like *vanA* plasmid in order to determine the occurrence of Inc18-like *vanA* plasmids in VRE and to examine the geographical distribution of these Inc18-positive VRE isolates.

MATERIALS AND METHODS

Bacterial isolate collection and species determination. The numbers and sources of VRE clinical isolates examined in this study are listed in Table 2. This study was conducted in three phases.

Phase 1 was a pilot study to compare the occurrences of VRE isolates with Inc18-like *vanA* plasmids in metropolitan Detroit, MI, and in two other areas: Chicago, IL, and Durham, NC. Isolates were requested from one health care institution in each of these three areas. Laboratories were asked to send approximately 30 VRE isolates, including approximately 20 *E. faecalis* and 10 *E. faecium* isolates that were recovered from either a clinical or surveillance culture during January 2002 to May 2006.

Phase 2 was an investigation of the occurrence of Inc18-like *vanA* plasmids in VRE isolates recovered from surveillance cultures collected in many different regions within the United States. A total of 1,269 VRE isolates were obtained from the "Strategies to Reduce Transmission of Antimicrobial Resistant Bacteria in Intensive Care Units" (STAR*ICU) trial, a cluster-randomized trial of infection control strategies to reduce the transmission of methicillin-resistant *S. aureus* (MRSA) and VRE in ICUs that was supported by the National Institute of Allergy and Infectious Diseases (http://clinicaltrials.gov/show/NCT00342745). The Institutional Review Boards (IRBs) at all sites waived the requirement for informed consent and Health Insurance Portability and Accountability Act

TABLE 2. Description of VRE isolates collected in this study

Study phase	Source of isolates	No. of VRE isolates by species			Total no. of isolates
		E. faecalis	*E. faecium*	Other spp.	
1	4 laboratories, 1 in IL, 1 in NC, 2 in MI	131	41	0	172
2	19 hospitals in 14 states, including 2 in MI	184	995	90	1,269
3	19 laboratories in MI	200	0	0	200
Total	42 facilities	515	1,036	90	1,641

TABLE 3. PCR primers used in this study

Primer name	DNA sequence (5′→3′)	Fragment size (bp)
vanA F	CATGAATAGAATAAAAGTTGCTGCAATA	1,032
vanA R	CCCCTTTAACGCTAATACGATCAA	
traA F	TAATCGCAATGGCTTCTTATC	475
traA R	TCTGCCCAATCTTTACGAAT	
repR F	GCTTCATGACGGCTTGTTA	565
repR R	TTGGCTGCTTTGACAGATTTA	
ddl F1	ATCAAGTACAGTTAGTCT	941
ddl R1	ACGATTCAAAGCTAACTG	
ddl F2	TAGAGACATTGAATATGCC	550
ddl R2	TCGAATGTGCTACAATC	
ORF18F	GGCAAATATAGTCAATTTTACTGAC	660
ORF19R	GACAAAACAGCTTAGCTACAGC	
ORF27F	CAGATGTAATTACAAATACTGTTGG	549
ORF28R	AAGTCCTGGAAGTTTAGGTGTTTT	

(HIPAA) authorization based on the criteria of the Federal Code of Regulations, Title 45, Part 46.116 (d) and section 164.512 (i) of the HIPAA privacy rule. As part of this study, stool or perianal swabs to screen for colonization with VRE were collected on admission and weekly from patients in 19 ICUs located in 14 states, including 2 institutions in Michigan, between June 2005 and August 2006. Swabs were processed for culture at the National Institutes of Health Clinical Center Microbiology Laboratory. VRE isolates were confirmed to be *vanA* positive, but the isolates were not identified to the species level. For the current study, all VRE isolates from the two Michigan institutions and between 50 and 70 randomly chosen VRE isolates from other institutions were tested for the Inc18-like *vanA* plasmid. Isolates were chosen so that no more than one isolate per patient was sampled, and since the isolates were not identified to the species level, no preference for species was made.

Phase 3 was an analysis of vancomycin-resistant *E. faecalis* isolates from Michigan. The Michigan Department of Community Health collected a large number of VRE isolates from various parts of the state between 1995 and 2002 as part of a surveillance program. In an effort to enrich for isolates with the Inc18-like *vanA* plasmid, only *E. faecalis* isolates were tested for the plasmid ($n = 200$).

Species identification of VRE was confirmed at the CDC by PCR amplification of species-specific ligase sequences (8), standard biochemical assays for *Enterococcus* identification (25), or 16S rRNA gene sequence analysis (21).

Methods for detection and characterization of Inc18-like *vanA* plasmids. Primer sequences for PCR assays are listed in Table 3. PCR assays were performed for the detection of *vanA* and the two Inc18-like, plasmid-specific genes *traA* and *repR* (8). Another PCR assay was developed to detect the Tn*1546* insertion site in the Inc18-like *vanA* plasmid. Specifically, primer pairs ORF18F/ORF19R and ORF27F/ORF28R were designed for detection of the Tn*1546* left- and right-side junctions, respectively. If needed, *E. faecalis* and *E. faecium* were identified by PCR detection of species-specific ligase (*ddl*) genes (primer pairs *ddl* F1 and R1 for *E. faecalis* and *ddl* F2 and R2 for *E. faecium*) (Table 3).

In phase 1, PCR for single genes was performed in a total volume of 50 μl consisting of 1.6 mM each deoxynucleoside triphosphate (dNTP; Applied Biosystems, Foster City, CA), 400 nM each primer, 1× buffer, 1 mM $MgCl_2$, 0.5 units of AmpliTaq gold enzyme (Applied Biosystems), and 2 μl of DNA extract (equaling 100 to 500 ng). PCRs were carried out in a GeneAmp PCR system 9700 (Applied Biosystems) with reaction cycles as follows: an initial denaturation step of 2 min at 94°C, 30 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation at 72°C for 7 min. In phase 2, a multiplex PCR assay for *traA*, *repR*, and the *ddl* genes from *E. faecalis* and *E. faecium* was performed using a Qiagen multiplex PCR kit (Valencia, CA) according to the manufacturer's instructions. PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems) with the following reaction cycles: an initial denaturation step of 2 min at 94°C, 30 cycles of 15 s at 95°C, 90 s at 55°C, and 90 s at 72°C; and a final elongation for 7 min at 72°C. The PCR products were visualized on a 1% agarose gel. In phase 3, a multiplex PCR assay for *traA* and *repR* was performed using the conditions described for the phase-2 multiplex assay. For *traA* and *repR* PCR assays, lysates of an *E. faecalis* JH2-2 transconjugant containing the Inc18-like plasmid pIP501 and *E. faecalis* JH2-2 without plasmid were used as positive and negative controls, respectively.

The Tn*1546 vanA* element arrangement was analyzed by restriction fragment length polymorphism (RFLP) as described by Clark et al. (4).

Susceptibility testing. Susceptibility to vancomycin and other antimicrobial agents was determined by reference broth microdilution (BMD) using in-house-prepared panels with cation-adjusted Mueller-Hinton broth (BD Biosciences, Sparks, MD). Susceptibility methods and interpretation were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (6).

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed by digesting genomic DNA with SmaI restriction enzyme using standard procedures for enterococci (28). TIFF images of PFGE gels were analyzed with BioNumerics, version 5.1 (Applied Maths, Austin, TX) using Dice coefficients plus unweighted-pair group method using average linkages (UPGMA) clustering.

Conjugation. Five milliliters of brain heart infusion (BHI) broth containing 25 μg/ml vancomycin or 25 μg/ml fusidic acid was inoculated with *traA*-positive donor isolates or the recipient *E. faecalis* JH2-2, respectively, and incubated overnight at 37°C. One hundred microliters of each culture was added to a new 5-ml BHI broth and incubated for 5 h before being filtered through a 0.45-μM Nalge filter under vacuum. The filter was then placed on a BHI agar plate and incubated for 18 h. The filter with overlying colonies was then removed from the agar and placed in BHI broth. Serial dilutions were prepared and plated on BHI selective plates containing 25 μg/ml each of vancomycin and fusidic acid. Controls were performed to detect breakthrough growth of the donor strain on fusidic acid-containing agar and of the recipient strain on vancomycin-containing agar. For three conjugation experiments, breakthrough growth of the donor was identified on fusidic acid-containing agar. These conjugations were repeated using 50 μg/m1 fusidic acid and 25 μg/ml rifampin to increase inhibition of the donor strains, and no breakthrough growth of the donor strains was identified.

DNA sequencing and analysis. To understand the genetic organization of the Inc18-like *vanA* plasmids from Michigan VRE isolates, three plasmids from three VRSA-associated VRE isolates were sequenced. The plasmids were sequenced on Applied Biosystem 3730XL sequencers with BigDye Terminator version 3.1 cycle sequencing kits (Applied Biosystems, Inc.). Plasmid sequencing was performed by primer walking with primers either purchased from IDT (Integrated DNA Technologies, Inc., San Diego, CA) or made at the CDC's core facility. Reaction mixtures were cleaned with Agencourt Cleanseq beads (Agencourt Bioscience Corporation, Beverly, MA). PhredPhrap and Consed software (University of Washington, WA) were used for base-calling of the sequence data and assembly of the data into continuous DNA sequences. DNA sequences were analyzed with Clone Manager, version 9 (Sci-Ed Software, Cary, NC).

Nucleotide sequence accession numbers. The nucleotide sequence data for plasmids pWZ7140 (47,277 bp), pWZ909 (42,602 bp), and pWZ1668 (48,365 bp) from VRSA cases 1, 4, and 5 associated with *E. fiecalis* were submitted to the National Center for Biotechnology Information Data Libraries (GenBank), and the sequences have been assigned accession numbers GQ484955, GQ484954, and GQ484956, respectively.

RESULTS

Completed sequence of plasmid pWZ909. In side-by-side comparisons, we found that plasmids PWZ7l40, pWZ909, and pWZ1668 have the same genetic organization and sequences, with few exceptions. The three plasmids share 100% homology over 42 kb of sequence; however, when compared to pWZ909, pWZ7140 has a 4.7-kb insertion (putative transposase gene) and pWZ1668 has two insertions of 5.4 kb and 0.6 kb (putative transposase gene and IS*456*, respectively). These plasmids also have a genetic organization similar to that of Inc18 plasmids pIP501 and pRE25 (Fig. 1). The map of a representative plasmid, pWZ909 (from VRSA case 4), is shown in Fig. 1. This plasmid has 44 putative open reading frames, and 16 are 100% homologous to the conjugative transfer region of pIP501. The major difference between pIP501 and pWZ909 is the insertion of a Tn*1546 vanA* transposon in pWZ909 (Fig. 1B). The DNA sequence of the transfer (*tra*) region, specifically *traA*, which encodes the nickase, is conserved in the Inc18 plasmid family (16, 24). The *traA* gene and another conserved gene, *repR* (coding for an essential replication protein) were chosen as plasmid-specific genetic targets for a PCR assay to detect Inc18-like plasmids.

The three sequenced plasmids demonstrate Tn*1546* charac-

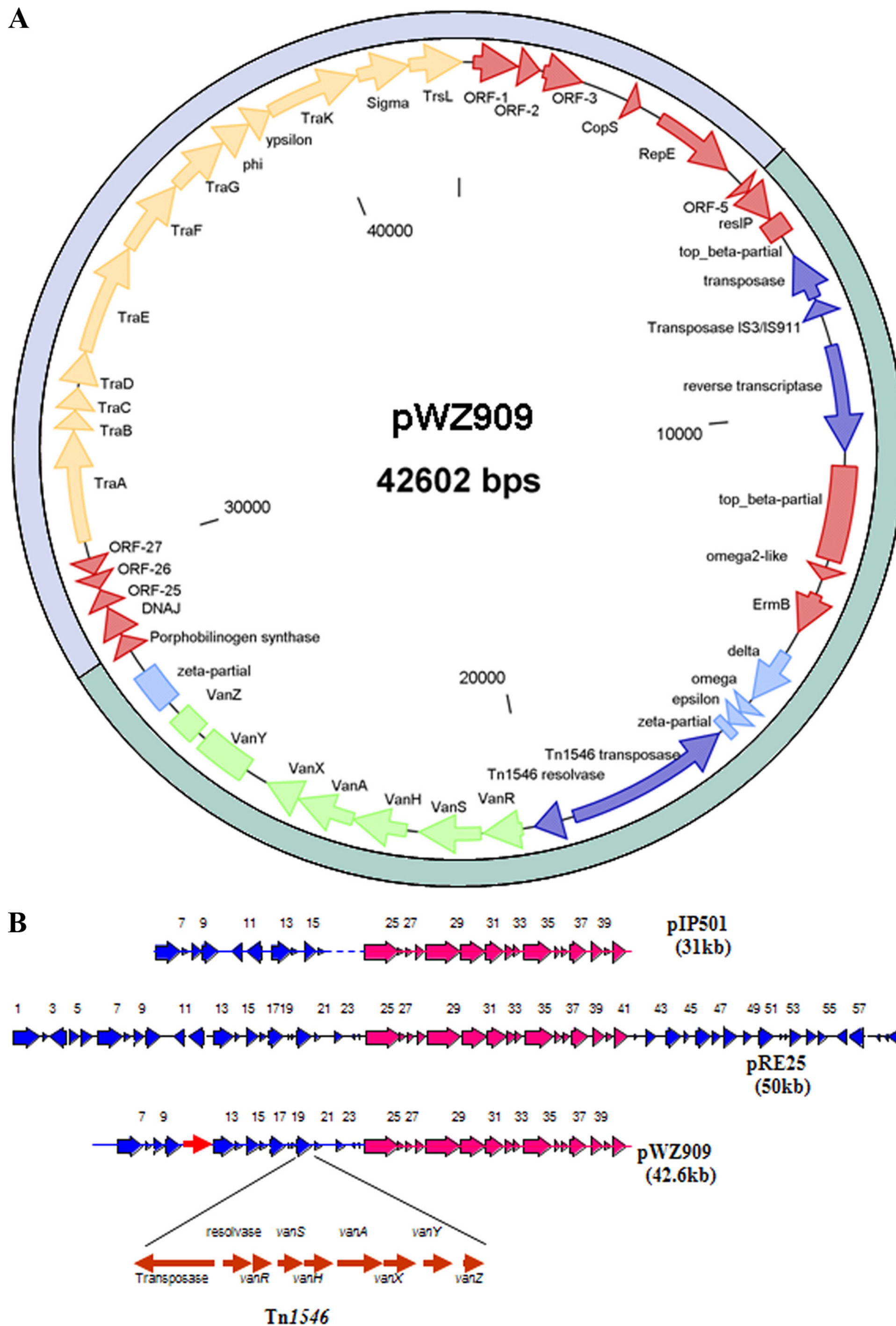

FIG. 1. (A) Circular map of the Inc18-like *vanA* plasmid in the *E. faecalis* isolate from VRSA case 4. Genes are shown as arrows; certain partial or nonfunctional genes are shown as boxes. The putative conjugative transfer region is in gold, the vancomycin resistance genes are noted in light green, transposon genes are noted in dark blue, and the putative replication genes are in red. ORF, open reading frame. (B) Comparison of genetic organization of pWZ909 with that of Inc18 plasmids pRE25 and pIP501. The conjugative transfer region of pWZ909 is very similar to that of plasmids pRE25 from *E. faecalis* and pIP501 from *Streptococcus agalactiae* (24). The putative transfer region is shown in red with black outline, antibiotic resistance genes and replication regions are in blue, and transposon Tn*1546* containing vancomycin genes is in red without outline.

teristics that were common among all VRSA-associated VRE isolates, and assays for these characteristics were used for surveillance of the Inc18-like *vanA* plasmid in other VRE isolates. One characteristic was the Tn*1546* insertion location; a PCR assay was developed to detect the presence of this same insertion location in other plasmids, and 4 of 5 Michigan VRSA-associated VRE isolates were positive by the assay (Table 1). The other common characteristic was a wild-type

TABLE 4. VRE isolates with Inc18-like plasmids from three different locations (phase 1)

Location	Total no. of isolates	Species (no. of isolates)	No. of isolates positive for:		
			vanA	*traA*	*repR*
Detroit, MI	111	*E. faecalis* (88)	68	13	13
		E. faecium (23)	22	0	0
Durham, NC	30	*E. faecalis* (21)	19	0	0
		E. faecium (9)	9	0	0
Chicago, IL	31	*E. faecalis* (22)	19	0	0
		E. faecium (9)	9	0	0
Total	172		146	13	13

TABLE 5. Characteristics of Inc18-like plasmids in VRE isolates from the STAR*ICU trial (phase 2)

Location	Species	No. of isolates (%)	No. (%) of isolates with		
			traA, *repR*, and *vanA*	Tn*1546* wild-type arrangement[a]	Tn*1546* insertion sites[b]
MI	*E. faecalis*	72 (19)	9	8	8
	E. faecium	291 (75)	3	3	3
	Other spp.	23 (6)	3	3	3
	Total	386	15 (3.9)	14	14 (3.6)
Outside MI	*E. faecalis*	112 (12.5)	3	0	0
	E. faecium	704 (80)	3	1	1
	Other spp.	67 (7.5)	0	0	0
	Total	883	6 (0.7)	1	1 (0.1)

[a] The wild-type arrangement of Tn*1546* was considered to be the prototype (2).
[b] Tn*1546* junction sites are the same as those of plasmids pWZ909, pWZ7140, and pWZ1668.

Tn*1546* arrangement that was demonstrated in 4 of 5 Michigan VRSA-associated VRE isolates by RFLP analysis.

Inc18 *vanA* plasmids in VRE isolates from three geographical locations (phase 1). The phase-1 isolates consisted of 172 VRE isolates: 88 *E. faecalis* and 23 *E. faecium* isolates from three southeastern Michigan laboratories; 22 *E. faecalis* and 9 *E. faecium* isolates from one Chicago, IL, laboratory; and 21 *E. faecalis* and 9 *E. faecium* isolates from one Durham, NC, laboratory (Table 4). All isolates were tested for *vanA*, *traA*, *repR*, and *Enterococcus*-specific genes (*ddl*) by PCR amplification. If the isolates were positive for *traA* and *repR*, then the Tn*1546* arrangement and insertion sites were analyzed by RFLP and PCR, respectively. Thirteen VRE isolates from metropolitan Detroit (11.7%) were positive for three genes: *vanA*, *traA*, and *repR* (Table 4). Another 77 VRE isolates were *vanA* positive but negative for both *traA* and *repR*. Of the VRE isolates from outside Michigan, none were positive for all three genes, *vanA*, *traA*, and *repR*, although 56 of 61 were positive for *vanA*. The 13 *traA*- and *repR*-positive isolates from Michigan had wild-type Tn*1546*-like elements, and the transposon was inserted at the same site as in pWZ909. All of the isolates that were positive for an Inc18-like *vanA* plasmid from Michigan were *E. faecalis*; none were *E. faecium*. Each isolate had a vancomycin MIC of at least 512 μg/ml. The results of conjugative experiments showed that all of these isolates were able to transfer the *vanA*-mediated vancomycin resistance to *E. faecalis* JH2-2, with *traA* and *repR* being cotransferred with *vanA* (data not shown). This suggests that all three genes were located on the same conjugative plasmid. The frequency of transfer for each conjugation was approximately 10^{-5} to 10^{-7} per donor cell; there was no significant difference in conjugation frequency observed among Inc18-like plasmid-carrying isolates.

Expanded survey of Inc18-like plasmids in VRE isolates (phase 2). A total of 1,269 VRE isolates from the STAR*ICU trial were tested for the presence of *traA*, *repR*, and the *Enterococcus* ligase genes, using a multiplex PCR assay. Isolates positive for *traA* and *repR* were then tested for *vanA* and the Tn*1546* arrangement and insertion site. All VRE isolates from two Michigan health care facilities were tested (n = 386), including 291 *E. faecium* isolates (75%), 72 *E. faecalis* isolates (19%), and 23 VRE isolates of other species (6%) (Table 5). Fifteen isolates were positive for the Inc18-like *vanA* plasmid: 9 were *E. faecalis*, 3 *E. faecium*, 2 *E. avium*, and 1 *E. raffinosus*. Of these, 14 isolates had plasmid Tn*1546* characteristics that were like those of pWZ909 (i.e., the same insertion site and arrangement). A total of 883 VRE isolates from 17 health care facilities in 14 other states were tested. These consisted of 704 *E. faecium* isolates (80%), 112 *E. faecalis* isolates (12%), and 67 VRE isolates of other species (8%). Three *E. faecalis* isolates and 3 *E. faecium* isolates were positive for an Inc18-like plasmid. One of these *E. faecium* isolates had Tn*1546* characteristics like those of pWZ909 (Table 5). Of the six VRE isolates from outside Michigan that were positive for the Inc18-like plasmid, two were from Ohio, two were from Georgia, one was from Arizona, and one was from Iowa.

All the plasmid-positive isolates had vancomycin MICs of at least 512 μg/ml. The results of conjugative assays demonstrated that all of these plasmid-positive isolates were able to transfer the *vanA*-mediated vancomycin resistance to *E. faecalis* JH2-2 via Inc18-like plasmids. No significant difference in conjugation frequency was observed between the six isolates and other Inc18-like plasmid-positive isolates.

Inc18-like *vanA* plasmid distribution within Michigan (phase 3). To examine the distribution of Inc18-like plasmids within Michigan, 200 isolates of *E. faecalis* from the Michigan Department of Community Health Laboratory collection were tested with a multiplex PCR assay for *traA* and *repR*. Isolates that were positive for these genes were tested for *vanA*, Tn*1546* arrangements, and the Tn*1546* insertion site. Of these 200 *E. faecalis* isolates, 11 (5.5%) were positive for Inc18-like *vanA* plasmids: 6 from blood samples, 3 from wound samples, and 2 from urine samples. All of the plasmids had pWZ909-like Tn*1546* characteristics. All 11 of the isolates were from three health care institutes located within a 30-mile radius of Detroit (Table 6).

PFGE patterns of VRE isolates with Inc18-like *vanA* plasmids. Typing by PFGE indicated that the majority of these VRE isolates were unrelated (Fig. 2). However, three pairs of *E. faecalis* isolates from the metropolitan Detroit area shared PFGE patterns that were indistinguishable. Also, one *E. faecium* isolate containing the Inc18-like *vanA* plasmid was identified outside Michigan and had a PFGE pattern that was indistinguishable from that of a Michigan *E. faecium* isolate carrying the same plasmid.

TABLE 6. Distribution of *E. faecalis* isolates with Inc18-like plasmids from Michigan State Health Department surveillance from 1995 to 2002 (phase 3)

Distance of source from Detroit (miles)	No. of *E. faecalis* isolates	No. (%) of plasmid-positive isolates	Geographic distribution (no. of plasmid-positive isolates/total no. of isolates)
<30	160	11 (6.8)	Detroit (6/78); 2 sites in Oakland county (5/78); 2 sites in Wayne county (0/4)
>30	40	0	14 sites (0/40)
Total	200	11 (5.5)	19 sites (11/200)

DISCUSSION

VRE isolates with VRSA-associated Inc18-like *vanA* plasmids were more common in Michigan than in other states. The best estimate of the occurence of Inc18-like *vanA* plasmids in VRE isolates, from phase 2, was 3.9% of isolates in Michigan and 0.7% of isolates from outside Michigan. However, there are two limitations of this estimate: first, more VRE isolates from the two Michigan institutions than from the institutions in other states were sampled, and, second, isolates from only 14 states were tested. It is thus possible that there are other geographic foci where VRE isolates with this plasmid are more prevalent. It is important to note that, although the plasmid was more common in Michigan, it was still rare in Michigan VRE isolates.

The exact characteristics of an Inc18-like *vanA* plasmid that are important for its transfer from *Enterococcus* spp. to *S. aureus* are unknown. It could be that not all Inc18-like *vanA* plasmids detected in this study are potential *vanA* donors to *S. aureus*. Because we did not know which characteristics were important, we looked for similarities between Inc18-like *vanA* plasmids and VRSA-associated plasmids (e.g., pWZ909) by assaying for the Tn*1546* insertion site and arrangement that are characteristic among Inc18-like *vanA* plasmids from VRSA cases. Most of the plasmids detected in Michigan VRE isolates shared these characteristics, while most of the plasmids detected in VRE isolates from outside Michigan did not. Only one isolate from outside Michigan, an *E. faecium* isolate from Iowa that also had the same PFGE pattern as an *E. faecium* isolate from Michigan, had pWZ909-like Tn*1546* characteristics. Data were not available to determine any possible epidemiologic link between the Iowa patient and Michigan patients.

The conjugation experiments in this study indicate that the Inc18-like *vanA* plasmids are able to transfer from one enterococcus isolate to another. The diversity of PFGE patterns among plasmid-positive isolates is consistent with these findings and suggests that Inc18-like *vanA* plasmids are being transferred between isolates in the natural environment as well. Among 21 analyzed Michigan *E. faecalis* isolates with Inc18-like plasmids, we found only three pairs of isolates with PFGE patterns that were indistinguishable from one another. These results suggest that the Inc18-like *vanA* plasmid has disseminated among *Enterococcus* spp. by horizontal dissemination of the plasmid rather than clonal spread of a single strain. This observation is consistent with the reports by Garcia-Migura et al. (12, 13), who reported Tn*1546 vanA* elements on Inc18-like plasmids in 45 of 150 different *E. faecium* isolates from farms (12). Their findings suggested that dissemination of vancomycin resistance by horizontal transfer could play an important role within livestock systems. The ability of these plasmids to be transmitted between strains is also supported by our conjugation experiment results, which showed that each of the plasmid-positive isolates tested was able to transfer vancomycin resistance to *E. faecalis* JH2-2.

Considering that most VRE isolates are *E. faecium* (3, 31), it was notable that, among VRE isolates collected from Michigan institutions in the STAR*ICU study (phase 2), the Inc18-like *vanA* plasmid was found more commonly in *E. faecalis* isolates (12.5%) than in *E. faecium* isolates (0.6%). The issue does not seem to be that the plasmid is species limited; besides being found in *E. faecalis* and *E. faecium*, the plasmid was also identified in two other species of enterococcus. We do not know why this plasmid was more common in *E. faecalis* isolates in Michigan. It could be that this is a relatively new *vanA* plasmid and that there are more *vanA*-negative *E. faecalis* than *vanA*-negative *E. faecium* isolates to serve as recipients. Although several reports have shown that pheromone response plasmids (including *vanA* and non-*vanA* plasmids) are more frequently found in *E. faecalis* isolates than in *E. faecium* iso-

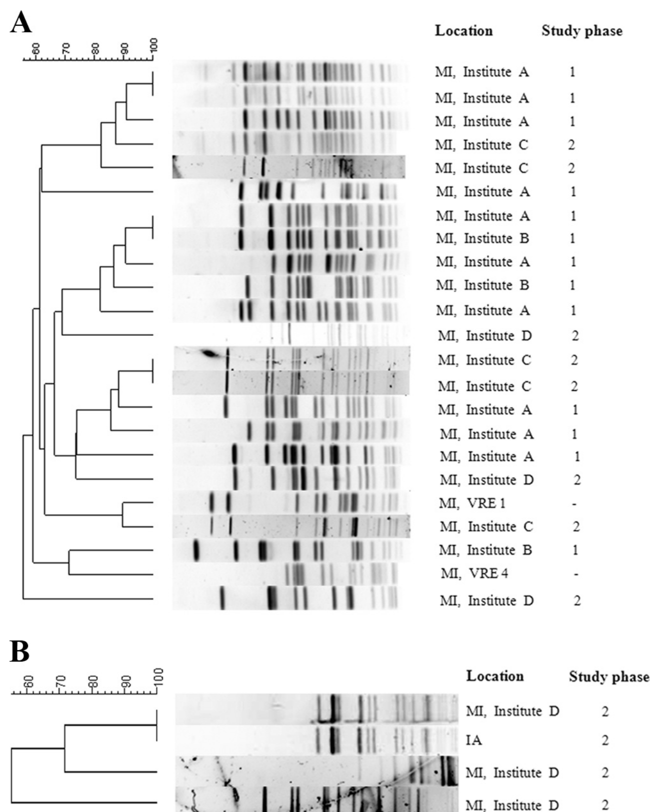

FIG. 2. PFGE analysis of SmaI-digested whole-chromosomal DNA of *E. faecalis* (A) and *E. faecium* (B) isolates with the Inc18-like *vanA* plasmid. The location and study phase of strain isolation are listed on the right. Isolates VRE1 and VRE4 were associated with VRSA cases 1 and 4, respectively.

lates and those of other *Enterococcus* species (5, 7, 11, 19, 32), Inc18-like plasmids are pheromone independent. We do not know if the species carrying the plasmid is important for *vanA* transfer from *Enterococcus* to *S. aureus*. One factor that may be important for *vanA* transfer is biofilm formation, and *E. faecalis* cells are better than cells of other *Enterococcus* spp. at biofilm formation (18). Regardless, the increased occurrence of the plasmid in *E. faecalis* isolates is consistent with the identification of vancomycin-resistant *E. faecalis* colonization or infection in 5 of the Michigan VRSA patients.

The findings in this study may be useful in predicting future VRSA occurrence. It is possible that, if VRE isolates with these plasmids increase in occurrence, the occurrence of VRSA will also increase. Periodic surveillance for these *vanA* plasmids among VRE isolates would be informative. Considering that VRSA isolates are so rare, there are likely other factors that are important for VRSA to occur. For example, there may be *S. aureus* and environmental factors that facilitate plasmid transfer. Additional studies are needed to identify those factors. However, regardless of exactly what factors are needed, our findings provide an additional public health rationale for controlling the spread of VRE. In additional to preserving treatment options for enterococcus infections, reducing the spread of VRE may be an important means of preserving the activity of vancomycin for the treatment of *S. aureus* infections, especially in areas where Inc18-like plasmids have begun to emerge.

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