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Vancomycin-Resistant *Staphylococcus aureus* Isolates Associated with Inc18-Like *vanA* Plasmids in Michigan▽

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Five of the seven cases of vancomycin-resistant *Staphylococcus aureus* (VRSA) infection identified to date have occurred in southeastern Michigan. VRSA isolates from the four most recent cases (all from Michigan) were characterized. The *vanA* gene was localized to a single plasmid in each VRSA isolate. The pulsed-field gel electrophoresis patterns of chromosomal DNA and the restriction profile of the plasmid demonstrated that the four isolates were unique and differed from the first three VRSA isolates. Vancomycin-resistant *Enterococcus* (VRE) isolates, all of which were *Enterococcus faecalis*, were recovered from case patients 4 to 6. Each VRE isolate transferred vancomycin resistance to *E. faecalis* JH2-2 by conjugation. PCRs for *vanA* and the Inc18-like plasmid genes *traA* and *repR* confirmed the presence of an Inc18-like *vanA* plasmid in all VRE isolates and transconjugants. An Inc18-like *vanA* plasmid was identified in the VRSA isolate from case patient 7. These findings suggest a role of Inc18-like plasmids as *vanA* donors.

The first *vanA*-mediated, vancomycin-resistant *Staphylococcus aureus* (VRSA) strain was isolated in a Michigan hospital in 2002 (5, 7). Since then, six additional occurrences have been reported: one each in Pennsylvania and New York and four in southeastern Michigan (3, 6, 28a, 29). The *vanA* plasmid from the first VRSA isolate was extensively characterized (35). The *vanA* gene was localized to a 57.9-kb plasmid which consisted of a pSK41-like *S. aureus* plasmid with an insertion of a Tn*1546*-like element carrying the *vanA* operon. The researchers hypothesized that this *S. aureus* isolate acquired *vanA* from an isolate of vancomycin-resistant *Enterococcus* (VRE). The patient with the first case of VRSA infection was also infected with a vancomycin-resistant *Enterococcus faecalis* isolate at the time of VRSA isolation.

The *vanA* plasmid from this *E. faecalis* isolate was characterized by Flannagan et al. (15). In this isolate, *vanA* was localized to a plasmid belonging to the Inc18 plasmid family. These plasmids are broad-host-range conjugative plasmids; pIP501 and pAMβ1 are two well-characterized examples of Inc18 plasmids. Flannagan et al. (15) demonstrated that the *E. faecalis vanA* plasmid was conjugative and not pheromone responsive. That was the first report of *vanA* on an Inc18-like plasmid.

Details of the VRSA isolate from case patient 2 (VRSA-2; Pennsylvania) and VRSA-3 (New York) have been reported elsewhere (29, 36). Case patient 2 had a history of VRE infection, but no isolate was available for characterization. The VRE isolate was either coisolated from the same site along with the VRSA isolate or colonized the patient at another site. For VRSA-3 (New York), both *Enterococcus faecium* and *E. faecalis* were isolated from the patient, but only the *E. faecium* isolate contained the same *vanA* plasmid as the VRSA isolate and thus was hypothesized to be the donor of *vanA* to *S. aureus* (36).

The occurrence of *vanA*-mediated resistance in *S. aureus* is uncommon; however, a disproportionate number of isolates (five of seven) have occurred in a limited geographic area. In three of the four most recent cases from southeastern Michigan, a vancomycin-resistant *E. faecalis* isolate was recovered from the patient in association with VRSA; in the sixth case, a vancomycin-resistant *Enterococcus avium* isolate was also identified. Although several explanations for this observation are possible, a prominent hypothesis is that the VRE isolates recovered in Michigan have common characteristics that may facilitate the transfer of *vanA*-mediated resistance to *S. aureus*. The objectives of this study were to determine the genetic interrelatedness of the seven VRSA isolates recovered to date, identify the genetic factors shared by the *Enterococcus* isolates recovered from three of the latest four cases of VRSA infection from southeastern Michigan, and determine whether the *Enterococcus vanA* plasmids could also be identified in the corresponding VRSA isolates.

MATERIALS AND METHODS

Bacterial isolates. All of the bacterial isolates analyzed in this study are listed in Table 1. To confirm the species identification of the isolates sent to the CDC, we used conventional biochemical analysis (2, 24).

Antimicrobial susceptibility testing. Susceptibility to vancomycin and other antimicrobial agents was determined by the reference broth microdilution method by 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 (formerly NCCLS) guidelines (10, 11). Quality control testing was performed daily by using strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212.

PFGE and MLST typing. Pulsed-field gel electrophoresis (PFGE) was performed by digesting genomic DNA with the SmaI restriction enzyme and following the standard procedures for *S. aureus* (25) or enterococci (26). TIFF images of the pulsed-field gels were analyzed by using BioNumerics software (Applied Maths, Austin, TX) and Dice coefficients. The relatedness of the *S. aureus* strains was established by using previously published standards to identify the isolates as USA types (25). Multilocus sequence typing (MLST) of VRSA-6

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TABLE 1. VRSA strains and associated VRE isolates in this study

Strain	Strain no.	Site	Mo/yr of isolation	MIC (μg/ml)[a]												
				CC	DAP	DOX	ERY	GM	OXA	LEV	LNZ	PEN	Q/D	RIF	STR	TEC	VAN
VRSA-4	HIP14300	Foot wound	02/2005	>16	≤0.5	≤0.25	>8	≤2	>16	>16	2	≥2	0.5	≤0.5	ND	16	256
E. faecalis	HIP14333	Rectum	02/2005	ND	ND	8	>8	>500	ND	>8	2	4	16	1	≤1,000	128	1,024
VRSA-5	HIP15178	Surgical wound	10/2005	>16	≤0.5	≤0.5	>8	≤2	>16	>16	4	>2	2	≤0.5	ND	8	512
E. faecalis	HIP15179	Co-isolated with VRSA	10/2005	ND	1	≤2	>8	>500	ND	8	4	2	8	2	>1,000	64	512
VRSA-6	AIS2006032	Foot wound	12/2005	>16	1	1	>8	≤2	>16	>16	2	>2	1	≤0.5	ND	16	1,024
E. faecalis	AIS2007003	Rectum	12/2005	ND	2	4	>8	>500	ND	>8	2	2	16	1	>1,000	64	>512
E. avium	AIS2007004	Rectum	12/2005	ND	1	8	>8	>500	ND	8	2	2	ND	1	≤1,000	8	256
VRSA-7	AIS2006049	Arm wound	10/2006	>16	2	≤1	>8	≤2	>16	8	2	>2	0.5	≤0.5	ND	16	512

[a] Abbreviations: CC, clindamycin; DAP, daptomycin; DOX, doxycycline; ERY, erythromycin; GM, gentamicin; OXA, oxacillin, LEV, levofloxacin; LNZ, linezolid, PEN, penicillin; Q/D, quinupristin-dalfopristin; STR, streptomycin; TEC, teicoplanin, TET, tetracycline, VAN, vancomycin; ND, not determined.

was performed by amplification and sequence determination of seven genetic loci. The primers and PCR conditions were the same as those described for *S. aureus* at www.mlst.net (14). Both strands of each PCR product were sequenced by using the same primers. Sequencing was performed with a CEQ DTCS Quick Start kit and a CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA).

Isolation and manipulation of plasmid DNA. Plasmids were prepared with a Qiagen Plasmid Midi or Maxi kit (Valencia, CA), according to the manufacturer's protocol, but with specific modifications for either *Staphylococcus* or *Enterococcus*. For *S. aureus*, the procedure was modified by the inclusion of lysostaphin (Sigma-Aldrich, St. Louis, MO) at a final concentration of 30 μg/ml in the cell lysis buffer. For the isolation of plasmids from *Enterococcus* species, lysozyme was added to a resuspension buffer at a final concentration of 5 mg/ml and the bacteria were incubated in the buffer at 37°C for 30 min before the lysis step. Also, the elution buffer was warmed to 65°C for the elution of plasmid DNA from the Qiagen cartridge.

PCR amplification of Inc18 and Tn*1546* elements. Six PCR primers were unique to this study (Table 2). PCRs for the detection of *vanA*, *traA*, and *repR* were prepared in a total volume of 50 μl, which consisted of 1.6 mM each deoxynucleoside triphosphate (Applied Biosystems, Foster City, CA), 400 μM each primer, 1× buffer, 1 mM $MgCl_2$, 0.5 U of AmpliTaq Gold Enzyme (Applied Biosystems), and 2 μl of DNA extract (which was equal to 100 to 500 ng of DNA). 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, 30 s at 55°C, and 30 s at 72°C; and a final elongation at 72°C for 7 min. The PCR products were visualized on an agarose gel.

Primers P1 to P19 and the reaction conditions previously reported by Arthur et al. (1) were used to characterize the Tn*1546*-like elements in the VRSA and VRE isolates. In addition, Tn*1546*-like elements were characterized by using the long PCR protocol of Woodford and Stigter (38). The long PCR products were digested with EcoRI, EcoRV, HindIII, and XbaI.

The PCR primers and conditions for the detection of erythromycin-mediated resistance were the same as those described previously (27).

Conjugation. Five filter mating experiments were performed in which the *vanA* donors were either *E. faecalis* HIP12467 (VRSA-1), HIP14333 (VRSA-4), HIP15179 (VRSA-5), or AIS2007003 (VRSA-6) or *E. avium* AIS2007004 (VRSA-6). The recipient strain was *E. faecalis* JH2-2. After overnight growth in brain heart infusion (BHI) broth with either 25 μg/ml vancomycin (donors) or 25 μg/ml fusidic acid (recipient), 100 μl of each culture was added to 5 ml of new BHI broth and incubated for 5 h. The mating mixtures were combined in a 20:1 ratio of donor to recipient (400 μl donor and 20 μl of recipient) and filtered through a Nalge 0.45-μm-pore-size filter under vacuum. The filter was removed from the filter unit and placed on a BHI agar plate, and the plate was incubated for 18 h. The filter with the overlying colonies was then removed from the agar and placed in 5 ml BHI broth, and the filter and broth were vortexed to remove the growth. Transconjugants were selected on BHI agar plates containing 25 μg/ml each of vancomycin and fusidic acid. The HindIII-digested plasmid DNA from the transconjugants was compared to the HindIII-digested plasmid DNA from the VRE donor to ensure that the transferred plasmid was the same as the donor plasmid.

TABLE 2. PCR Primers used in this study

Primer[a]	Sequence (5′ to 3′)	Fragment size (bp)
vanA F	CAT GAA TAG AAT AAA AGT TGC TGC AAT A	1,032
vanA R	CCC CTT TAA CGC TAA TAC GAT CAA	
traA F	TAA TCG CAA TGG CTT CTT ATC	475
traA R	TCT GCC CAA TCT TTA CGA AT	
repR F	GCT TCA TGA CGG CTT GTT A	565
repR R	TTG GCT GCT TTG ACA GAT TTA	

[a] F, forward; R, reverse.

Southern hybridization. To determine the locations of the *vanA* and *traA* genes, plasmid DNA was digested with HindIII and examined by Southern blot analysis. The DNA fragments were separated on an agarose gel and transferred to a Zeta-probe GT genomic blotting membrane (Bio-Rad, Hercules, CA). The blot was probed with a 1,032-bp *vanA* PCR product (the PCR is described above) that was labeled with horseradish peroxidase by using an ECL direct nucleic acid labeling and detection systems kit (Amersham Biosciences, Piscataway, NJ). Hybridization occurred at 42°C overnight (12 h), and washes were performed according to the manufacturer's instructions. For the nick translation experiment, the HindIII-digested plasmid fragments from the VRSA strains and the VRE transconjugants were separated on an agarose gel and transferred to the blotting membrane. The blot was probed with the HindIII-digested plasmid fragments from the corresponding VRE transconjugant that were labeled by use of a NEBlot Phototope probe labeling kit (New England Biolabs). The hybridization was visualized with a Phototope-Star detection kit (New England BioLabs).

Clumping assay. The clumping assays were performed by a microtiter serial twofold dilution method, as described previously (12). Briefly, a pheromone-containing supernatant was prepared from *E. faecalis* JH2-2. Cultures were prepared by diluting 1 ml of the overnight culture in 100 ml of fresh BHI broth and incubating the mixture at 37°C with shaking for 4 to 6 h. The culture was centrifuged at 8,000 × *g* for 10 min at 4°C, and the supernatant was then filtered through a 0.22-μm-pore-size filter unit (Nalgene, Rochester, NY) and boiled for 15 min (16). The ability of the supernatants to induce the clumping of *Enterococcus* strains was tested by mixing 50 μl of culture filtrate with 50 μl of fresh BHI broth and 25 μl of fresh log-phase *Enterococcus* cells. The mixtures were incubated for 2 to 3 h at 37°C with shaking and were examined for clumping. *E. faecalis* JH2-2 carrying pPD1 and *E. faecalis* JH2-2 carrying pAM373 were used as positive controls, and *E. faecalis* JH2-2 carrying pIP501 was used as a negative control.

RESULTS

VRSA-4, VRSA-5, VRSA-6, and VRSA-7 were positive for *vanA* by PCR; and the vancomycin MICs ranged from 256 μg/ml to 1,024 μg/ml (Table 1). Typing of the VRSA isolates by PFGE indicated that the four most recent VRSA isolates

TABLE 3. Summary of microbiological findings for seven VRSA cases

Case no.	State	VRSA PFT[a]	Associated VRE (possible *vanA* donor)	Evidence of Inc18-like *van A* plasmid		Characterization of VRSA *vanA* plasmid	Reference(s) or source
				VRE	VRSA		
1	MI	USA100	*E. faecalis*	Yes	No	*S. aureus* plasmid with Tn*1546* insertion	15, 35
2	PA	USA100	Unknown	Not applicable	No	Uncharacterized	29
3	NY	USA800	*E. faecium*	No	No	Maintained VRE *vanA* plasmid	36
4	MI	USA100	*E. faecalis*	Yes	Yes	Maintained VRE *vanA* plasmid	This work
5	MI	USA100	*E. faecalis*	Yes	Yes	Maintained VRE *vanA* plasmid	This work
6	MI	Not defined[b]	*E. faecalis* or *E. avium*	Yes	No	Probable *S. aureus* plasmid with Tn*1546* insertion	This work
7	MI	USA100	Unknown	Not applicable	Yes	Uncharacterized	This work

[a] PFT, pulsed-field type.
[b] The PFGE pattern is related to USA100, but it is outside of the 80% similarity range.

were unique (Fig. 1A). Comparison of these patterns to the patterns of the VRSA isolates from the first three cases revealed that VRSA-5 and VRSA-1 had indistinguishable patterns. The PFGE patterns for VRSA-4 and VRSA-5 placed these isolates in the USA100 lineage, the most common MRSA lineage in the health care setting (25). The PFGE pattern of VRSA-6 was just outside of the 80% similarity range for USA100, but by MLST this isolate was sequence type 5 (ST5). This is the same ST as VRSA-4 and VRSA-5 (CDC, unpublished data) (Table 3).

We analyzed VRSA plasmid DNA by restriction analysis to determine if the VRSA isolates differed in their total plasmid DNA contents and by Southern blot analysis to locate the *vanA* gene. The HindIII plasmid restriction patterns for VRSA-1 and VRSA-5 were different (Fig. 1B, lanes 1 and 5, respectively). Therefore, we concluded that VRSA-1 and VRSA-5 are independent isolates. The restriction patterns of the plasmids from VRSA-4, VRSA-5, and VRSA-7 indicated that these plasmids are related (Fig. 1B, lanes 4, 5, and 7, respectively). The location of *vanA* on a plasmid was previously reported for VRSA-1 to VRSA-3 (29, 35, 36). By using the *vanA* sequences as a probe, the *vanA* gene was localized by Southern blot analysis to a 7-kb HindIII fragment of plasmid DNA in VRSA-4 to VRSA-7 (data not shown).

Since vancomycin-resistant *E. faecalis* strains were isolated from all but the most recent Michigan VRSA case, the isolates were typed by PFGE to determine if a single strain was a common donor for all of the Michigan VRSA cases. Each vancomycin-resistant *E. faecalis* isolate demonstrated a unique PFGE pattern (Fig. 2A). For all four VRE isolates (three *E. faecalis* isolates and one *E. avium* isolate) associated with the recent Michigan VRSA isolates, *vanA*-mediated vancomycin resistance was transferred to *E. faecalis* JH2-2 by conjugation. The transfer efficiency ranged from approximately 1.2×10^{-4} to 6.3×10^{-6} transconjugants per recipient cell. Analysis of uncut and HindIII-digested plasmid DNA from the transconjugant demonstrated that a single plasmid was present in each transconjugant, and these plasmids ranged in size from approximately 30 kb to 45 kb (data not shown). In each case, only vancomycin resistance (*vanA* mediated) and erythromycin resistance (*ermB* mediated) were transferred to *E. faecalis* JH2-2 by conjugation. Plasmids from all of the VRE transconjugants were similar by restriction pattern analysis with either HindIII (Fig. 2B) or EcoRI (data not shown). Plasmids from both the *E. faecalis* isolates and the *E. avium* isolate associated with VRSA-6 had a pattern that was indistinguishable from the pattern of the VRE plasmid from case 4 [Fig. 2B, lanes 4, 6(a), and 6(b)].

Because the *vanA* VRE plasmid from the first VRSA case had previously been identified as an Inc18-like plasmid (15)

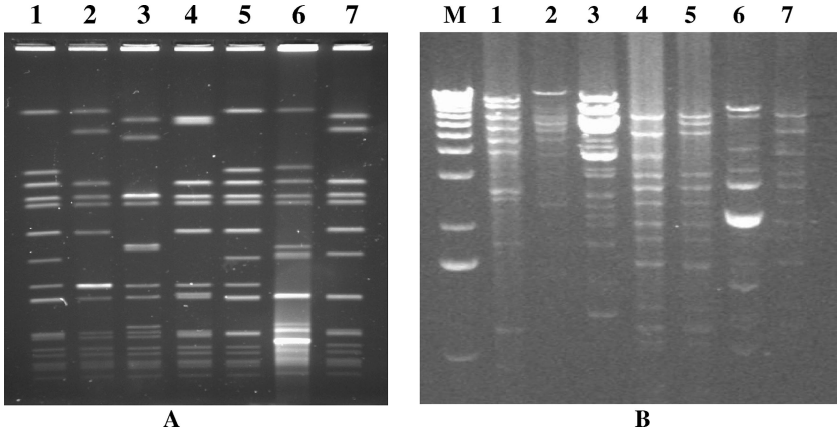

FIG. 1. PFGE analysis of VRSA genomic DNA (A) and restriction enzyme analysis of VRSA plasmid DNA (B). Each lane is labeled with the VRSA case number; lane M, 1-kb molecular marker. Genomic DNA was digested with SmaI, and plasmid DNA was digested with HindIII.

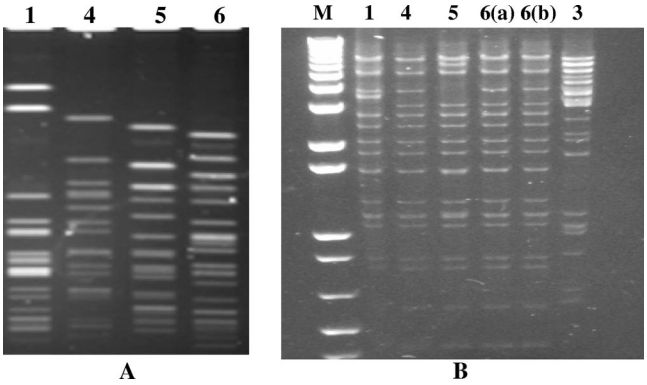

FIG. 2. PFGE analysis of *E. faecalis* genomic DNA (A) and restriction enzyme analysis of VRE *vanA* plasmids from the transconjugants (B). Each lane is labeled with the VRSA case number; lane M, 1-kb molecular marker. For VRSA-6, VRE isolates of two species were recovered; lane 6(a) is the plasmid from *E. faecalis* and lane 6(b) is the plasmid from the *E. avium*. *E. faecalis* genomic DNA was digested with SmaI. Although *E. faecalis* was isolated from four VRSA-infected patients, the PFGE patterns of these indicated that these were different strains. Plasmid DNA from the transconjugants was digested with HindIII. The restriction patterns of plasmids from the Michigan VRE isolates were similar, but the plasmid from the VRE isolate from patient 3 (New York) was different.

and because this plasmid appeared to be related by restriction analysis to the VRE *vanA* plasmids associated with more recent Michigan VRSA cases, we used PCR analysis to further characterize the latter plasmids by detection of two Inc18-specific genes, *traA* and *repR* (30). The *traA* gene encodes a DNA nickase conjugative protein, and *repR* encodes a replication regulatory protein. Plasmids from both the native VRE isolates and the corresponding strain JH2-2 transconjugants were tested; and all were found to be positive for *traA*, *repR*, and *vanA* by PCR (Table 3). In addition, Southern blot analysis located the *vanA* and *traA* genes on the same plasmid in each transconjugant (data not shown).

The VRSA-associated VRE isolates (isolates HIP14333, HIP15179, AIS2007003, and AIS2007004) were tested for pheromone-responsive clumping when they were exposed to an *E. faecalis* JH2-2 filtrate. None of the VRE isolates demonstrated clumping in the presence of the filtrate, suggesting that their *vanA* plasmids are not pheromone responsive.

To determine how much of the VRE *vanA* plasmid was maintained in the VRSA isolates, we isolated the VRE *vanA* plasmids from the transconjugants, labeled them by nick translation, and performed a Southern blot using *vanA* plasmid DNA as the probe and HindIII-digested plasmid DNA from the corresponding VRSA as the target. The sequence of the first VRSA plasmid, pLW1043, has been determined (35). The *S. aureus* plasmid had acquired Tn*1546*, presumably from the VRE plasmid. This transposon was the only portion of the VRE *vanA* plasmid identified in the VRSA *vanA* plasmid. Our sequence results from the hybridization experiment with VRSA-1 were consistent with the previously published plasmid sequence. The HindIII digestion of pLW1043 was expected to result in a 7-kb fragment containing the Tn*1546* sequence. This fragment was detected by Southern blot hybridization by using the corresponding VRE *vanA* plasmid as a probe (Fig. 3A). As a control, the VRE *vanA* plasmid probe was also used to detect HindIII fragments of the same plasmid from the VRE transconjugants, and all fragments were detected, indicating complete labeling of the plasmid DNA. The same hybridization experiment was performed with isolates from cases 4 to 6, as well as the corresponding VRE transconjugants. Hybridization of the VRSA-4 and VRSA-5 plasmids indicated that the VRSA isolates maintained the entire VRE *vanA* plasmid. Plasmids from VRSA-4 and VRSA-5 were positive for Inc18 *traA* and *repR* by PCR. In contrast, the VRE *van*A plasmid probe hybridized to a 7-kb HindIII restriction fragment of VRSA-6 plasmid DNA. This HindIII fragment was the same 7-kb fragment that hybridized to the *vanA* probe described above. This result is similar to the results seen for VRSA-1, suggesting that in VRSA-6 Tn*1546* inserted into an *S. aureus* plasmid and that no other VRE *vanA* plasmid DNA was maintained in the *S. aureus* isolate (Table 3). Plasmids from VRSA-1 and VRSA-2 were negative for *traA* and *repR* by PCR.

For case 7, no VRE was isolated from sites of possible colonization (nares and rectum). Since some of the VRSA isolates maintained the VRE plasmid, we isolated plasmids from the VRSA isolates and used PCR to test for Inc18-specific genes *traA* and *repR*, as well as for *vanA*. One plasmid

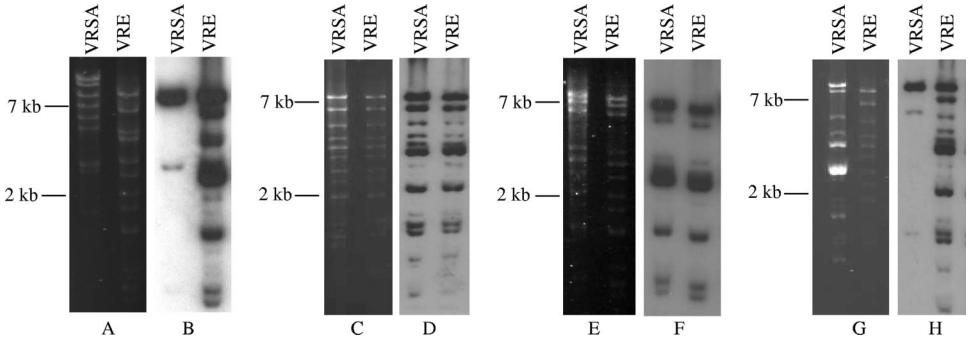

FIG. 3. Detection of VRE *vanA* plasmid DNA in VRSA isolates by Southern blot analysis. Southern blot analyses of each VRSA plasmid DNA were performed by using a biotin-labeled probe prepared from the corresponding VRE *vanA* plasmid from the transconjugant. For each VRSA case, the left panel is an ethidium bromide gel and the right panel is the Southern blot. Molecular size standards are indicated at the left. (A and B) Isolates from VRSA-1; (C and D) isolates from VRSA-4; (E and F) isolates from VRSA-5; (G and H) the VRSA and the vancomycin-resistant *E. faecalis* isolates from VRSA-6.

was identified in this isolate, and it was estimated to be 40 kb. The PCR for all three genes was positive, suggesting that an Inc18-like *vanA* plasmid was involved in this VRSA occurrence as well (Table 3).

Several different insertions and deletions within Tn*1546* have been reported in VRE and VRSA isolates (8, 18, 36, 37). The transposon sequences from the first VRSA isolate and the corresponding VRE isolate were identical, and there were no insertions or deletions relative to the sequence of the prototype Tn*1546* element (1, 35). To assess VRSA-4 to VRSA-7 and the corresponding VRE isolates from cases 4 to 6 for the presence of insertions or deletions within their Tn*1546*-like elements, we used a combination of primers to amplify 10 overlapping fragments of the entire element. The sizes of all PCR products were consistent with the size of the prototype Tn*1546* element. Also, the results of restriction analyses of the amplified Tn*1546*-like elements in each isolate were consistent with those for the prototype Tn*1546* element (data not shown). Therefore, all VRSA isolates from Michigan appeared to have the prototype transposon, whereas the transposon elements from the Pennsylvania and New York VRSA isolates each demonstrated unique insertions and deletions (8, 36).

DISCUSSION

We have identified an Inc18-like, *vanA*-encoding conjugative plasmid as a likely factor in the emergence of the five VRSA isolates that have been reported in southeastern Michigan since 2002. For each VRSA case, the isolate demonstrated a different restriction pattern for either the chromosomal or the plasmid DNA, so we conclude that each VRSA isolate was generated by a unique genetic event. However, each of the plasmids of the associated VRE isolates had similar restriction profiles, the same replication and conjugative proteins, and consistent Tn*1546*-like elements.

The Tn*1546*-like *vanA* element has been described on several different conjugative plasmids (13, 17, 19, 21–23, 28, 32), yet the transfer of *vanA*-mediated resistance to other bacteria, specifically, *S. aureus*, appears to be a rare event, with only seven VRSA isolates identified to date. It was anticipated that *vanA*, pheromone-responsive conjugative plasmids would be important for the occurrence of VRSA. *S. aureus* produces the pheromone cAM373, which can induce the clumping of *Enterococcus* carrying a cAM373-responsive plasmid, and the *vanA* gene was identified on such a plasmid in an isolate of *E. faecalis* (28). Also, *vanA* has been identified on other pheromone-responsive conjugative plasmids in both *E. faecium* and *E. faecalis*. It appears that all of the Michigan VRSA isolates described here occurred independently of pheromone-induced conjugation, since none of the vancomycin-resistant *E. faecalis* isolates associated with the Michigan VRSA isolates demonstrated pheromone-responsive clumping. Other environmental factors, e.g., the matrix of a biofilm, may play an important role in bringing the bacteria together in close proximity, where conjugation could take place.

The *vanA* gene has also been identified on non-pheromone-responsive conjugative plasmids. In *E. faecium*, *vanA* has been reported on pMG1-like plasmids (32). These plasmids demonstrate high-frequency conjugative transfer between enterococci (31, 32). The first report of a *vanA* Inc18-like plasmid was the characterization of the *vanA* plasmid in the *E. faecalis* isolate associated with the first VRSA case. Inc18-like conjugative plasmids (e.g., pIP501) characteristically demonstrate a broad host range. Conjugation between many different genera of gram-positive bacteria, in addition to at least one gram-negative species, *Escherichia coli*, has been demonstrated (9, 20, 33). The broad-host-range characteristic of Inc18-like plasmids is likely important for the conjugative transfer of *vanA*-mediated resistance from *Enterococcus* to *S. aureus*.

Little is known about the prevalence and geographical distributions of different *vanA* plasmids in VRE. If Inc18-like *vanA* plasmids are more common in Michigan than at other geographical locations, this may explain why five of the seven VRSA cases occurred in Michigan. It should be noted that the VRSA *vanA* plasmid in the isolate from the third VRSA case was identified in *E. faecium* (36). The *E. faecium vanA* plasmid is a conjugative plasmid, but it is negative for the Inc18 genes, *traA* and *repR* (W. Zhu, unpublished data). The nature of this plasmid is being investigated, but clearly, Inc18-like *vanA* plasmids are not the only plasmids that are capable of transferring vancomycin resistance from *Enterococcus* spp. to *S. aureus*.

S. aureus may have characteristics which allow some strains to be the more likely recipients of DNA transfer. All of the Michigan *S. aureus* recipients are part of the same clonal complex, ST5, and three of the four isolates clearly belong to the USA100 pulsed-field type. Even though they belong to a common lineage, there are differences in the PFGE patterns that identify each *S. aureus* recipient as unique. The association of isolates from the USA100, ST5 lineage with VRSA is likely because isolates within this lineage are the most common health care-associated MRSA strains (25). *S. aureus* strain RN4220 is often used in experimental studies because this strain can accept plasmid DNA from other species of bacteria by either conjugation or transformation. A study by Waldron and Lindsay demonstrated that a mutation in the *hsdR* gene, which encodes the Sau1 type I restriction-modification system, was responsible for RN4220's transformable phenotype (34). Whether the VRSA strains have similar mutations in the Sau1 type 1 restriction-modification system is being investigated.

The occurrence of VRSA appears to be either a one- or a two-step genetic event. The plasmid from the first VRSA isolate was sequenced and was found to be a previously recognized *S. aureus* plasmid containing a Tn*1546* insertion (35). The proposed model of resistance transfer was a two-step genetic event in which the *S. aureus* isolate acquired the *Enterococcus vanA* plasmid, Tn*1546* transferred from the *Enterococcus* plasmid to the *S. aureus* plasmid by transposition, and the *Enterococcus* plasmid was not maintained in the *S. aureus* recipient. In the third VRSA case, the entire *Enterococcus vanA* plasmid was maintained in the *S. aureus* recipient (36). This suggests a single genetic event in which the *vanA* plasmid is transferred from one isolate to the next, most likely by conjugation. In our analysis of the Michigan VRSA isolates, both outcomes were observed.

It is not clear how common VRSA will be in the future, but this is certainly a concerning antimicrobial resistance that should be prevented, if possible. An important aspect of VRSA prevention will be the control of VRE and methicillin-resistant *S. aureus* transmission. This is a challenging prospect, since both VRE and methicillin-resistant *S. aureus* are endemic in

most health care settings in the United States (4). It may be prudent to implement the most rigorous control measures for patient populations and at locations where VRE isolates with Inc18-like plasmids occur.

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