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 Tn1546-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 Smal 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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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 8800 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 lysozyme (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 Tn1546 elements.

Six PCR primers were unique to this study (Table 2). PCR for the detection of vanA, traA, and repR were prepared in a total volume of 50 μl, which consisted of 1.6 mM each deoxyribonucleoside triphosphate (Applied Biosystems, Foster City, CA), 400 μM each primer, 1X buffer, 1 mM MgCl2, 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). PCR conditions for 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 Tn1546 element. 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 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.

### 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 was 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...
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 \textit{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 \textit{vanA} on a plasmid was previously reported for VRSA-1 to VRSA-3 (29, 35, 36). By using the \textit{vanA} sequences as a probe, the \textit{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 \textit{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 \textit{E. faecalis} isolate demonstrated a unique PFGE pattern (Fig. 2A). For all four VRE isolates (three \textit{E. faecalis} isolates and one \textit{E. avium} isolate) associated with the recent Michigan VRSA isolates, \textit{vanA}-mediated vancomycin resistance was transferred to \textit{E. faecalis} JH2-2 by conjugation. The transfer efficiency ranged from approximately $1.2 \times 10^{-4}$ to $6.3 \times 10^{-6}$ 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 (\textit{vanA} mediated) and erythromycin resistance (\textit{ermB} mediated) were transferred to \textit{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 \textit{E. faecalis} isolates and the \textit{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 \textit{vanA} VRE plasmid from the first VRSA case had previously been identified as an Inc18-like plasmid (15)
and because this plasmid appeared to be related by restriction analysis to the VRE \textit{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, \textit{traA} and \textit{repR} \cite{30}. The \textit{traA} gene encodes a DNA nickase conjugative protein, and \textit{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 \textit{traA}, \textit{repR}, and \textit{vanA} by PCR (Table 3). In addition, Southern blot analysis located the \textit{vanA} and \textit{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 \textit{E. faecalis} JH2-2 filtrate. None of the VRE isolates demonstrated clumping in the presence of the filtrate, suggesting that their \textit{vanA} plasmids are not pheromone responsive.

To determine how much of the VRE \textit{vanA} plasmid was maintained in the VRSA isolates, we isolated the VRE \textit{vanA} plasmids from the transconjugants, labeled them by nick translation, and performed a Southern blot using \textit{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 \cite{35}. The \textit{S. aureus} plasmid had acquired Tn1546, presumably from the VRE plasmid. This transposon was the only portion of the VRE \textit{vanA} plasmid identified in the VRSA \textit{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 Tn1546 sequence. This fragment was detected by Southern blot hybridization by using the corresponding VRE \textit{vanA} plasmid as a probe (Fig. 3A). As a control, the VRE \textit{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 \textit{vanA} plasmid. Plasmids from VRSA-4 and VRSA-5 were positive for Inc18 \textit{traA} and \textit{repR} by PCR. In contrast, the VRE \textit{vanA} 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 \textit{vanA} probe described above. This result is similar to the results seen for VRSA-1, suggesting that in VRSA-6 Tn1546 inserted into an \textit{S. aureus} plasmid and that no other VRE \textit{vanA} plasmid DNA was maintained in the \textit{S. aureus} isolate (Table 3). Plasmids from VRSA-1 and VRSA-2 were negative for \textit{traA} and \textit{repR} by PCR.

For case 7, no VRE was isolated from sites of possible colonization (nares and rectum). Since some of the VRE isolates maintained the VRE plasmid, we isolated plasmids from the VRE isolates and used PCR to test for Inc18-specific genes \textit{traA} and \textit{repR}, as well as for \textit{vanA}. One plasmid...
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 \textit{vanA} plasmid was involved in this VRSA occurrence as well (Table 3).

Several different insertions and deletions within Tn1546 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 Tn1546 element (1, 35). To assess VRE-4 to VRSA-7 and the corresponding VRE isolates from cases 4 to 6 for the presence of insertions or deletions within their Tn1546-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 Tn1546 element. Also, the results of restriction analyses of the amplified Tn1546-like elements in each isolate were consistent with those for the prototype Tn1546 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, \textit{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 Tn1546-like elements.

The Tn1546-like \textit{vanA} element has been described on several different conjugative plasmids (13, 17, 19, 21–23, 28, 32), yet the transfer of \textit{vanA}-mediated resistance to other bacteria, specifically, \textit{S. aureus}, appears to be a rare event, with only seven VRSA isolates identified to date. It was anticipated that \textit{vanA}, pheromone-responsive conjugative plasmids would be important for the occurrence of VRSA. \textit{S. aureus} produces the pheromone cAM373, which can induce the clumping of \textit{Enterococcus} carrying a cAM373-responsive plasmid, and the \textit{vanA} gene was identified on such a plasmid in an isolate of \textit{E. faecalis} (28). Also, \textit{vanA} has been identified on other pheromone-responsive conjugative plasmids in both \textit{E. faecium} and \textit{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 \textit{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 \textit{vanA} gene has also been identified on non-pheromone-responsive conjugative plasmids. In \textit{E. faecium}, \textit{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 \textit{vanA} Inc18-like plasmid was the characterization of the \textit{vanA} plasmid in the \textit{E. faecalis} isolate associated with the first VRSA case. Inc18-like conjugative plasmids (e.g., pIP501) characterize demonstrate a broad host range. Conjugation between many different genera of gram-positive bacteria, in addition to at least one gram-negative species, \textit{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 \textit{vanA}-mediated resistance from \textit{Enterococcus} to \textit{S. aureus}.

Little is known about the prevalence and geographical distributions of different \textit{vanA} plasmids in VRE. If Inc18-like \textit{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 \textit{VRSA vanA} plasmid in the isolate from the third VRSA case was identified in \textit{E. faecium} (36). The \textit{E. faecium vanA} plasmid is a conjugative plasmid, but it is negative for the Inc18 genes, \textit{traA} and \textit{repR} (W. Zhu, unpublished data). The nature of this plasmid is being investigated, but clearly, Inc18-like \textit{vanA} plasmids are not the only plasmids that are capable of transferring vancomycin resistance from \textit{Enterococcus} spp. to \textit{S. aureus}.

\textit{S. aureus} may have characteristics which allow some strains to be the more likely recipients of DNA transfer. All of the Michigan \textit{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 \textit{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). \textit{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 \textit{hsdR} gene, which encodes the SauI type I restriction-modification system, was responsible for RN4220's transformable phenotype (34). Whether the VRSA strains have similar mutations in the SauI type I 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 \textit{S. aureus} plasmid containing a Tn1546 insertion (35). The proposed model of resistance transfer was a two-step genetic event in which the \textit{S. aureus} isolate acquired the \textit{Enterococcus vanA} plasmid, Tn1546 transferred from the \textit{Enterococcus} plasmid to the \textit{S. aureus} plasmid by transposition, and the \textit{Enterococcus} plasmid was not maintained in the \textit{S. aureus} recipient. In the third VRSA case, the entire \textit{Enterococcus vanA} plasmid was maintained in the \textit{S. aureus} recipient (36). This suggests a single genetic event in which the \textit{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 \textit{S. aureus} transmission. This is a challenging prospect, since both VRE and methicillin-resistant \textit{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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