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Plasmid Content of a Vancomycin-Resistant *Enterococcus faecalis* Isolate from a Patient Also Colonized by *Staphylococcus aureus* with a VanA Phenotype

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Vancomycin-resistant *Enterococcus faecalis* coisolated with vancomycin-resistant (VanA) *Staphylococcus aureus* was found to contain two plasmids, designated pAM830 (45 kb) and pAM831 (95 kb). pAM830, found to be conjugative and closely related to the Inc18 family of broad-host-range conjugative plasmids, encodes resistances to vancomycin (via a Tn*1546*-like element) and erythromycin; pAM831 encodes resistances to gentamicin, streptomycin, and erythromycin.

The first example of a vancomycin-resistant *Staphylococcus aureus* (VRSA) strain with the VanA phenotype was isolated during the summer of 2002 from a diabetic patient in Michigan (3, 28). Interestingly, a vancomycin-resistant *Enterococcus faecalis* (VRE) strain with a similar VanA phenotype was coisolated with the VRSA strain (coisolates were obtained from a foot ulcer and from the tip of a dialysis catheter), thus raising the question of whether the *S. aureus* isolate acquired resistance from the VRE strain. Enterococci and staphylococci are known to exchange genetic information, as was demonstrated previously (4, 26) with the transfer of the broad-host-range erythromycin-resistance plasmids pAMβ1 and related (Inc18) elements (16, 20, 32). There is even a report of plasmid-encoded transfer, in the laboratory, of *vanA* from *E. faecalis* to *S. aureus* (23). In addition, *S. aureus* is known to secrete a peptide with an activity resembling a known *E. faecalis* sex pheromone, cAM373 (4, 13). Indeed, an *E. faecalis vanA*-carrying plasmid, pAM368, was recently found to encode a response to cAM373 (27), thus raising concern about the potential uptake of *vanA* from enterococci by a pheromone-related process. The data reported here address the nature of the VanA-related trait in the VRE strain and show that it involves a Tn*1546*-like element (1) associated with a conjugative plasmid of the Inc18 family. We also present data relating to a vancomycin-sensitive, methicillin-resistant *S. aureus* (MRSA) nasal isolate believed to have been the host that acquired the *vanA* gene associated with the VRSA strain.

The strains and plasmids used or identified in the study are listed in Table 1. Plasmid characterization made use of CsCl-ethidium bromide buoyant density centrifugation and other previously described standard methodologies (13, 14, 25). With regard to the enterococcal strains, the MICs for various antibiotics are indicated in Table 2. The resistances of primary significance were to vancomycin, erythromycin, gentamicin, and streptomycin. The VRE strain was also hemolytic on horse blood agar and exhibited a bacteriocin activity using *E. faecalis* OG1X as the indicator. Figure 1A shows the results of pulsed-field gel electrophoresis analysis of chromosomal DNA preparations from both the *E. faecalis* foot isolate (DMC83006B) and the catheter isolate (WBH27862), as well as a vancomycin-sensitive derivative (discussed below) of the foot isolate; all are seen to be isogenic. (Fig. 1A also shows that key transconjugants generated in the study described below are isogenic with the recipient strain JH2-2.)

Transferable resistance traits of the VRE strain. Vancomycin resistance (Vm^r) was observed to transfer to *E. faecalis* JH2-2 from DMC83006B in overnight filter matings (4) at a frequency of 1.2×10^{-3} per donor (Table 3). A similar frequency was observed when selection was for erythromycin resistance (Em^r). The frequency was an order of magnitude higher when the recipient was *Enterococcus faecium*. When JH2-2 transconjugants selected on vancomycin were examined for unselected uptake of Em^r, all were found to have acquired this trait as well. In contrast, the transconjugants were sensitive to gentamicin. When the selected transconjugant SFV1 was used as a donor for a second round of transfer, movement of Vm^r occurred at a frequency of 2.1×10^{-4} per donor and again resulted in cotransfer of Em^r. When the SFV1 strain, as well as five additional transconjugants, were examined for plasmid content, they were found to harbor a 45-kb plasmid that was subsequently designated pAM830 (Fig. 1B, lane 6). Plasmid sizes were determined by summation of restriction fragments using agarose gel electrophoresis following separate digestions with *Bam*HI, *Eco*R1, or *Pst*I.

The pAM830 plasmid could also be visualized in the original

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Description or relevant characteristics[b]	Reference(s)
Strains		
Enterococcus faecalis		
DMC83006B	Clinical isolate, Vm^r Gm^r Sm^r Em^r Lv^r Hly^+ Bac^+	This study, 3, 28
WBH27862	Clinical isolate, Vm^r Gm^r Sm^r Em^r Lv^r Hly^+ Bac^+	This study, 3, 28
SF830Vs	Vm^s derivative of DMC83006B, Gm^r Sm^r Em^r Lv^r Hly^+ Bac^+	This study
SFV1	(DMC83006B X JH2-2), Rf^r Fa^r Vm^r Em^r	This study
SFG1	(SF830Vs X JH2-2), Rf^r Fa^r Gm^r Sm^r Em^r Bac^+	This study
JH2-2	Rf^r Fa^r	19
OG1RF	Rf^r Fa^r	9
OG1SS	Sm^r Sp^r	15
OG1X	Sm^r	17
OG1X/pAM373	Sm^r, responder to pheromone cAM373	7
OGIX/pAD1	Sm^r, responder to pheromone cAD1	17, 30
OG1-10/pAMβ1	Sm^r Em^r	6, 9
OG1X/pIP501	Sm^r Em^r Cm^r	33
Enterococcus faecium		
BM4105-RF	Rf^r Fa^r	24
BM4105-RF/pMG1	Rf^r Fa^r Gm^r	31
Staphylococcus aureus		
DMC82991A	Clinical isolate, Me^r	This study, 3, 28
DMC83006A	Clinical isolate, Me^r Vm^r (MIC = 1,024 μg/ml)	This study, 3, 28
879R4RF	Rf^r Fa^r	4
RN4220/pGO1	NARSA strain NRS106	29
SK5428	SK982/pSK41	11
Plasmids		
pAM830	Tra^+ Vm^r Em^r, 45 kb	This study
pAM831	Tra^+ Gm^r Sm^r Em^r Bac^+, 95 kb	This study
pAM829	Tra^+ Gm^r Km^r, 47 kb	This study
pAM373	Tra^+, responds to pheromone cAM373	4
pAD1	Tra^+, responds to pheromone cAD1	30
pCF10	Tra^+, responds to pheromone cCF10	8
pPD1	Tra^+, responds to pheromone cPD1	34
pAMβ1	Tra^+ Em^r	6
pIP501	Tra^+ Em^r Cm^r	21
pMG1	Tra^+ Gm^r	18
pGO1	Tra^+ Gm^r Km^r Tm^r	29
pSK41	Tra^+ Gm^r Km^r	12

[a] All clinical isolates are from the same patient. DMC83006B was isolated from a foot ulcer on 21 June 2002; WBH27862 was isolated from a catheter tip on 14 June 2002; DMC82991A was isolated from the nares on 21 June 2002; and DMC83006A was isolated from a foot ulcer on 21 June 2002.

[b] Cm, chloramphenicol; Em, erythromycin; Fa, fusidic acid; Gm, gentamicin; Km, kanamycin; Lv, levofloxacin; Me, methicillin; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Tm, trimethoprim; Vm, vancomycin; Bac, bacteriocin; Hly, Hemolysin; Tra, transfer; Tra^+, self transfer for pAM831 is unproven.

DMC83006B host, where it appears together with additional plasmid DNA (Fig. 1B, lane 3). It is noted that while the plasmid content of the two clinical VRE isolates is very similar, a few additional restriction fragments are present in the case of WBH27862. Sensitivity to vancomycin appeared spontaneously during growth of DMC83006B in the absence of drug; one of 25 colonies from nonselective medium represented a derivative that had lost the Vm^r trait and concomitantly lost the pAM830 plasmid. This strain, designated SF830Vs, remained resistant to gentamicin, erythromycin, and streptomycin and maintained a 95-kb plasmid (Fig. 1B, lane 4) that was subsequently designated pAM831. The latter DNA was found to transfer from SF830Vs to JH2-2 at a relatively low frequency (about 10^{-7}) when selection was for Gm^r or Em^r (Table 3). Considering the low transfer frequency, it remains unclear whether an unknown mobilizing factor in the donor aids in transfer of pAM831. Plasmid DNA from a transconjugant, SFG1, of such a mating is shown in Fig. 1B (lane 5) and is identical to plasmid DNA of eight additional Gm^r transconjugants examined, four from the DMC83006B donor and four from donor SF830Vs (data not shown). The data are consistent with the view that whereas pAM830 encodes Vm^r and Em^r, pAM831 encodes Gm^r, Em^r, and Sm^r. (There are Em^r determinants on both plasmids.) Bacteriocin activity was associated with pAM831, whereas an additional bacteriocin to which pAM831 does not provide immunity, hemolysin, and levofloxacin resistance traits of DMC83006B were not associated with either plasmid and thus are believed to be encoded on the host chromosome.

The *vanA* determinant of pAM830 is associated with a Tn*1546*-like transposon. Eight pairs of primers designed to generate PCR products that, taken together, overlap to span the entire sequence of the known VanA transposon Tn*1546* (1) produced data reflecting the expected size (10.8 kb) of this element on pAM830 (data not shown). Primers corresponding to regions close to the ends were used for sequencing outward to determine the presence of inverted repeats, the junctions,

TABLE 2. Antibiotic resistance levels for *E. faecalis* strains

Antibiotic	MIC[a] (μg/ml)					
	DMC83006B	WBH27862	SF830Vs	SFV1	SFG1	JH2-2
Vancomycin	>256	>256	2	>256	<2	1
LY33328	1	1	0.06	2	0.12	0.06
Gentamicin	>2,048	>2,048	>2,048	16	>2,048	16
Erythromycin	>512	>512	>512	>512	>512	<1
Streptomycin	>2,048	>2,048	>2,048	128	>2,048	128
Levofloxacin	>4	>4	>4	1	1	2
Ampicillin	0.5	0.5	0.5	0.5	0.5	2
Augmentin	<4	<4	<4	<4	<4	<4
Chloramphenicol	8	<4	8	8	8	8
Imipenem	<1	<1	<1	<1	<1	<1
Linezolid	2	1.5	1.5	1	1.5	2
Nitrofurazone	<32	<32	<32	<32	<32	<32
Rifampin	<1	<1	<1	>2	>2	>2
Tetracycline	<1	<1	<1	<1	<1	<1

[a] MICs were determined by broth microdilution using published guidelines (22) or by Etest (AB Biodisk).

and adjacent DNA. The ends of the element are identical to Tn*1546*, and the transposon is flanked by 5-bp direct repeats (TTCTT) presumed to reflect target site duplication. Blast analysis of the adjacent DNA revealed near-identity with sequences known to be present in the plasmids pAMβ1 and pIP501.

pAM830 is closely related to the Inc18 family of plasmids. pAM830 and pAM831 were used separately as probes in Southern analyses to determine their relationship to the following: (i) the pheromone-responding plasmids pAD1 and pAM373, (ii) the Inc18-type plasmids pAMβ1 and pIP501, and (iii) pMG1, representative of a group of conjugative plasmids commonly found to be associated with gentamicin and/or vancomycin resistance in *E. faecium* (18, 31). As shown in Fig. 2B, pAM830 exhibited strong homology with pIP501 (lane 4) and pAMβ1 (lane 5). In the case of pIP501, which was cleaved with *Hin*dIII (the other plasmids were cut with *Eco*RI), hybridiza-

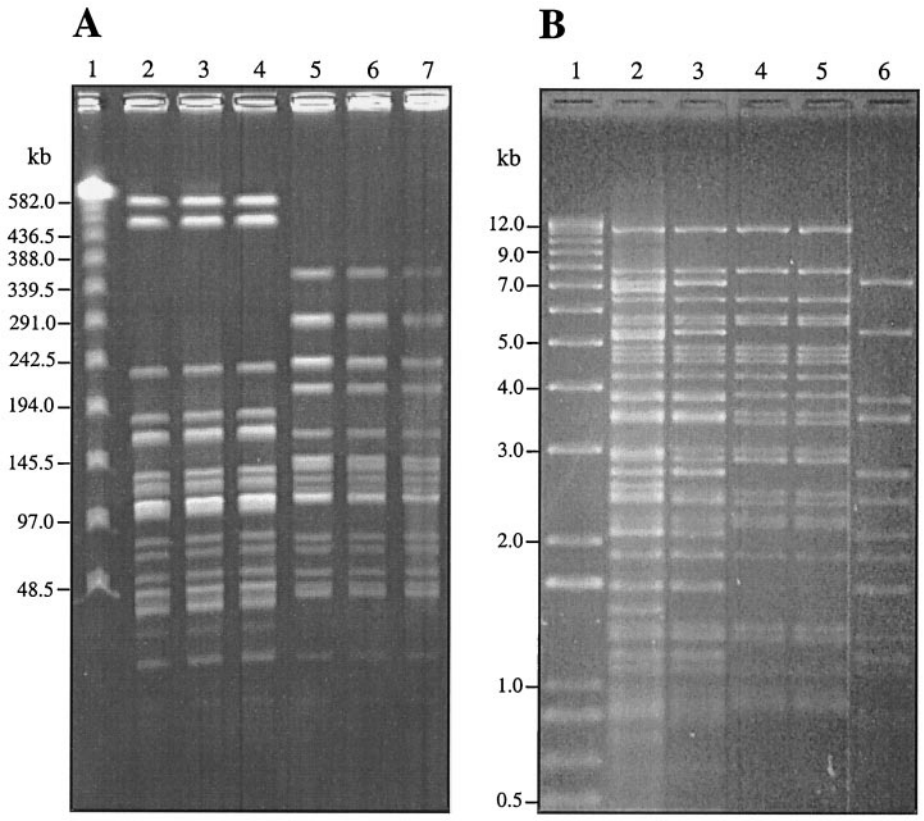

FIG. 1. Comparison of DNA from enterococcal strains. (A) Pulsed-field gel electrophoresis of genomic DNA digested with *Sma*I. (B) Plasmid DNA digested with *Hin*dIII. Lanes 1, molecular size marker; lanes 2, WBH27862; lanes 3, DMC83006B; lanes 4, SF830Vs; lanes 5, SFG1; lanes 6, SFV1; lane 7, JH2-2.

TABLE 3. Transfer of resistance

Donor	Recipient	Selection[a]	Frequency[b]	Appearance of nonselected markers in transconjugants[c]		
				% (No.) Vm^r	% (No.) Em^r	% (No.) Gm^r
E. faecalis DMC83006B	*E. faecalis* JH2-2	Vm	1.2×10^{-3}	*	100 (216/216)	0 (0/216)
		Em	1.3×10^{-3}	100 (250/250)	*	0 (0/250)
		Gm	4.0×10^{-7}	7 (7/106)	100 (106/106)	*
E. faecalis WBH27862	*E. faecalis* JH2-2	Vm	1.5×10^{-3}	*	100 (230/230)	0 (0/230)
		Em	1.8×10^{-3}	100 (234/234)	*	0 (0/234)
		Gm	8.6×10^{-7}	6 (3/52)	100 (52/52)	*
E. faecalis SF830Vs	*E. faecalis* JH2-2	Em	5.4×10^{-7}	NA	*	100 (52/52)
		Gm	3.3×10^{-7}	NA	100 (44/44)	*
E. faecalis SFV1	*E. faecalis* OG1X	Vm	2.1×10^{-4}	*	100 (217/217)	NA
		Em	3.6×10^{-4}	100 (261/261)	*	NA
E. faecalis SFG1	*E. faecalis* OG1SS	Gm	$<1.0 \times 10^{-8}$	NA	NA	*
E. faecalis DMC83006B	*E. faecium* BM4105-RF	Vm	1.0×10^{-2}	*	100 (164/164)	0 (0/164)
S. aureus DMC83006A	*E. faecalis* OG1RF	Vm	$<8.1 \times 10^{-8}$	*	NA	NA

[a] Selection for transconjugants involved antibiotics for the recipient markers, as well as one of the following as indicated: Vm, vancomycin (50 μg/ml); Em, erythromycin (10 μg/ml); Gm, gentamicin (100 μg/ml).
[b] Transconjugants per donor. Transfer frequency represents the mean average of three overnight filter matings, except in the case of the *S. aureus* mating for which transfer frequency is based on a four-hour filter mating.
[c] *, selected marker; NA, not applicable. Parenthetical values are number resistant/number total.

tion over the entire element is clearly evident. pAM831 did not exhibit such homology with the two Inc18 plasmids; only one to two bands were detected and with relatively low intensity. pAM830 also exhibited limited homology with pAM373 (one band; lane 3), and pAM831 exhibited limited homology with both pAD1 (one band; lane 2) and pAM373 (two bands; lane 3). pAM830 and pAM831 exhibited very limited homology to each other, and that which was observed may in part reflect that both carry Em^r determinants. Little, if any, homology with pMG1 was observed (lane 6).

The VRE strains do not exhibit a "pheromone" response to staphylococcal or enterococcal culture supernatants. Conjugative plasmids that confer a pheromone response in enterococci exhibit a characteristic clumping response that, using a microtiter dilution assay (10), can be used to quantitate the pheromone present in a given culture supernatant. Using this method, culture supernatants of the plasmid-free strains *E. faecalis* JH2-2 and *E. faecalis* OG1X did not generate a clumping response by the VRE isolates DMC83006B or WBH27862 or the transconjugant strain SFV1 carrying pAM830. These strains also did not respond to supernatants of the MRSA nasal isolate, although cAM373 activity (titer of 8) was detected, as well as activity (titer of 2) similar to that of cAD1 (10) produced by pSK41-type plasmids (11). The data indicate that a pheromone-responding plasmid is not present in the VRE strains.

Identification of a pSK41/pGO1-type plasmid in the *S. aureus* MRSA strain. Like the VRSA strain (J. M. Mohammed, L. Weigel, N. Clark, L. McDougal, P. Raney, A. Whitney, S.

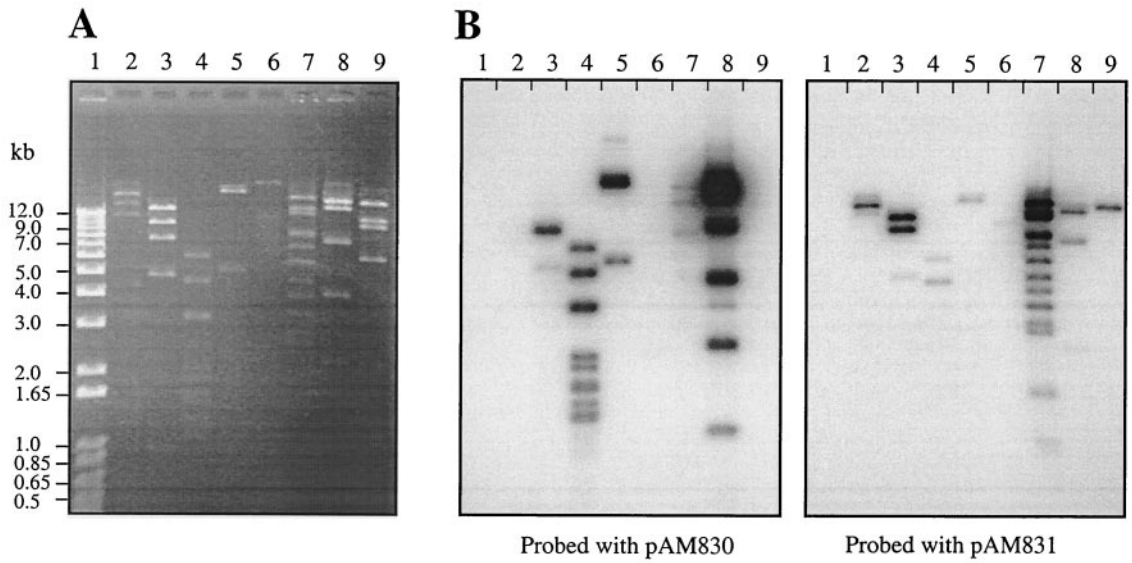

FIG. 2. Hybridization analysis of conjugative plasmids. An agarose gel with identically loaded halves, one of which is shown in panel A, was blotted to nitrocellulose, and separate halves (B) were probed with ^{32}P-labeled plasmids pAM830 and pAM831, as indicated. Lanes 1, molecular size marker; lanes 2, pAD1; lanes 3, pAM373; lanes 4, pIP501; lanes 5, pAMβ1; lanes 6, pMG1; lanes 7, pAM831; lanes 8, pAM830; lanes 9, pAM829. pIP501 was digested with *Hin*dIII; all others were digested with *Eco*RI.

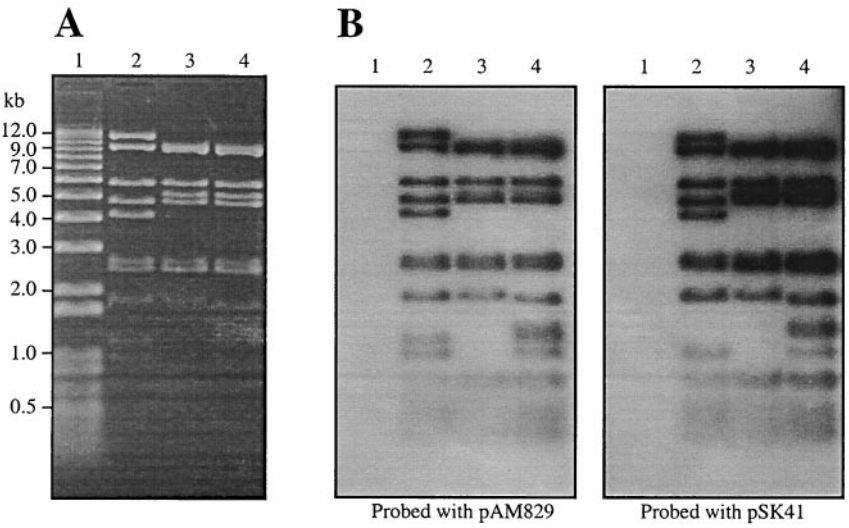

FIG. 3. Analysis of *S. aureus* plasmids. An agarose gel with identically loaded halves, one of which is shown in panel A, was blotted to nitrocellulose, and separate halves (B) were probed with the ^{32}P-labeled plasmids pAM829 and pSK41 as indicated. Lanes 1, molecular size marker; lanes 2, pAM829; lanes 3, pSK41; lanes 4, pGO1. All plasmids were digested with *Hin*dIII.

McAllister, M. Kellum, L. Jevitt, and F. C. Tenover, Abstr. Intersci. Conf. Antimicrob. Agents Chemother., abstr. LB-7, 2002), a vancomycin-sensitive nasal isolate of *S. aureus* (DMC82991A) exhibited the atypical characteristic of being weakly esculin positive. And in addition to being resistant to oxacillin, it was resistant to gentamicin (MIC, >100 μg/ml), kanamycin (MIC, >50 μg/ml), erythromycin (MIC, >10 μg/ml), and rifampin (MIC, >25 μg/ml). The strain harbors a 47-kb plasmid that has been designated pAM829 (Fig. 2A, lane 9). The VRSA strain contains a single plasmid that is essentially identical but with an additional segment carrying a *vanA* gene (F. Tenover and L. Weigel, personal communication). The pAM829 element showed limited homology when probed with pAM831, perhaps due to the presence of Gm^r genes on both, but no homology was detected with the VanA plasmid pAM830 (Fig. 2B, lane 9). When the strain was mated (overnight filter mating) with the *S. aureus* recipient 879R4RF with selection for transfer of the Gm^r trait, transconjugants were detected at a very low frequency (6.6×10^{-9}). Transconjugants were also kanamycin resistant but were sensitive to erythromycin.

pGO1 and pSK41 are members of a family of conjugative plasmids known to commonly carry Gm^r determinants (5, 12, 21). Therefore, the relationship of these plasmids to pAM829 was examined by performing a hybridization analysis using pSK41 and pAM829 as probes. As shown in Fig. 3B, strong homology between the plasmids is evident. Indeed, the agarose gel (Fig. 3A) showed a number of bands of common size for pAM829, pSK41, and pGO1. In addition, use of a pair of primers designed to amplify a 952-bp portion of the *traA* gene of pSK41 (2) resulted in generation of a 0.9-kb amplicon from pAM829 as well as from pSK41 and pGO1 (data not shown). The data are consistent with pAM829 being a member of the pSK41/pGO1 family of conjugative staphylococcal plasmids.

Conclusions. The Inc18-type conjugative element carrying the Tn*1546*-like transposon in the VRE strain is unrelated to the plasmid in the vancomycin-sensitive staphylococcal nasal isolate or, by inference, to the plasmid in the VRSA strain representing the same element but with additional DNA carrying *vanA*. The data do not conclusively show that the *vanA* determinant traveled on a plasmid from the VRE strain to the MRSA strain (e.g., on pAM830); however such an event can easily be envisioned to have occurred, followed by transposition of the resistance determinant to pAM829 and subsequent segregation of pAM830.

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