

# Methicillin-Susceptible, Vancomycin-Resistant *Staphylococcus aureus*, Brazil

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We report characterization of a methicillin-susceptible, vancomycin-resistant bloodstream isolate of *Staphylococcus aureus* recovered from a patient in Brazil. Emergence of vancomycin resistance in methicillin-susceptible *S. aureus* would indicate that this resistance trait might be poised to disseminate more rapidly among *S. aureus* and represents a major public health threat.

Acquisition of high-level vancomycin resistance by *Staphylococcus aureus* represents a major public health risk because this antimicrobial drug continues to be the first-line and most inexpensive therapy to treat methicillin-resistant *S. aureus* (MRSA) despite concerns about its clinical efficacy. Recently, we described vancomycin-resistant MRSA (VR-MRSA) recovered from the bloodstream of a patient in Brazil (1). VR-MRSA belongs to sequence type (ST) 8 and is phylogenetically related to the community-associated (CA) MRSA USA300 genetic lineage that has rapidly disseminated in the United States and the northern region of South America (USA300-Latin

American variant [USA300-LV]) (1,2). The *vanA* gene cluster in VR-MRSA was carried by a transferable staphylococcal plasmid (pBRZ01). We characterized a clinical isolate of vancomycin-resistant, methicillin-susceptible *S. aureus* (VR-MSSA) and document the in vivo transfer of the *vanA* gene cluster to 2 unrelated *S. aureus* strains causing bacteremia within the same patient.

## The Study

On August 28, 2012, a blood culture from a patient in Brazil was reported positive for 2 isolates of MSSA while the patient was receiving daptomycin therapy (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/10/14-1914-Techapp1.pdf>). One MSSA isolate was susceptible to all antimicrobial drugs tested (VS-MSSA). The second isolate (VR-MSSA) had a vancomycin MIC of 256 µg/mL and was also resistant to gentamicin (Table 1). Both isolates were susceptible to daptomycin (MIC 0.5 µg/mL). Thirteen days earlier, 2 MRSA isolates, 1 of which was resistant to vancomycin (VR-MRSA), were recovered from the blood of the same patient (online Technical Appendix) (1). The daptomycin MICs for both MRSA strains were also 0.5 µg/mL.

Bacterial strains used in this study (Table 1) were grown in brain–heart infusion broth and agar. Plasmid pBRZ01 was transferred by using filter mating (3) and VR-MSSA and VR-MRSA as donors and VS-MSSA, VS-MRSA, and RN4220RF as recipients (Table 1). Transconjugants were selected on brain heart infusion medium containing vancomycin (32 µg/mL) and fusidic acid (25 µg/mL). Colonies from each mating experiment were subjected to digestion with *SmaI* and pulsed-field gel electrophoresis to investigate genetic relatedness (1). Plasmids carrying the *vanA* gene cluster were detected by using S1 nuclease digestion followed by hybridization with a *vanA* probe (4).

Whole-genome sequencing of VR-MSSA, VS-MSSA, and 2 representatives of the Chilean/Cordobes clone (M1, M91) was performed by using MiSeq PacBio RS II (Illumina, San Diego, CA, USA) to close the VR-MSSA genome (5) (online Technical Appendix). Phylogenetic analysis was performed by using the maximum-likelihood framework within RAxML v7.4.2 (6). For cell wall analysis, extraction and separation of peptidoglycan precursors was performed as described (7).

The PFGE patterns of both isolates (VR-MSSA and VS-MSSA) were indistinguishable, and in vitro growth rates were similar (Figure 1, panel A). S1 nuclease analyses indicated that VR-MSSA harbored a plasmid of ~55

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**Table 1.** *Staphylococcus aureus* strains used in analysis of methicillin and vancomycin resistance, Brazil\*

Strain	Strain characteristics	MIC, µg/mL		Reference
		Vancomycin	Gentamicin	
VS-MRSA	Isolated from the bloodstream of a patient in Brazil	0.5	0.5	(1)
VR-MRSA	Isolated from the blood of the same patient above and carrying <i>vanA</i> -containing pBRZ01	>256	32	(1)
VS-MSSA	Isolated from the blood of the same patient 13 d after isolation of VR-MRSA	1	0.75	This study
VR-MSSA	Isolated from the same blood culture as VS-MSSA	256	48	This study
RN4220-RF	Laboratory strain of <i>S. aureus</i> used as recipient for mating experiments; fusidic acid and rifampin-resistant	1	1	(1)
Transconjugant 1†	Transconjugant obtained from a mating experiment using VR-MSSA as donor and VS-MRSA as recipient	>256	48	This study
Transconjugant 2†	Transconjugant obtained from a mating experiment using VR-MRSA as donor and VS-MSSA as recipient	>256	64	This study
Transconjugant 3†	Transconjugant obtained from a mating experiment using VR-MSSA as donor and RN4220-RF as recipient	>256	64	This study

\*VS-MRSA, vancomycin-susceptible, methicillin-resistant *S. aureus*; VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA, vancomycin-resistant, methicillin-susceptible *S. aureus*.

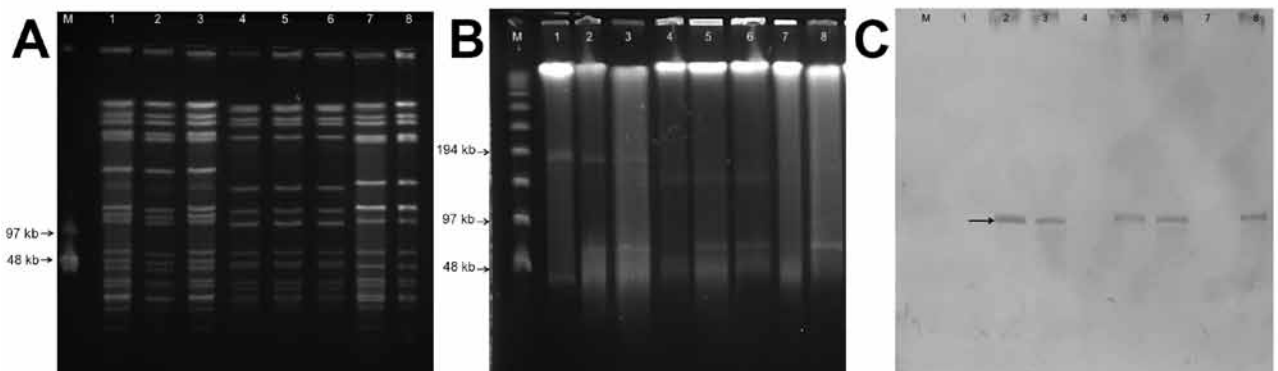
†A fusidic acid-resistant derivative was generated for mating experiments. All mating experiments were performed on brain heart infusion agar in the presence of vancomycin (32 µg/mL) and fusidic acid (25 µg/mL) to select for transconjugants.

kb, which yielded a positive result when hybridized with a *vanA* probe (Figure 1, panels B, C) and was similar in size to the previously described *vanA*-containing plasmid pBRZ01 identified in the same patient (1). pBRZ01 of VR-MSSA was readily transferred to *S. aureus* RN4220-RF (efficiency =  $3 \times 10^{-5}$ /donor). In vitro conjugative transfer of pBRZ01 between MRSA and MSSA strains recovered from the patient's bloodstream was also readily achieved with efficiencies ranging from  $4.3 \times 10^{-7}$ /donor to  $2.5 \times 10^{-6}$ /donor. Acquisition of the pBRZ01 by corresponding strains resulted in resistance to vancomycin and gentamicin (Table 1).

Genome sequencing (online Technical Appendix) showed that VR-MSSA and VS-MSSA belong to clonal

complex (CC) 5 (sequence type ST5) and harbor staphylococcal protein A (Spa) type t002. VS-MSSA and VR-MSSA have the characteristic CC5 genetic traits described by Kos et al. (8). The genome of VR-MSSA has a 2,906,602-bp chromosome and 3 extrachromosomal elements, including a plasmid of 55,713 bp identical to the previously described *vanA*-carrying pBRZ01 (1), which also harbors *aac(6')-aph(2'')*, which confers gentamicin resistance.

Comparison of the core genomes of VR-MSSA and VS-MSSA showed only 20 single-nucleotide polymorphism differences, which suggested a close genetic relationship and probably representing the same organism that acquired pBRZ01. Phylogenetic analysis (Figure 2) confirmed that VR-MSSA is not a derivative of VR-MRSA (1)



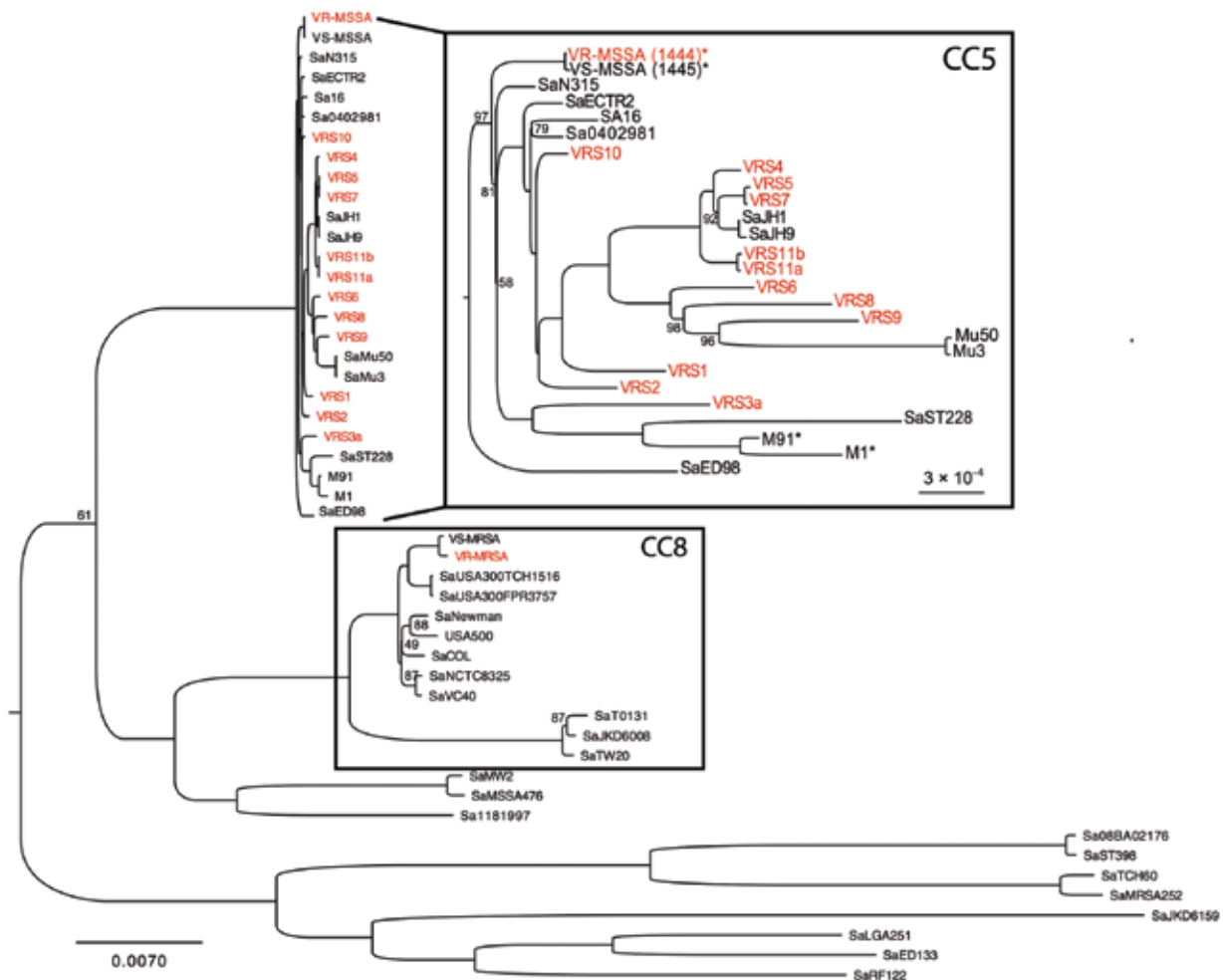
**Figure 1.** Molecular typing of *Staphylococcus aureus* strains, Brazil. A) *Sma*I digestion of total DNA, followed by pulsed-field gel electrophoresis. Lane M, lambda ladder (molecular masses are indicated in kilobases on the left); lane 1, vancomycin-susceptible, methicillin-resistant *S. aureus* (VS-MRSA) isolated from the blood of a Brazilian patient (1); lane 2, vancomycin-resistant MRSA (VR-MRSA) isolated from the same patient and blood culture (1); lane 3, transconjugant 1 obtained from a mating experiment using vancomycin-resistant MSSA (VR-MSSA) as donor and VS-MRSA as recipient; lane 4, vancomycin-susceptible MSSA (VS-MSSA) isolated from the blood of the same patient 13 days after isolation of VR-MRSA; lane 5, VR-MSSA isolated at the same time as VS-MSSA; lane 6, transconjugant 2 obtained from a mating experiment using VR-MRSA as donor and VS-MSSA as recipient; lane 7, *S. aureus* RN4220 RF, lane 8, transconjugant 3 obtained using VR-MSSA as donor and RN4220 RF as recipient. B) S1 digestion of total DNA using the same strains shown in panel A. C) Hybridization with *vanA* probe using the same strains shown in panel A. Arrow indicates a positive signal for the *vanA* gene.

(isolated days before from the same patient) and emphasized the relationship of this strain to other vancomycin-resistant *S. aureus* and MRSA isolates with intermediate susceptibility to vancomycin (VISA).

We analyzed the pool of cytoplasmic peptidoglycan precursors of VR-MSSA grown in the absence or presence of 50 µg/mL of vancomycin for induction of the *vanA* cluster (Table 2). Tandem mass spectrometry analysis identified 3 nucleotide precursors ending in D-alanyl-D-alanine (UDP-MurNAc-pentapeptide), D-alanyl-D-lactate (UDP-MurNAc-pentadepsipeptide), and D-Ala (UDP-MurNAc-tetrapeptide). Upon induction with vancomycin, UDP-MurNAc-pentapeptide was not detected, and UDP-MurNAc-pentadepsipeptide accounted for most of the

precursors (Table 2). These results indicate that the *van*-encoded enzymes required for incorporation of D-Lac into the precursors were fully functional in VR-MSSA. Our results also show that the *vanA* cluster was inducible by vancomycin in the *S. aureus* host because only a small proportion of the precursors (4%) ended in D-Lac in the absence of the drug.

Analyses of cell wall mucopeptides from VR-MSSA showed 2 modifications of the L-Ala<sup>1</sup>-γ-D-Glu<sup>2</sup>-L-Lys<sup>3</sup>-D-Ala<sup>4</sup>-D-Ala<sup>5</sup> stem peptide that are highly conserved in *S. aureus* strains, namely the amidation of the α-carboxyl of D-Glu<sup>2</sup> to form D-iGln<sup>2</sup> and the addition of a pentaglycine side chain on the ε-amino group of L-Lys<sup>3</sup> by the Fem amino-acyltransferases (9). Induction of the *vanA* gene cluster



**Figure 2.** Phylogenetic analyses of *Staphylococcus aureus* strains, Brazil. Whole-genome phylogenetic tree (dataset = 325,732 single-nucleotide polymorphisms, gamma-based log likelihood – 1909607.06950) of the *S. aureus* species showing position of vancomycin-resistant, methicillin-susceptible *S. aureus* (VR-MSSA) and vancomycin-susceptible MSSA (VS-MSSA) isolates sequenced for this study. Vancomycin-resistant *S. aureus* (VRSA) strains are shown in red. Numbers on branches are bootstrap values based on 1,000 resampling iterations. All branches without numbers had bootstrap values of 100%. Branch lengths are proportional to number of nucleotide substitutions per site (scale bars). Inset labeled CC5 is expanded to emphasize the polyphyly of VRSA strains. \*Genomes sequenced for this study. M1 and M91 are members of the Chilean/Cordobes clone that is widespread in Latin America (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/10/14-1914-Techapp1.pdf>). CC, clonal complex.

**Table 2.** Relative abundance of peptidoglycan precursors in *Staphylococcus aureus* strains, Brazil\*

Precursor	Monoisotopic mass		Abundance (%) in corresponding strains		
	Observed	Calculated	VS-MSSA	VR-MSSA (not induced)	VR-MSSA (induced)
UDP-MurNaAc-tetrapeptide	1,078.35	1,078.35	27	32	29
UDP-MurNAC-pentapeptide	1,149.37	1,149.39	73	64	ND
UDP-MurNAC-pentadepsipeptide	1,150.37	1,150.37	ND	4	71

\*Bacteria were grown on brain heart infusion (BHI) broth (not induced) or BHI supplemented with vancomycin (50 µg/mL). VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA, vancomycin-resistant, methicillin-susceptible *S. aureus*; ND, not detected.

led to 2 major modifications. First, stem peptides ended in D-Ala<sup>4</sup>, indicating that the peptidyl- D-Ala<sup>4</sup>- D-Ala<sup>5</sup> target of vancomycin, and D-Ala<sup>4</sup>-D-Lac<sup>5</sup> termini, were fully eliminated. Second, the pentaglycine side chain was frequently missing (online Technical Appendix), indicating that replacement of D-Ala by D-Lac at the extremity of peptidoglycan precursors might have impaired the ability of Fem transferases to add Gly on L-Lys<sup>3</sup>.

## Conclusions

In this study, we demonstrated that the *vanA*-containing pBRZ01 plasmid previously described in MRSA was acquired by an invasive MSSA isolate within the same patient. Our findings also suggest that a *vanA*-containing plasmid (pBRZ01) was horizontally acquired at least twice during a short period by distinct *S. aureus* lineages within the same host (MRSA belonging to ST8 and an ST5 MSSA). VR-MSSA belongs to the ST5 lineage of CC5, a major hospital-associated lineage (10). The prevalent hospital-associated lineages circulating in Brazil are ST5 (New York/Japan and Pediatric clones), ST239 (Brazilian clone) and ST1 (USA400 clone) (11), and recent epidemiologic data showed replacement of the endemic Brazilian (ST239) clone by ST5 strains (11–13). Moreover, VR-MSSA is related to ST5 vancomycin-resistant *S. aureus* strains recovered in the United States (8) and to VISA isolates, including Mu50 and the hetero-VISA strain Mu3, initially recovered in Japan (14). It remains unclear why CC5 strains appear more likely to exhibit vancomycin resistance.

Our biochemical analysis indicates that the *vanA* gene cluster is fully functional in VR-MSSA, which leads to vancomycin-inducible production of D-Lac ending precursors and elimination of D-Ala- D-Ala containing peptidoglycan, as found in the enterococci (15). Our results also revealed a defect in side chain synthesis, although this did not prevent the synthesis of a functional and highly cross-linked peptidoglycan in VR-MSSA.

In summary, we report the *in vivo* acquisition of high-level vancomycin resistance in a bloodstream MSSA isolate. Of note, *vanA*-containing pBRZ01 was maintained even after the selective pressure of vancomycin had been removed, raising serious concerns about the possibility of further spread of resistance to this agent. However, no other MSSA strains containing this plasmid have been isolated so far in Brazil.

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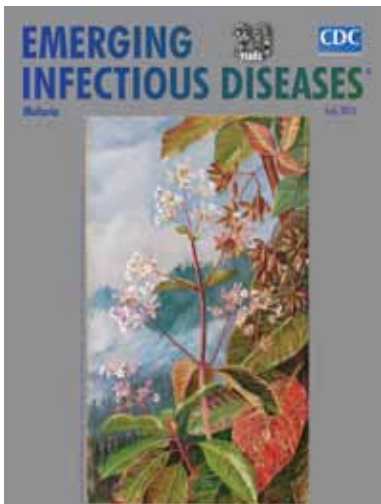
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## July 2015: Malaria



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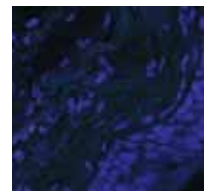
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# Methicillin-Susceptible, Vancomycin-Resistant *Staphylococcus aureus*, Brazil

## Technical Appendix

### Case-Patient Summary

The patient was a 35-year-old man with mycosis fungoides, cocaine addiction, diabetes mellitus, and a history of repetitive skin and soft tissue infections. He was first hospitalized and treated for leg cellulitis in November 2011 and readmitted for recurrent skin and soft tissue infections and worsening concurrent conditions in June 2012. During his hospitalization, repetitive febrile episodes developed, and he had blood cultures positive for different *Staphylococcus aureus* isolates. The clinical course of the patient, *Staphylococcus aureus* isolates, and antimicrobial drugs provided are summarized in Technical Appendix Figure 1. Further details can be found in a prior publication by Rossi et al. (1).

### Genome Sequencing

MiSeq assembly was performed by using ABySS (2), and PacBio assembly was performed by using the HGAP2 v2.1 de novo assembly pipeline (Pacific Biosciences, Menlo Park, CA, USA). Comparison of single-nucleotide polymorphisms (SNPs) between genomes used in this study was performed by using the short read alignment to the *S. aureus* genome for strain N315 as a reference and the Burrows-Wheeler Alignment tool (<http://bio-bwa.sourceforge.net>). SNP calls were detected by using samtools (<http://samtools.sourceforge.net>), and SNPs were identified as high quality if they were unambiguous and had a q score  $\geq 20$ . For preassembled genomes available from public databases, we used whole-genome alignment with reference to the N315 genome by using the show-snps utility of NUCmer (<http://mummer.sourceforge.net>). We created phylogenetic datasets by combining results of both SNP calling techniques above. We excluded potentially repeated regions from the reference genome that had  $>80\%$  nucleotide similarity over 100 bp on the basis of BLAST

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the genome against itself. All locations in the genome annotated as mobile genetic elements were also excluded.

### **Phylogenetic Methods**

Maximum-likelihood phylogenies were constructed by using the POSIX-threads version of RAxML v8.0.19 (3). For SNP data, we used an ascertainment bias correction and a general time-reversible substitution model accounting for among-site rate heterogeneity by using the gamma distribution and 4 rate categories (ASC\_GTRGAMMA model) for 100 individual searches with maximum parsimony random-addition starting trees. Node support was evaluated with 1,000 nonparametric bootstrap pseudoreplicates and filtering the optimal maximum-likelihood tree through the bootstrap trees so that node support values shown indicate the percentage proportion of bootstrap trees that contained a given internode branch.

### **Peptidoglycan Precursor and Cell Wall Analyses**

Extraction of peptidoglycan precursors was performed as described (4). Separation of precursors by reversed-phase, high-performance liquid chromatography was conducted by using a C18 column (Nucleosil 4.6 × 250 mm; Macherey-Nagel, Hoerd, France). Peaks were collected and precursors were identified by mass spectrometry (Qstar Pulsar I; Applied Biosystems, Courtaboeuf, France) (4). The peptide moiety of the precursors was sequenced by tandem mass spectrometry (4). Relative abundance of precursors was estimated by the percentage of the integrate peak area at 262 nm. Peptidoglycan was prepared as described (5), and covalently attached proteins were removed from peptidoglycan by digestion with pronase and trypsin. Muropeptides were obtained by digestion with lysozyme and mutanolysin. The ether bond internal to N-acetylmuramic acid was cleaved with 3% ammonia, and the resulting lactoyl peptides were separated by reversed-phase, high-performance liquid chromatography for sequencing by tandem mass spectrometry (Qstar Pulsar I).

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<http://dx.doi.org/10.1074/jbc.M407149200>

**Technical Appendix Table 1.** Genome statistics for *Staphylococcus aureus*, Brazil\*

Strain	Coverage	No. contigs	Mean subread length, bp	Read length N50/assembly N50	NCBI Bioproject no.
VR-MSSA (HP022)	800x	1,437	NA	NA/189054 bp	PRJNA262896
VS-MSSA (HP023)	575x	1,438	NA	NA/91,499 bp	PRJNA262928
M1 (HP012)	250x	1,813	NA	NA/8,727 bp	PRJNA262670
M91 (HP013)	85x	1,808	NA	NA/46,912 bp	PRJNA262672
VR-MSSA (PacBio)	81.1x	9†	4,955	6,305 bp/ 2.04 Mbp	PRJNA262896

\*NCBI, National Center for Biotechnology Information; VR-MSSA vancomycin-resistant, methicillin-susceptible *S. aureus*; NA, not applicable; VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*.

†Manual polishing and additional assembly resulted in 4 contigs (1 closed circular chromosome and 3 extrachromosomal elements).

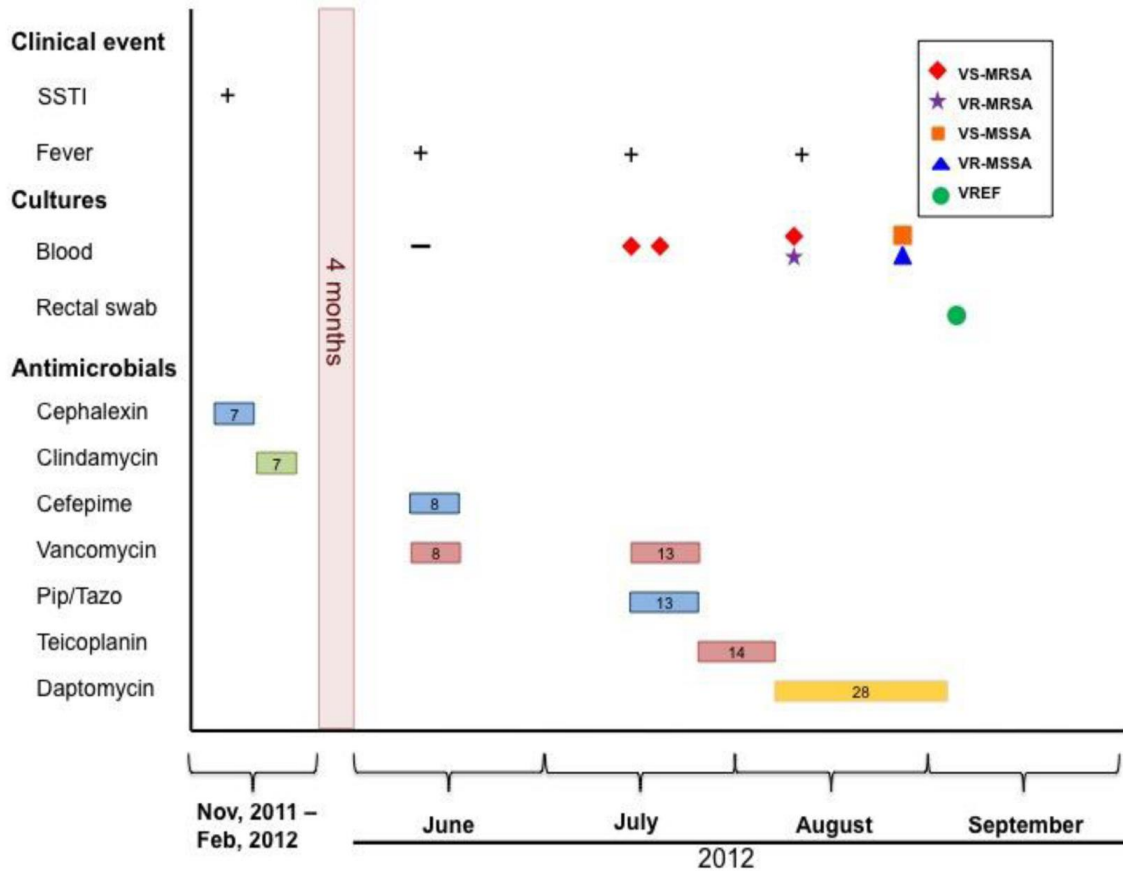
**Technical Appendix Table 2.** Mass of mucopeptide from vancomycin-susceptible and vancomycin-resistant, methicillin-susceptible *Staphylococcus aureus*, Brazil\*

Strain (growth condition)	R substituent of mucopeptide		Value	Monoisotopic mass of mucopeptide, atomic mass units					
	R1	R2		Monomer, n = 0	Dimer, n = 1	Trimer, n = 1	Tetramer, n = 2	Pentamer, n = 3	Hexamer, n = 5
VS-MSSA	D-Ala- D-Ala	Gly <sub>5</sub>	Calculated	844.40	1,599.76	2,355.12	3,110.47	3,865.83	4,621.18
			Observed	844.42	1,599.79	2,355.18	3,110.55	3,865.92	4,621.38
VR-MSSA (induced)†	D-Ala	H	Calculated	488.26	1,243.62	1,998.97	2,754.33	3,509.68	4,265.04
			Observed	488.25	1,243.6	1,998.97	2,754.34	3,509.71	4,265.08
	D-Ala	Gly <sub>5</sub>	Calculated	773.37	1,528.72	2,284.08	3,039.44	3,794.79	4,550.14
			Observed	773.38	1,528.73	2,284.08	3,039.46	3,794.83	4,550.17

\*VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA vancomycin-resistant, methicillin-susceptible *S. aureus*.

†Induction was performed with 10 µg/mL of vancomycin.

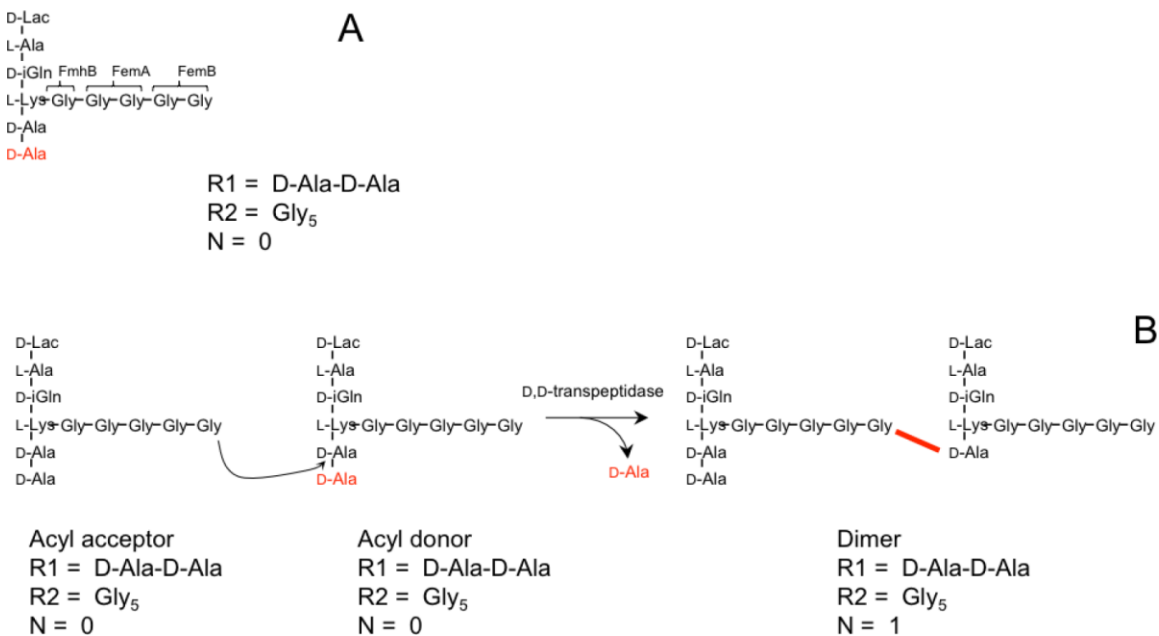




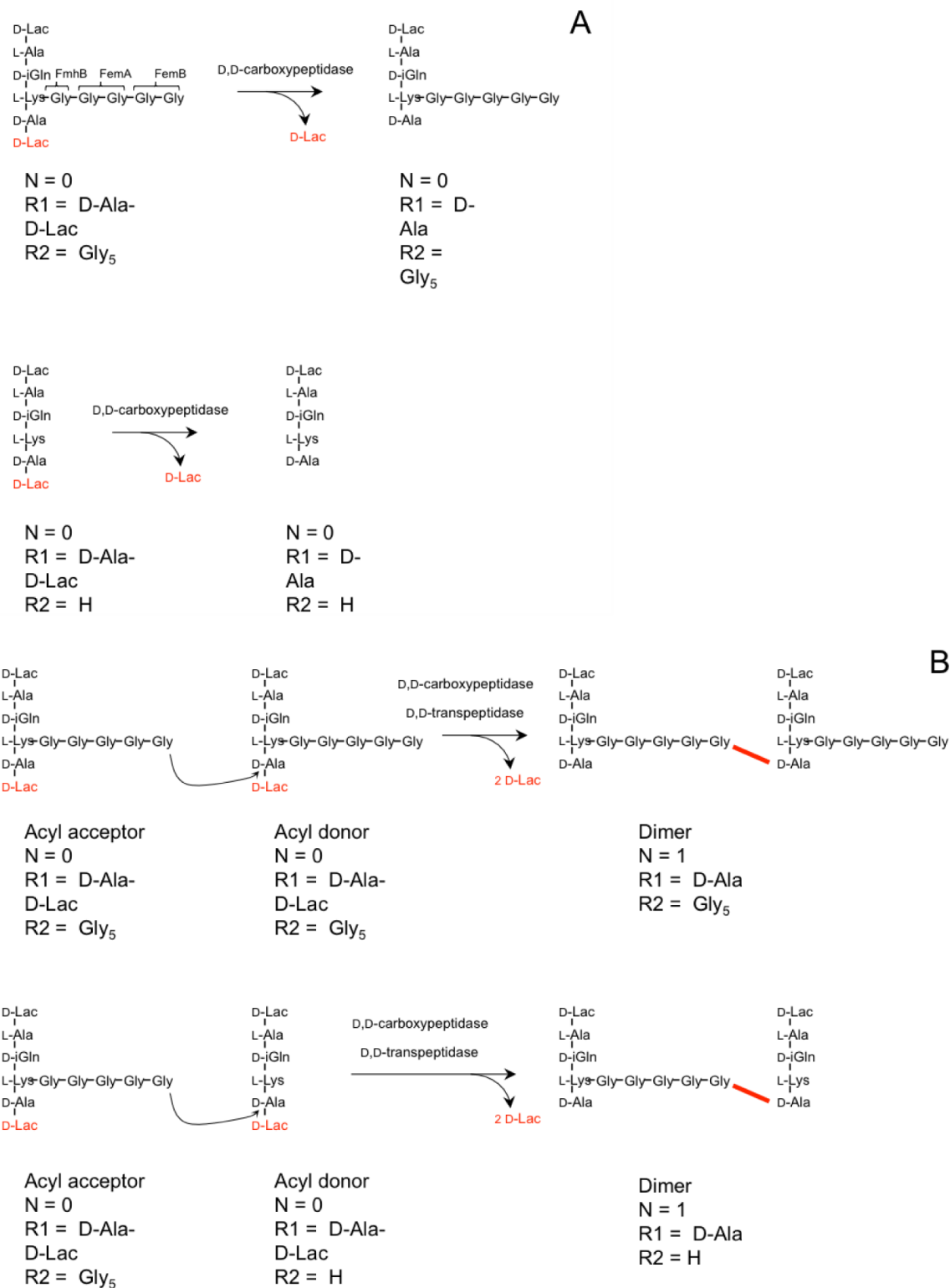
**Technical Appendix Figure 1.** Clinical course timeline of the patient, Brazil. Drugs used are indicated by colored rectangles:  $\beta$ -lactams in blue (cephalexin, cefepime, and piperacillin/tazobactam [Pip/Tazo]), clindamycin in green, glycopeptides in pink (vancomycin and teicoplanin), and daptomycin in yellow. The number in each rectangle corresponds to the number of days of treatment with the drug. Drugs are shown in the order in which they were added to therapy. The final days of hospitalization are not included. SSTI, skin and soft-tissue infection; VS-MRSA, vancomycin-susceptible, methicillin-resistant *Staphylococcus aureus*; VR-MRSA, vancomycin-resistant, methicillin-resistant *S. aureus*; VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA vancomycin-resistant, methicillin-susceptible *S. aureus*; VREF, vancomycin-resistant *Enterococcus faecalis*.



**Technical Appendix Figure 2.** Diversity in the structure of mucopeptides from *Staphylococcus aureus*, Brazil. Diversity of mucopeptides is generated by variations at the C-terminus ( $R_1 = \text{OH}$  or  $\text{D-Ala}^4\text{-D-Ala}^5$ ), at the N terminus ( $R_2 = \text{H}$  or  $\text{D-Gly}_5$ ) and by the extent of oligomerization (from  $N = 0$  for monomers to  $N = 6$  for heptamers).



**Technical Appendix Figure 3.** Mucopeptides from vancomycin-susceptible, methicillin-susceptible *Staphylococcus aureus*, Brazil. A) Main monomers. The side-chain is assembled by aminoacyl transferases of the Fem family that sequentially add the first (FmhB), second, and third (FemA), and fourth and fifth (FemB) Gly residues. B) Dimer generated by D,D-transpeptidation. The D,D-transpeptidases cleave the  $\text{D-Ala}^4\text{-D-Ala}^5$  peptide bond of the acyl donor and link the carbonyl of  $\text{D-Ala}^4$  to amino group located at the extremity of the side chain of the acyl acceptor.



**Technical Appendix Figure 4.** Mucopeptides from vancomycin-resistant, methicillin-susceptible *Staphylococcus aureus* grown in the presence of 10 µg/mL vancomycin, Brazil. A) Main monomers. The C-terminal D-Lac is cleaved by D,D-carboxypeptidase and is not found in mature

peptidoglycan. Most (62%) of the mucopeptide monomers did not contain any side-chain ( $R_2 = H$  instead of  $Gly_5$ ) because of impaired activity of FmhB with D-Lac ending precursors. B) Dimer generated by D,D-transpeptidation. All cross-links contain  $Gly_5$  because unsubstituted stem peptides ( $R_2 = H$ ) are not used as acyl acceptors by D,D-transpeptidases.

### Tree

#NEXUS

begin taxa;

dimensions ntax = 50;

taxlabels

VRS3a

HP022

HP023

SaED98

SaMSHR1132

Sa08BA02176

SaST398

SaTCH60

SaMRSA252

SaJKD6159

SaLGA251\

SaED133

SaRF122

SaMW2

SaMSSA476

Sa1181997

SaT0131  
SaJKD6008  
SaTW20  
VSSA  
VRSa  
SaUSA300TCH1516  
SaUSA300FPR3757  
SaNewman  
USA500  
SaCOL  
SaNCTC8325  
SaVC40  
SaN315  
SaECTR2  
Sa16  
Sa0402981  
VRS10  
VRS4  
VRS5  
VRS7  
SaJH1  
SaJH9  
VRS11b  
VRS11a

VRS6

VRS8

VRS9

SaMu50

SaMu3

VRS1

VRS2

SaST228

HP013

HP012

;

end;

begin trees;

```
tree tree_1 = [&R] (((VRS3a:8.244799231807832E-4,(((HP022:3.96736443382444E-6,HP023:2.250523383219172E-5):3.411632724734676E-4,(SaED98:9.703221642786964E-4,((SaMSHR1132:0.5632346423186245,((Sa08BA02176:5.105885699270055E-4,SaST398:5.538524020539065E-4):0.023187580060007828,(SaTCH60:0.0018606904107206922,SaMRSA252:0.0023361910795642177):0.0228694576846738):0.0208677517984276,(SaJKD6159:0.043048096207328516,((SaLGA251:0.012751949141110169,SaED133:0.014657491012749579):0.007702308459562556,SaRF122:0.020706149045281765):0.005715274275517768):0.005352806350899746):0.014270643684360873):0.004215184179548835,(((SaMW2:8.148223984244278E-4,SaMSSA476:9.399261875994422E-4):0.011723988543628189,Sa1181997:0.012031146247175885):0.003472808070423307,(((SaT0131:0.0011290280300672703,SaJKD6008:4.616415737154414E-4):2.4208588870377014E-4,SaTW20:5.876500448633564E-
```

4):0.011876794547025669,(((VSSA:2.3017304230714717E-  
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4):0.0017108374225468953,(SaUSA300TCH1516:5.690969067851791E-  
5,SaUSA300FPR3757:7.414423176218751E-  
5):0.0012672330396069006):5.448881866674404E-  
4,(((SaNewman:9.578627969952902E-  
4,USA500:0.0015003244427622417):4.515479337227684E-  
4,SaCOL:0.001221232134307728):3.865704697744097E-  
5,(SaNCTC8325:3.3883278053041993E-4,SaVC40:3.1202602748559656E-  
4):8.030801728358954E-4):1.5673146668809766E-  
4):0.002729247117285292):0.009758518105999302):0.004378187341032908):0.011153  
156773090636):1.0540166871535802E-4):2.024831647401745E-  
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6,(SaECTR2:1.7884162073543815E-4,((Sa16:3.063593398554345E-  
4,Sa0402981:1.4522808953175689E-4):5.25613854399233E-  
6,(VRS10:1.463713478899369E-4,((((VRS4:1.2530559741665855E-  
4,((VRS5:2.535180723808072E-5,VRS7:1.038618732320479E-  
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5):6.151791798841402E-5,(VRS11b:1.720642232484988E-  
5,VRS11a:1.323680283108371E-5):1.7233451943842544E-4):4.229759968813017E-  
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4,HP013:8.712211399490469E-5,HP012:4.732197689535684E-4);

end;

**Matrix**

Large file, available from the authors.