Fitness Cost of VanA-Type Vancomycin Resistance in Methicillin-Resistant \textit{Staphylococcus aureus}\textsuperscript{\dag}

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We have quantified the biological cost of VanA-type glycopeptide resistance due to the acquisition of the resistance operon by methicillin-resistant \textit{Staphylococcus aureus} (MRSA) from \textit{Enterococcus} sp. Exponential growths of recipient strain HIP11713, its transconjugant VRSA-1, VRSA-5, and VRSA-6 were compared in the absence or, except for HIP11713, in the presence of vancomycin. Induction of resistance was performed by adding vancomycin in both the preculture and the culture or the culture at only 1/50 the MIC. In the absence of vancomycin, the growth rates of the vancomycin-resistant \textit{S. aureus} (VRSA) strains were similar to that of susceptible MRSA strain HIP11713. When resistance was induced, and under both conditions, there was a significant reduction of the growth rate of the VRSA strains relative to that of HIP11713 and to those of their noninduced counterparts, corresponding to a ca. 20% to 38% reduction in fitness. Competition experiments between isogenic VRSA-1 and HIP11713 mixed at a 1:1, 1:100, or 100:1 ratio revealed a competitive disadvantage of 0.4% to 3% per 10 generations of the transconjugant versus the recipient. This slight fitness burden can be attributed to the basal level of expression of the \textit{van} genes in the absence of induction combined with a gene dosage effect due to the presence of the \textit{van} operon on multicyclic plasmids. These data indicate that VanA-type resistance, when induced, is highly costly for the MRSA host, whereas in the absence of induction, its biological cost is minimal. Thus, the potential for the dissemination of VRSA clinical isolates should not be underestimated.

\textit{Staphylococcus aureus} is one of the most common causes of hospital- and community-acquired infections, and treatment of staphylococcal diseases is complicated by the organism’s innate ability to become resistant to chemotherapy (15). Vancomycin is the drug of choice to treat infections due to methicillin-resistant \textit{S. aureus} (MRSA), but an increase in vancomycin use has led to the emergence of two types of glycopeptide-resistant \textit{S. aureus} strains. The first one, designated glycopeptide-intermediate-resistant \textit{S. aureus} (GISA), is associated with a thickened and poorly cross-linked cell wall, resulting in an accumulation of \textalpha{-}alanyl-\textalpha{-}alanine (\textalpha{-}Ala-\textalpha{-}Ala) targets in the periphery that sequester glycopeptides (9). The second type, designated vancomycin-resistant \textit{S. aureus} (VRSA), is due to the acquisition of the \textit{vanA} operon carried by transposon Tn1546 from \textit{Enterococcus} sp., resulting in high-level resistance (4, 5). VanA-type resistance results in the synthesis of a new cell wall utilizing precursors ending in \textalpha{-}alanyl-\textgamma{-}lactate (\textalpha{-}Ala-\textgamma{-}Lac) that have 1,000-fold less affinity for glycopeptides associated with the elimination of the susceptible \textalpha{-}Ala-\textgamma{-}Ala-containing precursors to which vancomycin binds (8). The expression of resistance is regulated by a two-component system (VanS-VanR) that allows the inducible expression of the \textit{vanA} operon in response to the presence of glycopeptides, vancomycin, or teicoplanin in the culture medium (3, 10). Since 2002, nine MRSA strains that were highly resistant to glycopeptides that harbor the \textit{vanA} gene cluster on two types of plasmids have been reported in the United States. The first group is exemplified by transconjugant strain VRSA-1, which was isolated together with susceptible recipient strain HIP11713 and vancomycin-resistant \textit{Enterococcus faecalis} donor strain DMC83006B from the foot ulcer of a diabetic patient (23, 24). The \textit{vanA} operon was acquired in two steps: first, plasmid pAM830, carrying Tn1546, was transferred by conjugation from \textit{E. faecalis} DMC83006B (12) to MRSA strain HIP11713, and Tn1546 was then transposed on resident plasmid pAM829, generating plW1043 (pAM829::Tn1546) (20, 24). Strains VRSA-5 and VRSA-6, which belong to the second group, acquired and subsequently stably maintained an Inc18-like enterococcal plasmid carrying Tn1546 (26). We have studied these three clinical isolates that are representative of the two VRSA classes.

Antibiotic resistance, by acquisition of a mobile genetic element or by mutation, is often associated with a reduced fitness of the bacterial host (1). More-fit variants can be selected during further evolution after either a loss of resistance or the occurrence of a compensatory mutation that restores bacterial fitness (16). The biological cost is one of the major indirect factors that determines the stability and dissemination of antibiotic resistance. Study of the fitnesses of MRSA and GISA strains revealed a decrease in the growth rates of the two types of strains (17). Worldwide dissemination of MRSA clones has been associated with their ability to compensate for the cost of harboring the staphylococcal chromosomal cassette \textit{mec} element (11). In certain GISA isolates, the deletion of the \textit{mec} gene can partially compensate for the fitness cost imposed by vancomycin resistance, suggesting that the simultaneous resistance to \textbeta{-}lactams and glycopeptides is highly costly for \textit{S. aureus} (17). High-level vancomycin resistance is associated

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with a sophisticated dual biochemical mechanism mediated by seven genes, vanR/SHAXYZ, which combines the synthesis of modified late peptidoglycan precursors with the elimination of the chromosomal pathway for the synthesis of the susceptible cell wall. The biological cost resulting from this combinatorial mechanism of resistance to glycopeptides on the host is predicted to be high, which is consistent with the fact that only a few strains of VRSA have been isolated. Considering the major public health problem that would result from VRSA dissemination, we have evaluated the fitnesses of clinical isolates VRSA-1, VRSA-5, and VRSA-6.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The S. aureus strains used in this study were obtained through the Network on Antimicrobial Resistance in Staphylococcus aureus. MICs were determined by dilution in Mueller-Hinton broth (Difco Laboratories, Detroit, MI). The strains were grown in brain heart infusion (BHI) broth or on agar at 37°C.

Growth rate. Growth rates were determined in microplates coupled to an eMS reader (Labsystems, Cergy Pontoise, France) (13). Each strain was grown overnight at 37°C with 1/50 the MIC of vancomycin (Merck & Cie, Lyon, France) or without vancomycin. The cultures were diluted 1:20 into 20 ml of BHI broth with or without vancomycin and grown at 37°C with shaking. At the beginning of the stationary phase, the cultures were diluted 1:1,000 in order to inoculate approximately 10^6 bacteria into 200 μl of BHI broth in a 96-well microplate (Greiner Bio-one, Courtaboeuf, France), which was then incubated at 37°C with regular shaking. Absorbance was measured at 600 nm every 3 min. Each strain was assayed in four independent cultures in two separate experiments. Growth rates were determined mathematically by regressing the natural logarithm of population density (N) against time (t) using only those time points over which population density increased exponentially (between optical density values at 600 nm of 0.1 and 0.2). μ = [ln(Nt) - ln(N0)]/(t - t0). Relative growth rates were calculated as the ratio of the growth rate of resistant transconjugant strain VRSA-1 versus recipient strain HIP11713 or of the induced versus the noninduced resistant strains.

Growth competition experiments. Fitness was also determined in competition experiments between recipient HIP11713 and transconjugant VRSA-1 in vancomycin-free BHI medium at ratios of 1:1, 1:100, and 100:1 (11, 14). To preconcentration each population to the competition environment, individual strains were grown exponentially at 37°C in BHI broth to an optical density at 600 nm of 0.1. The cultures were diluted 1,000-fold, and 5 × 10^5 CFU of VRSA-1 were mixed with 5 × 10^3 CFU of HIP11713 in 2 ml of BHI medium. Where indicated, the ratio of VRSA-1 to HIP11713 to its competitor was reduced to 1:100 by decreasing the inoculum of the corresponding strain to 5 × 10^4 CFU/ml. The mixed culture was transferred into fresh broth every 10 generations over 5 to 14 cycles. At each generation cycle, the total number of viable cells was determined by plating aliquots onto nonselective plates, and the proportion of resistant cells was deduced by replica plating of an average of 100 colonies on plates containing 10 μg/ml of vancomycin. Each mixed culture was performed three times in two independent experiments. At the end of every competition experiment, the phenotypes of vancomycin-resistant and -susceptible populations were controlled by disk-agar diffusion. Relative fitness was expressed as the competition index (CI), calculated as the ratio of the mean CFU in two independent competition experiments between the resistant and susceptible strains at t/g divided by the same ratio at t0 (7). The selection coefficient, s, of VRSA-1 was then calculated as the slope of the linear regression model x = ln(CI)/ln(d), where d is the dilution factor (13, 14). The selection coefficient estimates the difference between the relative fitnesses of the two competitors over the entire competition experiment.

Determination of plasmid copy number. Quantitative PCR of the rrs, ddr, rpoB, vanA, and vanH genes was performed in a LightCycler apparatus using the LightCycler Fast Start DNA MasterPLUS SYBR green I kit (Roche Diagnostics GmbH, Mannheim, Germany) with the following specific primer pairs: qPCRrs1 (5'-AGGTAACCGCCTAAAGGCA-3') and qPCRrs2 (5'-ACGGATACGGCCCTCA-3'), qPCRdd1 (5'-CCTTTTACGTTGCTC-3') and qPCRdd2 (5'-GATCTGTTGCTGCTGTTG-3') and qPCRrpoB2 (5'-CATTGTTGCTAGACCTTCC-3'), qPRVvanA1 (5'-TCTAGCTTTCGCACTG-3') and qPCRvanA2 (5'-ACCCAAAAGCGCGGAGTA-3') and qPCRvanH1 (5'-CCTGGGTCGATXATX-3') and qPCRvanH2 (5'-CTGCAATACCGGCTTGGCT-3'). Data were analyzed by use of a method described previously by Peirson et al. (19). The plasmid copy number was determined as the ratio between vanA or vanH and rpoB or ddr. The rrs genes were used for confirmation of the copy number of the genes.

Statistical analysis. Mean values and standard deviations were calculated using Excel version 11.3.7 software (Microsoft Corporation, WA). A Student's t test was used to evaluate differences between means, with a significant probability at a P value of <0.001.

RESULTS AND DISCUSSION

Growth rate reduction and increased lag phase in the presence of vancomycin. Growth kinetics of recipient HIP11713, transconjugant VRSA-1, VRSA-5, and VRSA-6 were measured in independent cultures in medium with or without vancomycin (Fig. 1). The growth kinetics in the presence of vancomycin were assessed in two ways: either the VanA-type resistance of the three VRSA strains was induced by adding vancomycin (1/50 the MIC) in the preculture and in the culture or vancomycin was added only in the culture. It was previously shown that resistance is induced by this subinhibitory vancomycin concentration (20, 21). In the first type of experiment, VanA-type resistance was already induced at the onset of the culture and fully expressed during growth rate measurement, whereas in the second type, induction of resistance occurred during monitoring of growth.

Bacterial fitness can be measured as the growth rate of individual bacterial populations determined during the exponential phase in monoculture (Fig. 1A) (1, 14). In the absence of vancomycin, the growth rates of VRSA-1 (0.0157 ± 0.0003), VRSA-5 (0.0160 ± 0.0006), and VRSA-6 (0.0156 ± 0.0008) were similar to that of vancomycin-susceptible MRSA HIP11713 (0.0162 ± 0.0003) (Table 1). The growth rate ratio of VRSA-1 relative to that of HIP11713 in the absence of vancomycin was not significantly different (0.97 ± 0.01; P < 0.001 by t test) from 1 (Fig. 1B), indicating that the fitness reduction of the resistant strain due to the acquisition of transposon Tn1546 was minimal in the absence of an inducer in the environment.

Since the initial inoculum (10^5 CFU) was low, this allowed the detection of small differences in the lag phases of the various strains. The presence of vancomycin in the culture led to an increase in the lag phase of the three VRSA isolates (Fig. 1A). The longest lag phase was observed for all three strains when resistance was induced during the culture and may correspond to the time required by the host to synthesize a new resistant cell wall and to eliminate the chromosomal susceptible pathway of peptidoglycan synthesis (5). It was previously shown that the phenotypic expression of glycopeptide resistance requires a nearly complete elimination of the chromosomal susceptible pathway of cell wall synthesis (2, 22). The growth delay was slightly more pronounced for VRSA-5 and VRSA-6, which presented a lower resistance level than that for VRSA-1. A very long lag phase in the presence of vancomycin for two other VRSA clinical isolates, VRSA-2 and VRSA-3, was reported previously (20, 21). However, this was attributed, at least in part, to a loss, at a high frequency, of the enterococcal plasmids carrying Tn1546 in the two strains (20, 21). This is in contrast with VRSA-5 and VRSA-6, in which the resistance plasmids are extremely stable (21; data not shown).

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When resistance was induced, there was a statistically significant reduction in growth rates of the three VRSA strains (Table 1). For every strain, the levels of growth rate reduction observed in the presence of vancomycin were similar under the two inducing conditions (Fig. 1A). Under both conditions of induction by vancomycin, the relative growth rates of VRSA-1 were 0.62 ± 0.008 and 0.63 ± 0.017 compared to recipient strain HIP11713 and noninduced VRSA-1, respectively, which correspond to approximate reductions in fitness of 38% and 37% (Fig. 1B and C). Strains VRSA-5 and VRSA-6 had relative growth rates of 0.80 ± 0.029 and 0.67 ± 0.043 when grown under both inducing conditions compared to their respective noninduced counterparts: this corresponds to 20% and 33% reductions in fitness, respectively (Fig. 1C). The level of reduction in fitness paralleled the level of resistance to vancomycin (Table 1). To account for the various levels of resistance of the three VRSA strains, the copy numbers of the plasmids versus that of the chromosome were determined by quantitative PCR of vanA and vanH relative to the chromosomal ddl, rpoB, and rrs genes, which are present at 1, 1, and 5 copies, respectively. In three independent experiments, the relative copy numbers of vanA and vanH were evaluated to be ca. 6 in VRSA-1, 2 in VRSA-5, and 3 in VRSA-6 (Table 2). The higher reduction in the growth rate observed with VRSA-1 is probably due to the higher copy number of S. aureus resident plasmid pLW1043 (pAM829::Tn1546) (24), which in turn leads to higher levels of resistance because of the gene dosage effect (Table 2). The longer lag phase and the reduction in growth rate observed when the vanA cluster is induced could lead to a lack of detection of the VRSA clinical isolates using automated techniques (20, 21). In summary, the growth rate of the three VRSA strains was similar to that of recipient strain HIP11713.

![Fig. 1](https://example.com/figure1.png)

**TABLE 1. Properties of the strains used**

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>MIC (µg/ml)^a of:</th>
<th>Avg growth rate (min⁻¹) ± SD^b</th>
<th>−/−</th>
<th>−/+</th>
<th>+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIP11713</td>
<td>Vancomycin</td>
<td>2</td>
<td>0.0162 ± 0.0003</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Teicoplanin</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VRSA-1</td>
<td></td>
<td>2,048</td>
<td>0.0157 ± 0.0003</td>
<td>0.0101 ± 0.0003</td>
<td>0.0098 ± 0.0003</td>
</tr>
<tr>
<td>VRSA-5</td>
<td></td>
<td>1,024</td>
<td>0.0160 ± 0.0006</td>
<td>0.0130 ± 0.0003</td>
<td>0.0127 ± 0.0005</td>
</tr>
<tr>
<td>VRSA-6</td>
<td></td>
<td>1,024</td>
<td>0.0156 ± 0.0008</td>
<td>0.0106 ± 0.0006</td>
<td>0.0104 ± 0.0004</td>
</tr>
</tbody>
</table>

^a Determined by broth dilution.

^b Exponential growth rate measured in the absence (−/−) or in the presence of 1/50 MIC of vancomycin in the culture (−/+ or in the preculture and the culture (+/+); shown are averages of two independent experiments ± standard deviations. NA, not applicable.
in the absence of vancomycin, whereas when resistance was induced by vancomycin, a statistically significant reduction in the growth rate was observed for the three strains, indicating that VanA-type resistance is associated with a biological cost for the bacterial host.

In *S. aureus*, various resistances due to target modification following either mutation, resulting, for example, in fusidic acid (16) or linezolid resistance (6), or as the result of the acquisition of a mobile genetic element such as what occurs in methicillin resistance (11) were previously shown to be costly for the host. The level of reduction in fitness varies according to the type of genetic event. For example, the reduction in the growth rate reported for methicillin resistance in the absence of the drug is similar to that observed in this study for induced glycopeptide resistance, indicating that the expression of the two mechanisms imposes a similarly high impact on fitness (11).

**Transconjugant VRSA-1 is slightly less competitive than the HIP11713 recipient.** Comparison of the exponential growth rates of two bacterial populations is insufficient to evaluate the global fitness burden. A more sensitive and accurate estimate is obtained by competition experiments in the absence of antibiotic that allow comparisons of the entire growth cycle during several generations; a difference in the two competitors reflects differences in lag phase, exponential growth rate, or survival in stationary phase (14). Transconjugant VRSA-1 was mixed with recipient HIP11713 at an initial ratio of 1:1 and subcultured over 14 cycles of growth (Fig. 2A). A slight, but significant, difference between the susceptible and resistant populations was observed after 9 days of competition (*P* < 0.001 by *t* test). The slight decrease in the rate of growth of the resistant strain versus the susceptible strain observed after the first transfer could be associated with a higher natural death rate during the stationary phase. The selection coefficient, which provides an estimate of the percent difference in relative fitnesses between the two competitors over the entire experiment, indicated that VRSA-1 has a competitive disadvantage of ca. 2% per 10 generations compared to HIP11713 (Fig. 2A). The transconjugant was thus slightly less fit than the recipient strain when they were mixed under the same environmental conditions in the absence of selective pressure. This disadvantage in competition could be attributed to the fitness burden imposed on the host by the presence of *Tn1546* on multicopy plasmid pLW1043 (pAM829::*Tn1546*); *Tra*<sup>+</sup> *Gm*<sup>+</sup> *Tc*<sup>+</sup> *Tc*<sup>+</sup> *Vm*<sup>+</sup>; 57.9 kb.

### Table 2. Characteristics of plasmids

<table>
<thead>
<tr>
<th>Host</th>
<th>Characteristic&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Avg copy no. ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>VRSA-1</td>
<td><em>S. aureus</em> pLW1043 (pAM829::<em>Tn1546</em>); <em>Tra</em>&lt;sup&gt;+&lt;/sup&gt; <em>Gm</em>&lt;sup&gt;+&lt;/sup&gt; <em>Tc</em>&lt;sup&gt;+&lt;/sup&gt; <em>Tc</em>&lt;sup&gt;+&lt;/sup&gt; <em>Vm</em>&lt;sup&gt;+&lt;/sup&gt;; 57.9 kb.</td>
<td>6.3 ± 1.3 6.2 ± 1.2</td>
</tr>
<tr>
<td>VRSA-5</td>
<td>Enterococcus Incl8-like; <em>Tra</em>&lt;sup&gt;+&lt;/sup&gt; <em>Em</em>&lt;sup&gt;+&lt;/sup&gt; <em>Tc</em>&lt;sup&gt;+&lt;/sup&gt; <em>Vm</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.3 ± 0.3 2.4 ± 0.3</td>
</tr>
<tr>
<td>VRSA-6</td>
<td>Enterococcus Incl8-like; <em>Tra</em>&lt;sup&gt;+&lt;/sup&gt; <em>Tc</em>&lt;sup&gt;+&lt;/sup&gt; <em>Vm</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.7 ± 0.4 2.6 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average data for three independent experiments ± standard deviations.

<sup>b</sup> *Tra*, transfer; *Em*, erythromycin; *Gm*, gentamicin; *Tc*, teicoplanin; *Tp*, trimethoprim; *Vm*, vancomycin.

In conclusion, a major reduction in growth rate was observed for the three VRSA strains when the *vanA* operon was induced by vancomycin, indicating that the expression of resistance is highly costly for the host. In the absence of vancomycin, this biological cost was only minimal because of the inducible regulation of resistance expression mediated by the two-component VanS-VanR system. The slight fitness burden observed in the absence of induction could be attributed to the basal level of *van* gene expression (2) combined with a gene dosage effect (Table 2). This fitness reduction was more easily detectable in competition experiments than when growth rates in monocultures were determined. The competition experiments indicated that in the absence of selective pressure, the transconjugant is more rapidly eliminated than the MRSA recipient, although this fitness burden remains to be evaluated in vivo in an animal model. This could explain the observed low dissemination of the VRSA clinical isolates that remain localized mainly in Michigan. The high incidence of enterococci harboring Incl8-like vancomycin resistance plasmids in this state has recently been associated with this local emergence (18, 26). Low dissemination of VRSA has also been attributed in part to the high instability of certain enterococcal plasmids in MRSA isolates (20, 21, 25). However, the risk of dissemination of VRSA strains should not be underestimated, since for patients not treated with vancomycin, the slight biological cost associated with resistance could lead to the selec-
tion of compensatory mutations that restore the fitness of the host (13, 16).

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REFERENCES