Generic intravenous antibiotics are accepted for clinical use solely by fulfilling the requirement of pharmaceutical equivalence (i.e., having similar concentrations of the active ingredients), from which therapeutic equivalence (i.e., similar efficacy and safety) is assumed. However, our research group has shown that this assumption is not straightforward, and many pharmaceutically equivalent generics fail in vivo, suggesting that other factors, such as stability of the active pharmaceutical ingredient (API), excipients, and apparently innocent impurities may have a role in determining in vivo efficacy (15, 21, 23).

In the case of vancomycin, we demonstrated that despite similar or even higher concentrations of the API, indistinguishable in vitro activity, and “bioequivalent” pharmacokinetics, generic products killed significantly fewer bacteria (several orders of magnitude) in a murine thigh infection model and in some cases displayed the Eagle effect (paradoxical antagonistic effect at the highest dose) (21). Considering that “dead bugs don’t mutate” (19) and that vancomycin resistance in S. aureus is a growing concern, manifested by isolation of vancomycin-intermediate Staphylococcus aureus ([VISA] MIC of 4 to 8 mg/liter) and vancomycin-resistant S. aureus (VRSA) strains (MIC of >16 mg/liter), we aimed to determine if the in vivo exposure to generic bioequivalent products with inferior bactericidal efficacy favored the emergence of resistance in S. aureus.

(Preliminary results of this work were presented at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 27 to 30 September 2006 [14]).

MATERIALS AND METHODS

Strain. A clinical methicillin-resistant S. aureus (MRSA) strain from the blood of a liver transplant patient with persistent bacteremia was recovered after 10 days of treatment with generic vancomycin and stored at −70°C under the identification code S. aureus GRP-0109 (for a full description and discussion of the case, see Rodriguez et al. [13]). The identity of the isolate was confirmed by coagulase and mannitol fermentation tests. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) or Trypticase soy agar (TSA) (Difco, Becton Dickinson) was used for routine liquid and solid cultures, respectively. For population analysis profiles (PAP) brain heart infusion agar (BHIA) was employed (Difco, Becton Dickinson) (see below). All bacterial counts were expressed as log_{10} CFU.

Susceptibility testing. Vancomycin minimal inhibitory and bactericidal concentrations (MIC and MBC) were determined by broth microdilution according to the CLSI, using S. aureus ATCC 29213 as a control (3). For PAP, a log-phase culture of 8 to 9 log_{10} CFU/ml was plated on BHIA plates containing 0, 1, 2, 3, 4, and 5 mg/liter of vancomycin (Vancocin CP; Eli Lilly [Lilly]) in triplicate and incubated aerobically at 37°C for 48 h following the methodology described by Hiramatsu et al. (8). The area under the vancomycin concentration versus the log_{10} CFU/ml curve (AUC) was calculated with Prism, version 5.0 (GraphPad, San Diego, CA).
TABLE 1 Vancomycin products

<table>
<thead>
<tr>
<th>Product name (manufacturer)</th>
<th>Batch/lot no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin CP (Eli Lilly, Mexico)</td>
<td>A050370, A048213</td>
</tr>
<tr>
<td>Vancomycin USP (APP, USA)</td>
<td>121384</td>
</tr>
<tr>
<td>Vancomicina USP (Abbott, USA)</td>
<td>09993Z7, 09993Z8, 18879Z7, 19236TB21</td>
</tr>
<tr>
<td>Vancomicina Proclin (Laboratories Northia, Argentina)</td>
<td>8441, 8690, 8872</td>
</tr>
</tbody>
</table>

CA), and the resistance frequency at each concentration was determined by dividing the number of CFU that grew in antibiotic-containing agar by the total population in antibiotic-free plates. Population analysis profiles were performed with *S. aureus* GRP-0109 after it was isolated from the patient (baseline PAP) and after 5, 10, 11, and 12 cycles of treatment with innovator or generic vancomycin (postexposure PAP) or sterile saline (control).

**Autolysis assay.** Triton X-100-induced autolysis of *S. aureus* GRP-0109 was measured with the assay described by Gustafson et al. (7): a log-phase culture containing 8.0 log_{10} CFU/ml was centrifuged at 13,000 rpm for 10 min at 4°C, the supernatant was removed, and the pellet was suspended in sterile chilled water. After another centrifugation under the same conditions, the pellet was resuspended in 0.05 M Tris-HCl adjusted to a pH of 7.4 until the culture reached an optical density at 580 nm (OD_{580}) of 1.00. Triton X-100 was added to a final concentration of 0.05% (vol/vol), and the tube was incubated statically at 37°C for 4 h. Every 30 min the absorbance was measured by spectrophotometry (Spectro 22; Labomed, Culver City, CA) and registered as a percentage of the initial OD. *S. aureus* ATCC 29213 was used as the control strain. Three independent assays were performed in duplicate.

**Antibiotics.** The innovator product of vancomycin from Eli Lilly (Mexico) and generics from Abbott (Chicago, IL), American Pharmaceutical Partners (APP) Los Angeles, CA), and Proclin (Laboratories Northia, Buenos Aires, Argentina) were purchased at reputable drugstores in groups of two mice per product (innovator and three generics) plus a nontreated intragroup control. A separate group of two animals per treatment group received saline injections (infected but untreated controls). After 24 h, the animals were processed as described above to determine bacterial growth and antimicrobial effect and to prepare the inoculum for the next passage. The cycle was repeated until 12 passages were completed; follow-up of mutant selection after the fifth passage allowed the researchers to stop vancomycin exposure at this point to prevent unnecessary suffering of experimental animals without compromising the statistical power of the study.

**Data analysis.** (i) **Autolysis assay.** Absorbance data from the Triton-X assay were expressed as percentages of the original OD values and analyzed by linear regression with Prism, version 5.0, to compare the slopes of *S. aureus* and *ATCC 29213* by the overall test.

(ii) **Population analysis profile and resistance frequency.** The area under the vancomycin concentration versus the log_{10} CFU/ml curve was obtained with Prism, version 5.0, for each treatment group. The intensity of the effect (IE) was calculated as the difference between the AUCs of the control and treated groups by the following formula: IE = AUC_{control} - AUC_{treated}.

As each group yielded a set of values, means were compared by analysis of variance (ANOVA), followed by Dunnnett’s posthoc test. Additionally, the resistance frequency (RF) at 1, 2, and 3 mg/liter of vancomycin was determined by dividing the number of CFU growing in antibiotic-containing agar by the total number of bacteria in the population (grown in antibiotic-free agar) and expressed in negative logarithms. CFU counts at 4 and 5 mg/liter were below the limit of detection, so no exact RF could be calculated. Changes in RF (final RF — initial RF) were obtained for each group and concentration and compared by Student’s t test.

**RESULTS**

**Initial susceptibility profile.** *S. aureus* GRP-0109 was identified by automatic testing (Vitek 2; bioMérieux, Marcy l’Étoile, France) as resistant to penicillin G, oxacillin, macrolides, lincosamides, and aminoglycosides and susceptible to vancomycin, rifampin, tetracyclines, and sulfonamides. Table 2 shows the confirmation for the presence of resistance as measured by the broth microdilution assay on January 25, 2018 by guest http://aac.asm.org/ Downloaded from

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/liter)</th>
<th>MBC (mg/liter)</th>
<th>Susceptibility of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Resistant</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>8</td>
<td>8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>Resistant</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>2</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

**TABLE 2 Antibiotic MICs and MBCs for *S. aureus* GRP-0109 by broth microdilution**
results from broth microdilution susceptibility testing to penicillin G, oxacillin, gentamicin, and vancomycin.

Figure 1 displays the initial PAP with the respective resistance frequencies for vancomycin from 1 to 5 mg/liter. As frequencies at concentrations of ≥4 mg/liter were <−6.0 logs, a heterogeneous VISA (hVISA) phenotype was ruled out, but comparison with the PAP of reference strain \textit{S. aureus} ATCC 29213 showed an altered susceptibility pattern with increased resistant subpopulations.

**Autolytic profile.** Control strain ATCC 29213 exhibited a 45.3% decrease in absorbance after exposure to Triton X-100, while GRP-0109 displayed a reduction of 21.2%, indicating resistance to autolysis in this strain relative to its control. The difference in autolysis profiles is shown in Fig. 2 as two independent linear regressions with significantly different slopes (\( P < 0.0001 \)), indirectly confirming cell wall alterations of \textit{S. aureus} GRP-0109.

**Postexposure susceptibility changes.** Selection of less susceptible cells at 2 mg/liter was evident after 5 cycles for one vancomycin product (corresponding to Proclin after the blinding code was opened) and 10 cycles for two products (APP and Abbott). Resistance to vancomycin at 3 mg/liter required 11 cycles to appear (Proclin). After cycle 12, the same three vancomycin products were consistently selecting cells resistant to 2 and 3 mg/liter. One product (corresponding to Lilly) was characterized for the opposite tendency; i.e., it suppressed resistant subpopulations.

Figure 3 shows the PAP after 12 passages \textit{in vivo}. Innovator vancomycin (Lilly) reduced resistant subpopulations (indicated by a smaller AUC and a left shift of the PAP curve compared with the baseline) while generics enriched them, as shown by greater AUCs or right shift of the PAP curves along the \( x \)-axis. The global impact of the exposure can be seen in the intensity of effects in Fig. 4 comparing treatment groups with the control. Lilly was the only product that reduced the AUC (\( I_E = 0.22 \)) while generics significantly enlarged it (\( I_E \) values of −1.83, −2.68, and −6.07 for APP, Abbott, and Proclin, respectively; \( P < 0.0001 \) by ANOVA). Figure 5 shows the change in resistance frequencies (final RF − initial RF) after \textit{in vivo} exposure at concentrations from 1 to 3 mg/liter: Lilly reduced resistant cells at all concentrations by approximately 1 order of magnitude while generics enriched them at 2 mg/liter (from 10 to 1,000-fold), and Proclin also increased cells growing at 3 mg/liter by 10-fold. In the mock-treated control group (sterile saline injections), all subpopulations diminished by approximately 1 log\(_{10}\), reaching resistance frequencies similar to those of the susceptible strain ATCC 29213 (Fig. 1).

\[ \begin{array}{|c|c|c|c|} \hline \text{Resistance frequency (log}_{10}) & \text{VAN mg/L} & \text{GRP-0109} & \text{ATCC 29213} \\ \hline 1 & -1.15 & 2.16 & 1.01 \\ 2 & -5.42 & -6.02 & 0.60 \\ 3 & -6.49 & -8.96 & 0.40 \\ 4 & -7.16 & -7.16 & - \\ 5 & -5.09 & ND & - \\ \hline \end{array} \]

**FIG 2** Triton X-100 autolysis assay. Compared with \textit{S. aureus} ATCC 29213, the strain GRP-0109 exhibited a reduced autolysis profile (45% versus 21%), one of the first steps toward vancomycin resistance.

**FIG 1** Vancomycin population analysis profile of \textit{S. aureus} GRP-0109 after being isolated from a patient with persistent bacteremia and unsuccessful generic treatment, indicating altered susceptibility in comparison with strain ATCC 29213: 10 times more cells were able to grow at 1 mg/liter of vancomycin, 4 times more grew at 2 mg/liter, and 2.5 times more grew at 3 mg/liter (resistance frequency data at right).

**FIG 3** Pre- and postexposure PAP of \textit{S. aureus} GRP-0109 (AUC in parentheses). Values for the initial isolate are plotted. Treatment with innovator vancomycin (Lilly) caused a down and left curve shift, indicating a reduction of the less susceptible subpopulations, which is sharply different from three generics, which had higher AUCs and up and/or right displacement of the curve, (especially Proclin), due to resistant subpopulation enrichment. The control saline group exhibited a down and left displacement, consistent with reversion of unstable resistance associated with reduced fitness. The limit of detection for all of the postexposure isolates was 10 CFU/ml, and for the GRP-0109 initial strain the limit was 0 CFU/ml.
Reversion of resistance phenotype. After the 12 passages were completed, the strains were kept in agar plates at 4°C with monthly subcultures. After 2 months, PAP were performed again, and the results were similar to those with the control sterile saline group, indicating reversion of resistance and recovery of the preexposure pattern of susceptibility (data not shown). Unfortunately, this was an unexpected outcome, and no additional tests could be performed on the isolates.

DISCUSSION

We demonstrated previously that generic products of vancomycin had inferior efficacy in vivo despite pharmaceutical equivalence, bioequivalence, and indistinguishable MIC and MBC values (13, 21). The same problem was demonstrated with oxacillin (15) and gentamicin (23), confirming that current criteria for approval of generic antibiotics do not ensure therapeutic equivalence and that the impact of other factors, such as antagonistic impurities, the quality of excipients, or in vivo instability of the API may cause therapeutic failures in generic antibacterials that are otherwise “equivalent” to their respective innovators. The data shown in this paper reveal that resistance, the natural consequence of suboptimal treatment of infections, is efficiently promoted by inequivalent generics of vancomycin.

Suboptimal efficacy of vancomycin generics led to the hypothesis that exposure to these products could select for less susceptible subpopulations of S. aureus. We chose an MRSA strain (S. aureus GRP-0109) from a patient treated unsuccessfully for 10 days with generic vancomycin (13). The patient did not have risk factors associated with persistent bacteremia: endocarditis, retained devices, metastatic foci of infection, septic shock, diabetes mellitus, or a strain with a vancomycin MIC of \(\geq 2\) mg/liter (10, 12, 22); and, notably, 48 h after the patient was switched to the innovator drug, blood cultures became sterile, strongly suggesting that the therapeutic failure was due to the generic vancomycin. As shown by the Triton X-100 assay, S. aureus GRP-0109 had an altered autolysis profile (Fig. 2), one of the first steps toward vancomycin resistance (2), and exhibited a right-shifted PAP curve compared with that of S. aureus ATCC 29213 (Fig. 1). Considering that GRP-0109 was not the initial isolate of the patient but was recovered after 10 days of therapy, we do not know with certainty if these resistance features predated treatment or were induced by it; however, the fact that they were exacerbated under further generic exposure in mice with the same product (Abbott) and others (APP and Proclin), while they reverted under innovator pressure and saline “treatment,” favors the hypothesis that generic vancomycin induced them in both the patient (10-day exposure) and the animal model (12-day exposure).

The spontaneous resistance frequency of our strain to vancomycin (i.e., at 4 mg/liter) was \(-7.18\) logs (1 resistant mutant for every 15.8 million cells) (Fig. 1). Considering that the inoculum in the in vivo model was 7.0 log_{10} CFU/g (below the resistance frequency), it is not surprising that we could not find hVISA or VISA levels of resistance (as currently defined by regulatory agencies) after exposure, but we did find significant enrichment of subpopulations growing at vancomycin concentrations of 1 (MIC), 2 (susceptibility breakpoint), and 3 (nonsusceptible) mg/liter, all with resistance frequencies greater than \(-7.0\) logs. As mentioned above, these alterations reverted once the antibiotic pressure was withdrawn, suggesting an adaptive or unstable nature.

Sieradzki and Tomasz (18) reported that exposure of a susceptible S. aureus strain to subinhibitory concentrations of vancomycin induces a transitory VISA-like phenotype characterized by reduced autolysis and increased cell wall thickness. They found that vancomycin molecules trapped in the outer layers of the cell wall (i.e., bound to free, unprocessed D-Ala-D-Ala termini) inhibit the action of murein hydrolases and lysostaphin, blocking access to their substrates by steric hindrance. If generics contain more impurities and degradation products than the innovator (like

FIG 4 Overall changes in the AUC (intensity of the effect \(I_2\) after exposure to innovator (Lilly) and generic (APP, Abbott, and Proclin) vancomycin products compared with the control group. Positive values indicate smaller AUCs, i.e., a reduction of less susceptible subpopulations with Lilly, while negative values indicate greater AUCs, i.e., enrichment of resistant subpopulations, with APP, Abbott, and Proclin (ANOVA, \(P < 0.0001\); all comparisons of a generic versus the innovator compound had a \(P\) value of \(<0.05\) by Dunnett’s posthoc test).

FIG 5 Changes in resistance frequencies (RFs) to 1, 2, and 3 mg/liter of vancomycin after in vivo exposure to innovator vancomycin (Lilly), generic versions (APP, Abbott, and Proclin), or sterile saline. At 1 mg/liter, compared to initial values (GRP-0109), Lilly reduced the RFs by almost 10-fold, while generics induced no significant change. At 2 mg/liter Lilly also reduced the RFs, but generics significantly increased them 10- to 1,000-fold. At 3 mg/liter, again Lilly reduced the RFs, APP and Abbott did not change the baseline RF, and Proclin significantly increased it by 1 order of magnitude. In the saline group RFs were reduced about 1 log_{10} at all concentrations. The asterisk indicates that the postexposure value is significantly different from the preexposure value (Student’s t-test): \(P\) values of 0.0002 and 0.0005 for Lilly and saline at 1 mg/liter, respectively; \(P\) values of 0.0258, 0.0012, 0.0002, \(<0.0001\), and 0.0029 for Lilly, APP, Abbott, Proclin, and saline at 2 mg/liter, respectively; \(P\) values of 0.0140, 0.0152, and 0.0094 for Lilly, Proclin, and saline at 3 mg/liter, respectively. CFU counts at 4 mg/liter and higher were below the limit of detection.
CDP-1, that also binds D-Ala-D-Ala), more molecules may be trapped in the cell wall, blocking lysis and clogging the peptidoglycan layers, as described by Cui et al. (4), which reduces the diffusion of factor B to the lethal target in the outer membrane. This could explain the lower bactericidal efficacy and favor the enrichment of subpopulations with higher MICs carrying mutations in regulatory genes of the cell wall synthesis and stress response (9). Last year (21), we reported that generics were ineffective at 1,200 mg/kg per day; it is remarkable that Proclin, the generic with the greatest impact on resistance in this paper, was precisely the one that displayed the greatest Eagle effect at this dose. It suggests that further a generic is from the innovator in terms of in vivo efficacy, the greater is its power to select for resistant subpopulations.

As mentioned earlier, the strains reverted to a more susceptible phenotype after passages without vancomycin, precluding additional experiments to define their resistance mechanisms. The reversion of VISA strains was first reported by Boyle-Vavra et al. after 15 daily passages in antibiotic-free medium, probably due to the high metabolic burden and reduced fitness associated with vancomycin resistance, which is maintained only under continuous selective pressure (1). Future projects in the same line would include electron microscopy to measure the thickness of the cell wall (5) after innovator and generic product exposure, cell wall composition analysis, evaluation of the cell wall stimulus response (20) to different versions of vancomycin, and whole-genome sequencing to identify potential mutations contributing to resistance (11).

Our results suggest that the use of inequivalent generic products of vancomycin can contribute to resistance and therapeutic failures as even minor MIC increments have a huge impact on clinical outcome (17). Intermediate vancomycin resistance in S. aureus is a slow, progressive process; thus longer treatment courses with generic vancomycin (as seen in humans) can probably lead to the isolation of hVISA and VISA strains. Considering the enormous amount of generic antibiotics prescribed in the world, the fact that no proof of in vivo efficacy is currently required for their approval, and our previous results of therapeutic inequivalence, these preliminary data of resistance enrichment by generic antibiotics reinforce the suggestion that more stringent criteria for generic approvals should be required. We are currently working to expand this hypothesis to different antibiotic-bacterium combinations, especially those with rapid development and well-defined mechanisms.

ACKNOWLEDGMENTS
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We have no relation, current or past, with the manufacturers of any of the vancomycin products used.

REFERENCES