The site of action of *C. difficile* is the large intestine, a milieu filled with vast numbers of different species of anaerobic flora that, through their metabolites, maintain a physiological pH ranging from 5.5 to 7 (11). Disruption of the gut flora by antibiotic therapy can therefore lead to pH changes, which can affect the pH-dependent activities of many antibiotics (4, 8, 21). Other environmental variables, such as divalent cation concentrations (including calcium and magnesium) and bacterial density, can also influence the antimicrobial activities of compounds. The dependence of the antibacterial activity on these factors is an important consideration, particularly for an unabsorbed antibiotic such as fidaxomicin that localizes and targets bacteria in the gut, where these parameters can vary greatly with diet and disease state.

The *in vitro* activities of antimicrobial compounds (expressed as the MICs) under conditions with such environmental variables are also important factors to be considered when a methodology for future *in vitro* testing is designed. Brucella agar, which is recommended by the Clinical and Laboratory Standards Institute (CLSI) (18) for use for MIC determination, is not standardized, and the consistency of the divalent cation concentrations has not been established. Moreover, the pH of the medium used under anaerobic conditions in a glove box may also vary with different gas mixtures, as the CO₂ concentration in the gas mixture has the propensity to acidify the medium and can thus be a significant source of variability. Macrolides, as an example, show elevated MICs in the presence of CO₂ (8). The inoculum size may also be difficult to standardize, given the variety of atmospheric conditions available for anaerobic susceptibility testing (H₂/CO₂ generator, evacuation/replacement method, or anaerobic chamber) and the duration of organism exposure to the aerobic atmosphere during benchtop manipulations.

In the study described here, we examined the anti-*Clostridium difficile* activity of fidaxomicin by comparing the MIC values obtained in the presence of different concentrations of divalent cations, pHs ranging from 6 to 8, inoculum density ranges of over 3 orders of magnitude, and various commercial lots of brucella broth. *C. difficile* laboratory strains ATCC 9689, ATCC 700057, ATCC 43255, and ATCC 17857 and *Eubacterium lentum* laboratory strain ATCC 43055 were obtained from the American Type Culture Collection (ATCC); and the MIC values were determined by the CLSI agar dilution method (18). Broth microdilution is not a CLSI-validated method for testing the MICs of *Clostridium*; however, due to the potential inaccuracy of measuring the pH of solid agar after equilibration inside the anaerobic chamber, both broth and agar dilution methods were used and the results were compared for the assessment of the pH effects. With the exception of drug dilution, all MIC testing steps for both methods were performed inside a glove box under anaerobic conditions (10% H₂, 5% CO₂, 85% N₂). Microtiter plates with diluted drugs were equilibrated for a minimum of 3 h inside the glove box, prior to addition of the inocula. All MIC testing runs were performed at least in duplicate. When the values for replicate runs varied, the mode was presented, or if the values for the replicate runs were evenly split between two values, the higher value was reported.

**Inoculum effect.** To evaluate the effect of the inoculum density on the susceptibility of *C. difficile* to fidaxomicin, a suspension of ~10⁸ CFU/ml was prepared and serially diluted by 10-fold factors to obtain culture densities of 10⁵ to 10⁸ CFU/ml. The delivery of 2 μl to the agar medium yielded inoculum densities that ranged from 10⁷ to 10⁸ CFU/ml (10⁵ to 10⁷ CFU/spot). The fidaxomicin MIC for *C. difficile* strains ATCC 9689 and ATCC 700057 (MIC values, 0.063 and 0.125 μg/ml, respectively) remained the same at all the concentrations tested, whereas the vancomycin MICs increased progressively with increasing inoculum concentrations, with the highest in-
TABLE 1. In vitro activity of fidaxomicin in supplemented brucella agar with different divalent cation concentrations

<table>
<thead>
<tr>
<th>Cation</th>
<th>Drug</th>
<th>Cation content in brucella agar medium (mg/liter)</th>
<th>MIC (μg/ml) for C. difficile:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATCC 700057</td>
<td>ATCC 9689</td>
</tr>
<tr>
<td>Calcium</td>
<td>Fidaxomicin</td>
<td>33</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Fidaxomicin</td>
<td>21</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>21</td>
<td>1</td>
</tr>
</tbody>
</table>

oculum density showing a 4-fold rise in MIC over that obtained with the lowest inoculum density. Similarly, reproducible MIC values with other C. difficile strains have been observed in our laboratory (data not shown) at different inoculum concentrations.

Cation effect. The concentrations of the calcium and magnesium divalent cations in commercial brucella agar were determined by Laboratory Specialists, Inc., to be 21 and 33 mg/liter, respectively. Additional amounts of divalent cations were added (in the form of either calcium chloride or magnesium chloride) to obtain media with calcium ion concentrations of 21, 30, and 57 mg/liter or magnesium ion concentrations of 33, 45, and 75 mg/liter. The fidaxomicin MIC values for C. difficile ATCC 9689 and ATCC 700057 remained identical (0.063 and 0.125 μg/ml, respectively) for all cation concentrations tested (Table 1). Quality control susceptibility testing was conducted with vancomycin in medium with unaltered cation levels and consistently produced the expected MIC value of 1 μg/ml.

Commercial lot variation effect. Three different lots of supplemented brucella agar medium were used on three separate days to compare the reproducibility of the fidaxomicin MIC values with each lot for three C. difficile strains (ATCC 9689, ATCC 43255, and ATCC 17857). The MIC assays were controlled by testing the activity of clindamycin against the quality control organism, Eubacterium lentum. As an internal control, the activity of metronidazole against C. difficile, which in our laboratory has been about 0.25 to 0.5 μg/ml, was monitored. The fidaxomicin MICs were unaffected by the different medium lots and remained within one 2-fold dilution of each other (Table 2).

pH effect. The susceptibility of C. difficile (ATCC 9689 and ATCC 700057) to fidaxomicin was evaluated over a pH range of 6 to 8 by both the CLSI agar and the CLSI broth microdilution methods. With the agar dilution method, the fidaxomicin MIC was determined over a pH range of 6.2 to 8.0. In order to achieve the desired anaerobic pH for susceptibility testing, two different buffers [100 mM NaH2PO4 and N-Tris(hydroxymethyl)methyl-3-aminopropansulfonic acid (TAPS)] were added to the media at pH 7 and 8, respectively. To compensate for the reduction of the pH in the medium inside the anaerobic glove box (which occurred even with buffered media), the titer in the medium in ambient air, which was above the desired anaerobic pH, was first determined. Following equilibration inside the chamber with 5% CO2 for 3 h, the anaerobic pH was verified with a portable pH meter with a flat-bottomed pH probe. Vancomycin, used as a control, was tested only at about pH 7. The results showed no increase in the fidaxomicin MIC between pH 6.2 and pH 7; however, the fidaxomicin MIC increased by 8-fold with treatment at the highest pH (pH 7.9). This increase in the MIC was verified by repeating the MIC run at the highest pH (pH 8.0).

The fidaxomicin MICs, obtained by the agar dilution method, were verified by the broth microdilution method over a pH range of 6 to 8. Since the pH of the unbuffered brucella broth, the titer of which was determined in ambient air over a pH range of 5 to 9, dropped significantly over a pH range of 5 to 7.5 inside the glove box environment, buffer was added in the subsequent experiments to resist the pH shifts caused by anaerobic equilibration. Addition of buffer either as 10 mM or 100 mM (NaH2PO4 · H2O, pH 7.0; morpholinopropanesulfonic acid, pH 8.0; or TAPS, pH 9.0 [the pH values are those in ambient air]) to the broth medium (with a pH above 6) resulted in final anaerobic pH ranges of from 6 to 7.6 and 6 to 8.1, respectively. Data from all experiments verified that the MIC values for both fidaxomicin and vancomycin increased with increasing pH. While the organism grew poorly at pH 5.0, at pHs above 6.5, the log of the MIC values for both drugs increased in a roughly linear fashion with the increase in the pH; the MIC values for both drugs at pH 7.5 and pH 8.1 were 8- to 16-fold those obtained by treatment at the lowest pH, pH 6 (Fig. 1).

Overall, with both methods of susceptibility testing and across various concentrations of buffer salts, the fidaxomicin and vancomycin MIC values increased with increasing pH. The high MIC values at basic pH, which has also been reported for other drugs (4, 21), may be due to increasing deprotonation of the phenolic hydroxyl groups of both compounds, forming a charged species that is expected to be less able to permeate bacterial cells. In contrast, under more acidic conditions, the antibiotics will be mostly protonated and should thus permeate the cell membrane more efficiently. It is unlikely that MIC

TABLE 2. In vitro activity of fidaxomicin tested with three different lots of media

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Metronidazole (μg/ml)</th>
<th>Clindamycin (μg/ml)</th>
<th>Fidaxomicin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lot 1</td>
<td>Lot 2</td>
<td>Lot 3</td>
</tr>
<tr>
<td>C. difficile (ATCC 9689)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C. difficile (ATCC 43255)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C. difficile (ATCC 17857)</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Eubacterium lentum (ATCC 43055)</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
</tr>
</tbody>
</table>
trends are the result of the effect of pH on the organism density, as the level of growth (monitored by visual examination) did not always rise with increasing pH. Similarly, it is unlikely that MIC trends are the result of the interaction of the drug with buffer at the high concentrations used. Additional experimentation with various concentrations of buffers (at physiological pH) by the checkerboard method (against aerobic organisms, to avoid the shift in the pH from that in the physiological pH) by the checkerboard method (against aerobic organisms, to avoid the shift in the pH from that in the atmosphere of the glove box with a high CO2 concentration) did not always rise with increasing pH. Similarly, it is unlikely that MIC trends are the result of the effect of pH on the organism density, as the level of growth (monitored by visual examinations in the human intestine).

REFERENCES
14. Reference deleted.

FIG. 1. Effects of pH on broth MIC values of fidaxomicin (A) and vancomycin (B). The results were compiled from three experiments with C. difficile strains ATCC 9689 (open shapes) and ATCC 700057 (filled shapes).