The effect of a meropenem-ciprofloxacin combination (MCC) on the susceptibility of multidrug-resistant (MDR) *Pseudomonas aeruginosa* (MRPA) clinical isolates was determined using checkerboard and time-kill curve techniques. Structural changes and differential gene expression that resulted from the synergistic action of the MCC against one of the *P. aeruginosa* isolates (1071-MRPA) were evaluated using electron microscopy and representational difference analysis (RDA), respectively. The differentially expressed, SOS response-associated, and resistance-associated genes in 1071-MRPA exposed to meropenem, ciprofloxacin, and the MCC were monitored by quantitative PCR. The MCC was synergistic against 25% and 40.6% of MDR *P. aeruginosa* isolates as shown by the checkerboard and time-kill curves, respectively. The morphological and structural changes that resulted from the synergistic action of the MCC against 1071-MRPA were a summation of the effects observed with each antimicrobial alone. One exception included outer membrane vesicles, which were seen in a greater amount upon ciprofloxacin exposure but were significantly inhibited upon MCC exposure. Cell wall- and DNA repair-associated genes were differentially expressed in 1071-MRPA exposed to meropenem, ciprofloxacin, and the MCC. However, some of the RDA-detected, resistance-associated, and SOS response-associated genes were expressed at significantly lower levels in 1071-MRPA exposed to the MCC. The MCC may be an alternative for the treatment of MDR *P. aeruginosa*. The effect of this antimicrobial combination may be not only the result of a summation of the effects of meropenem and ciprofloxacin but also a result of differential action that likely inhibits protective mechanisms in the bacteria.

**Pseudomonas aeruginosa** is a highly successful opportunistic pathogen that displays intrinsic multidrug resistance and has a great ability to acquire further resistance mechanisms (1, 2). Limited classes of antibiotics can be used for the treatment of *P. aeruginosa* infection (3). The emergence of multidrug-resistant (MDR) *P. aeruginosa* (MRPA) has prompted the search for new agents or alternative therapeutics (1, 4, 5), including antimicrobial combinations (6–9), including antimicrobial combinations.

Synergistic interactions between drugs have been investigated to improve therapeutic results, decrease the potential toxicity of antimicrobial agents, and prevent the emergence of bacterial resistance (6, 10, 11). Several studies have evaluated the effects of a meropenem-ciprofloxacin combination (MCC) in *P. aeruginosa* (12–14), including MDR isolates (8, 15). Despite some differences in the results, likely attributable to variations in techniques and bacterial isolates, the MCC represents a promising antipseudomonal therapeutic option (12–14). Although β-lactams and fluoroquinolones represent classic antimicrobials with previously known mechanisms of action, the molecular basis of synergism between these classes of drugs has not yet been elucidated.

Thus, because of high rates of resistance observed in Brazilian *P. aeruginosa* isolates (16) and the consequent possibility of using antimicrobial combination therapy (17), the present study sought to identify bacterial cell structure changes and differential transcript results using electron microscopy (EM) and representational difference analysis (RDA), respectively, in a MDR *P. aeruginosa* isolate exposed to a synergistic antimicrobial combination (MCC) selected from among MDR clinical isolates from a Brazilian university hospital.

**MATERIALS AND METHODS**

**Bacterial strains.** The isolates were selected after screening 75 *P. aeruginosa* isolates from several biological sources from patients admitted to a university hospital in northwestern Paraná, Brazil, from January 2007 to July 2009. A total of 32 genetically distinct MDR *P. aeruginosa* isolates resistant to at least one of the two antimicrobials studied (meropenem and ciprofloxacin) were tested. The resistance profiles and genetic relationships were previously determined (18). The selected isolates were stored in tryptic soy broth (Difco Laboratories, Sparks, MD) with 15% glycerol at −80°C. *P. aeruginosa* ATCC 27853 was used as a control for antimicrobial activity and the synergism tests.

**Determination of MIC.** The MICs of meropenem (lot no. 09405C-1; AstraZeneca, Cotia, Brazil) and ciprofloxacin (lot no. 0001396108; Sigma-Aldrich, Steinheim, Germany) were determined by microdilution using cation-adjusted Mueller-Hinton broth (CAMHB; Difco) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (19), and categorical susceptibility was based on CLSI breakpoints (20). All of the determinations were performed in triplicate.

**Determination of structural changes.** The isolates were selected after screening 75 *P. aeruginosa* isolates from several biological sources from patients admitted to a university hospital in northwestern Paraná, Brazil, from January 2007 to July 2009. A total of 32 genetically distinct MDR *P. aeruginosa* isolates resistant to at least one of the two antimicrobials studied (meropenem and ciprofloxacin) were tested. The resistance profiles and genetic relationships were previously determined (18). The selected isolates were stored in tryptic soy broth (Difco Laboratories, Sparks, MD) with 15% glycerol at −80°C. *P. aeruginosa* ATCC 27853 was used as a control for antimicrobial activity and the synergism tests.

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Checkerboard assay. The MCCs were assessed in microtiter plate checkerboard assays with CAMHB (21). Both drugs were tested alone and combined at concentrations up to four times higher and lower than the MICs of the drugs tested alone. The final inoculum concentration of the *P. aeruginosa* isolates tested was approximately 5.0 × 10⁵ CFU/ml, and the plates were incubated at 35°C for 18 h. The tests were performed in triplicate. Fractional inhibitory concentrations indices (FICIs) were calculated (21). Synergy was defined as FICI ≤ 0.5, no interaction was defined as FICI > 0.5 and ≤ 4, and antagonism was defined as FICI > 4 (22).

Time-kill curve assay. Time-kill curve assays for all of the *P. aeruginosa* isolates were performed in CAMHB. Meropenem, ciprofloxacin, and the MCC were tested at concentrations of 0.5× and 0.25× MIC for each isolate. All of the assays included a growth control with no antimicrobial. The final inoculum used was 10⁵ CFU/ml. The antimicrobial and growth control tubes were continuously shaken on an orbital incubator at 35°C. Aliquots (0.1 ml) were removed at 0, 3, 6, 9, 12, and 24 h and serially diluted in sterile saline solution to avoid antibiotic carryover. Afterward, 20 μl of each dilution was inoculated in tryptic soy agar (TSA; Difco) in triplicate. The plates were incubated at 35°C for 18 to 24 h, colony counts were performed, and the lowest limit of detection was 50 CFU/ml. All of the time-kill assays were performed in duplicate. *P. aeruginosa* ATCC 27853 (susceptible to meropenem and ciprofloxacin) was tested as a control strain. Data from at least two independent experiments were averaged and plotted as log₁₀ CFU/ml versus time (h) for each time point over 24 h. Synergism was defined as a reduction ≥ 2 log₁₀ CFU/ml with the MCC compared with the most active single antibiotic at 24 h, with the number of surviving organisms in the presence of the combination being ≥2 log₁₀ CFU/ml below the starting inoculum (21). Bactericidal activity was defined as a reduction of ≥3 log₁₀ CFU/ml in the colony count from the starting inoculum at 24 h (9).

SEM and TEM. A clinical isolate (1071-MRPA) for which the MCC showed a synergistic action was selected for the evaluation of morphological and structural changes. The 1071-MRPA cells were exposed to 0.5× MIC of meropenem, ciprofloxacin, and the MCC for 3 and 12 h and fixed for at least 2 h with 2.5% glutaraldehyde (Sigma)–0.1 M cacodylate buffer (Electron Microscopy Science, Hatfield, PA) at 4°C. Subcultures on TSA were made to ensure that no contaminating organisms were present. Prior to subculture, dilutions were performed to avoid antibiotic carryover. The electron microscopy experiments were performed in duplicate on different days. For scanning electron microscopy (SEM), the treated cells were placed on a glass support with poly-L-lysine (Sigma), dehydrated in acetone, and embedded in EMbed resin (EMS). Ultrathin 60-nm-thick sections were analyzed using the Phred, Crossmatch, and CAP3 (http://asparaginacealibrary.embbrasp.br/phph/) programs. Sequences with at least 100 nucleotides and Phred quality ≥ 20 were considered for further analysis. The ESTs were grouped in clusters represented by contigs and singlets. The screened sequences were compared to the GenBank (http://www.ncbi.nlm.nih.gov) nonredundant (nr) database from the National Center for Biotechnology Information (NCBI) using the BLASTX algorithm (25) with an E value cutoff at 10⁻⁸.

qPCR. To confirm and quantify the relative transcript levels of the selected RDA products, quantitative PCR (qPCR) was performed using T1, T2, T3, and D cDNA and StepOnePlus real-time PCR systems (version 2; Applied Biosystems, Carlsbad, CA). Transcripts detected by RDA1 (meropenem exposure), RDA2 (ciprofloxacin exposure), and RDA3 (MCC exposure) with redundancy above 10 were tested, including three genes related to meropenem or ciprofloxacin resistance (*mexA, ampC, and oprD*) and one gene related to the SOS response (*lexA*). The primers were designed using Primer Express software (version 3.0, Applied Biosystems), with the exception of the resistance and *lexA* genes (Table 1). The primer concentrations were adjusted to provide amplification efficiencies of approximately 90% to 110% for all of the experiments. PCR was performed using SYBR Select master mix (Applied Biosystems) with 1 μl of cDNA and 400 nM each primer in a total volume of 10 μl. PCR thermal cycling was performed at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. A melting curve analysis was performed to confirm a single PCR product. The cycle threshold (*Ct*) values for the triplicate PCRs and duplicate biological assessments for each cDNA sample were averaged, and then 2⁻ΔΔ*Ct* values were calculated (26). A negative control containing all of the reagents except cDNA was also included. The *rpsL* gene was used to normalize all of the reactions.

The *P. aeruginosa* antimicrobial growth-free cDNA driver (D) was used as a reference sample. The genes were considered up- or downregulated when the amounts of the transcripts were at least 2 times higher or lower than those present in the reference sample (D) (27).

RNA isolation and cDNA synthesis. Overnight TSA (Difco) growth of 1071-MRPA was transferred to four vials that contained CAMHB (Difco), and the vials were incubated at 35°C to obtain growth with an optical density at 600 nm (OD₆₀₀) of 0.4. Meropenem, ciprofloxacin, and the MCC were separately added at a final concentration of 16 μg/ml (corresponding to 0.5× MIC). Three vials containing antimicrobial were referred to as testers (test 1 [T1], T2, and T3, respectively). A vial without antimicrobial was used as a control and referred to as a driver (D). Sixty minutes after the addition of the antimicrobials, treated and control bacterial cells were harvested at 4,700 rpm for 10 min and washed twice with RNase-free ultrapure water. Total RNA was extracted from 1071-MRPA cells for each experimental condition (T1, T2, T3, and D) using an RNAeasy minikit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Quantification purity and quality assessments were performed with a Qubit2.0 fluorometer (Invitrogen, Carlsbad, CA) and on 1% agarose gels, respectively. Contaminating DNA was removed by treatment with RNase-free DNase I (Invitrogen) and subjected to conventional PCR to ensure that all DNA was removed as assessed by agarose gel. First-strand cDNA synthesis was performed with Superscript III reverse transcriptase (RT) (Invitrogen) using 1 μg of DNase-treated total RNA, 0.8 μM oligo(dT) primer (Integrated DNA Technologies, Coralville, IA), and 0.8 μM Random Primers (Invitrogen). Sequentially, the second strand was synthesized using Random Primer and Platinum Taq DNA polymerase.

High Fidelity (Invitrogen) according to the manufacturer’s instructions.

cDNA RDA. Approximately 1 μg of T1, T2, T3, and D cDNAs was digested with Sau3AI (Promega, Madison, WI), and the RDAs for meropenem (RDA1), ciprofloxacin (RDA2), and MCC (RDA3) were performed according to the method of Leite et al. (23), with modification, in rounds of subtractions and amplifications. Three tester-driver hybridizations (1:10, 1:20, and 1:10 ratios) were performed consecutively for each RDA condition. A reverse cDNA-RNA experiment was performed as a control under the same conditions as described above in which the tester cultures were not exposed to the MCC and the driver cultures were exposed to MCC. The final RDA products were purified using a QIAquick PCR purification kit (Qiagen), cloned into pGEM-T-Easy vector (Promega), and transformed into *Escherichia coli* DH5α competent cells. Plasmidial DNA was extracted as previously described (24). Sequencing was performed with universal M13 primers (Promega) and standard fluorescence-labeling dye terminator protocols. The samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare Life Science, Piscataway, NJ) for automated analysis. Expression sequence tags (ESTs) were analyzed using the Phred, Crossmatch, and CAP3 (http://asparaginacealibrary.embbrasp.br/phph/) programs. Sequences with at least 100 nucleotides and Phred quality ≥ 20 were considered for further analysis. The ESTs were grouped in clusters represented by contigs and singlets. The screened sequences were compared to the GenBank (http://www.ncbi.nlm.nih.gov) nonredundant (nr) database from the National Center for Biotechnology Information (NCBI) using the BLASTX algorithm (25) with an E value cutoff at 10⁻⁸.

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The *P. aeruginosa* antimicrobial growth-free cDNA driver (D) was used as a reference sample. The genes were considered up- or downregulated when the amounts of the transcripts were at least 2 times higher or lower than those present in the reference sample (D) (27).
Data analysis. Group comparisons (meropenem, ciprofloxacin, and MCC data determined by qPCR and transmission electron microscopy) were made using one-way analysis of variance (ANOVA) with SAS 9.0 software (SAS OnlineDoc 9; SAS Institute, Cary, NC), followed by the Tukey post hoc test. Values of $P < 0.01$ were considered statistically significant.

RESULTS

Clinical isolate susceptibilities and combination assays. All of the $P. aeruginosa$ isolates were MDR and collected from several biological sources from patients admitted in different hospital units (18). The MIC$_{50}$ and MIC$_{90}$ of meropenem and ciprofloxacin for $P. aeruginosa$ were 4 and 32 $\mu$g/ml and 32 and 64 $\mu$g/ml, respectively. Seventeen of the 32 isolates (53.1%) were resistant to ciprofloxacin. Five of the isolates (15.6%) were resistant to meropenem, and 10 of the isolates (31.3%) were resistant to both antimicrobials (Table 2).

The checkerboard titrations yielded a synergistic FICI ($\leq 0.5$) for eight (25.0%) of the isolates tested. For the remainder of the isolates, the antimicrobial combination displayed no interaction (Table 2). In all of the kill-curve assays, the viable counts in the control with and without the antimicrobial agents tested at 0 h were close to the initial inoculum count, thus ensuring that an antimicrobial carryover effect was avoided. The results of the time-kill assays showed a $2-\log_{10}$ decrease in viable counts for the MCC at 0.5 $\times$ MIC, regardless of the time of bacterial growth, in 31 (96.9%) of the isolates. This decrease was more apparent with 12 h of exposure (87.5% of the isolates). However, synergism as previously defined (21) was observed in 13 (40.6%) of the isolates tested (Table 2). With combinations of 0.25 $\times$ MIC plus 0.5 $\times$ MIC for each antimicrobial and 0.25 $\times$ MIC for both antimicrobials, a $2-\log_{10}$ reduction of viable counts was observed in 10 (31.3%) of the isolates between 6 and 12 h of exposure and in 1 isolate at 24 h of exposure (data not shown). Bactericidal activity was observed in only four (12.5%) of the isolates with the MCC at 0.5 $\times$ MIC (Table 2). Representative time-kill curves for ATCC strains and MDR clinical isolates using 0.5 $\times$ MIC of the meropenem, ciprofloxacin, and MCC are illustrated in Fig. 1a and b, respectively. The time-kill curve of the ATCC strain showed a

| Primer | Primer sequence (5’–3’)a | GenBank accession no. or reference |
|--------|--------------------------|----------------------------------|
| OprF   | F—GGGGCTTCGATGCTTTACTT   | NP_250468.1                      |
|        | R—CGTCATGGTACCTACCCGTTAGGA |                                  |
| Serine acetyltransferase | F—CCGGTGCCTGCGCATCTAC | ZP_15787657.1                   |
|        | R—AAACATCTAAATGACCCGAGAAAAAGA |                              |
| Hydrolase | F—CGAATTTCTTTGTCGCGTAA   | ZP_10486838.1                    |
|        | R—AGGCCCCGCTATGGTATC     |                                  |
| Alanine racemase | F—CGGATGCCGTTGGTTCTAT    | ZP_01680398.1                   |
|        | R—GAGCCCCGCGATACCGTTATC  |                                  |
| Uracil-DNA glycosylase | F—CAGCCCCAGGATGTTGATGA | ZP_01682025.1                   |
|        | R—TCATATCGAGCGGGTGTGT    |                                  |
| Hypothetical protein I | F—TACCCGAAATGGCGACAGCAT   | ZP_22178892.1                   |
|        | R—CTTTTTCACGCAGCGACTTT   |                                  |
| Hypothetical protein II | F—TACCGGCGACGCTACACA     | ZP_07663463.1                   |
|        | R—CGGCTTTGGAACCCCTT      |                                  |
| Hypothetical protein III | F—GGATTAGCCCTTAAAGCATTGA | YP_001949471.1                 |
|        | R—CAATACGGCCGACTTCCA     |                                  |
| rpsL   | F—GCAAGGGCATGGTCGACAAGA  | Dumas et al. (55)                |
|        | R—CGCTGTGGCTTCGGAGGTTTGA |                                  |
| ampC   | F—CGGCTCGGTGAGCAAGCAGCTTC | Dumas et al. (55)               |
|        | R—AGTCCGGATCTGCGCCGTTGTC |                                  |
| mexA   | F—CGACCAGGCCTAGGCGGAACGCC | Dumas et al. (55)               |
|        | R—GGAGACCTTCAGGCGGCTGCGC |                                  |
| oprD   | F—ATCTAAGCGCATAAACGCATGAGG | Dumas et al. (55)             |
|        | R—GCCGAAGCGATATATAATCAAACG |                                   |
| lexA   | F—GGATCAATCCCGCTTCTCTC    | Liu et al. (56)                 |
|        | R—AAGCGTTTTTACGCGGATCTC   |                                  |

a F, forward; R, reverse.
2-log₁₀ reduction of viable counts between 9 and 24 h of exposure (Fig. 1). No reduction ≥ 2-log₁₀ CFU/ml below the starting inoculum was observed with 0.25× MIC against all 32 clinical isolates (data not shown) or with 0.25× (data not shown) and 0.5× MIC (Fig. 1) against the ATCC strain, indicating no synergism.

Morphological and structural changes. Because of the favorable results from the time-kill curves and checkerboard assay, one MDR *P. aeruginosa* clinical isolate (1071-MRPA) was selected for electron microscopy assays after exposure to 0.5× MIC of meropenem, ciprofloxacin, and the MCC at 3 h (i.e., the start of the decrease in viable counts) and 12 h (i.e., the time with a greater decrease in viable counts). Scanning electron microscopy indicated that the effect of ciprofloxacin on cell morphology was less evident than that seen with cells treated with meropenem. Rounding and a wrinkled appearance were observed in 1071-MRPA cells exposed to meropenem (Fig. 2b and f) and ciprofloxacin (Fig. 2c and g), respectively, mainly at 12 h of antimicrobial exposure. The changes caused by the MCC were observed mainly at 12 h of treatment and appeared to be a summation of the observed changes in 1071-MRPA caused by meropenem and ciprofloxacin exposure (i.e., rounding and cell wrinkling; Fig. 2d and h). Structures similar to those of outer membrane vesicles (OMVs) were observed in 1071-MRPA cells exposed to ciprofloxacin at 3 h (Fig. 2c, inset). Transmission electron microscopy was used to observe and quantify changes inside and on the wall of 1071-MRPA cells treated with meropenem, ciprofloxacin, and the MCC. Rounding cells and cell wall disintegration were observed in meropenem-exposed cells (Table 3 and Fig. 3b and f). A low electrondensity of areas without content was observed in ciprofloxacin-treated cells (Table 3 and Fig. 3c and g). Changes in MCC-treated cells were a summation of the effects (cellular rounding and areas without content) observed with each antimicrobial alone (Table 3 and Fig. 3d and h). Cellular changes were observed mainly at 12 h with the different treatments. Outer membrane vesicles were observed in 1071-MRPA cells subjected to the three different treatments, but a significantly greater number of OMVs was observed in ciprofloxacin-treated cells at 3 h (Table 3 and Fig. 3c and inset). A significant decrease in the amount of OMVs was observed in MCC-exposed cells at 3 and 12 h (Table 3 and Fig. 3d and h).

Differentially expressed genes. After three rounds of subtractive cDNA RDA hybridization, several DNA fragments were de-

### TABLE 2 MIC, checkerboard, and time-kill curve results of the multidrug-resistant *Pseudomonas aeruginosa* isolates tested

| Isolate | MIC (µg/ml) | Checkerboard result* | Time-kill curve (time [h] for decrease of ≥2-log₁₀ CFU/ml compared with the most active single antibiotic)** |
|---------|-------------|----------------------|---------------------------------------------------------------|
|         | Ciprofloxacin | Meropenem | FICI | Interpretation |                                      |
| 596     | 32          | 16          | 0.75 | NI             | 6–24*                               |
| 603     | 32          | 16          | 0.50 | S              | 9–12                                |
| 632     | 1           | 16          | 0.65 | NI             | 6–24*                               |
| 751     | 32          | 8           | 1.00 | NI             | 9–24*                               |
| 754     | 64          | 4           | 2.00 | NI             | 9–24*                               |
| 756     | 32          | 64          | 0.37 | S              | 24                                  |
| 758     | 32          | 0.25        | 1.00 | NI             | 12                                  |
| 765     | 64          | 2           | 2.00 | NI             | 12                                  |
| 766     | 0.5         | 64          | 1.00 | NI             | 9–24*                               |
| 783     | 32          | 1           | 0.75 | NI             | 12–24                               |
| 784     | 1           | 128         | 0.65 | NI             | 12                                  |
| 780     | 16          | 4           | 0.50 | S              | 12–24*                              |
| 787     | 512         | 2           | 0.75 | NI             | 12                                  |
| 790     | 32          | 32          | 1.00 | NI             | 12                                  |
| 803     | 0.5         | 32          | 0.75 | NI             | 6–24*                               |
| 805     | 16          | 16          | 0.50 | S              | 9                                   |
| 819     | 32          | 4           | 1.00 | NI             | 12                                  |
| 884     | 512         | 2           | 1.00 | NI             | 12–24                               |
| 907     | 8           | 2           | 1.00 | NI             | 9–24*                               |
| 909     | 512         | 2           | 1.00 | NI             | 24                                  |
| 915     | 0.25        | 32          | 0.63 | NI             | 9–12                                |
| 921     | 32          | 16          | 0.75 | NI             | 12                                  |
| 952     | 16          | 4           | 1.00 | NI             | 12                                  |
| 1001    | 16          | 2           | 0.50 | S              | 6–24*                               |
| 1004    | 32          | 4           | 0.75 | NI             | No decrease                         |
| 1018    | 32          | 4           | 0.31 | S              | 6–24*                               |
| 1020    | 512         | 32          | 0.19 | S              | 6–24*                               |
| 1037    | 32          | 2           | 0.75 | NI             | 12–24                               |
| 1038    | 16          | 2           | 2.00 | NI             | 9–24*                               |
| 1048    | 32          | 2           | 0.75 | NI             | 9–24                                |
| 1071    | 32          | 32          | 0.19 | S              | 6–24*                               |
| 1072    | 16          | 32          | 0.75 | NI             | 6–12                                |
| ATCC    | 0.25        | 1           | 0.75 | NI             | 9–24**                              |

* FICI, fractional inhibitory concentration index; NI, no interaction; S, synergism.

** Data represent results determined with 0.5× MIC of meropenem and ciprofloxacin alone and in combination (MCC). *, synergistic activity; **, synergistic and bactericidal activity.
ected that indicated differential expression. A total of 91, 86, and 89 clones were successfully sequenced, and the RDA1 (meropenem exposure), RDA2 (ciprofloxacin exposure), and RDA3 (MCC exposure) expressed sequence tag data are summarized in Table 4. The most redundant contigs were matched with serine acetyltransferase and OprF in RDA1, with cell wall hydrolase and alanine racemase in RDA2, and with alanine racemase plus a hypothetical protein in RDA3 (Table 4). The singlets formed from ESTs of the different RDAs did not match with the databases and were not considered for further studies. In the reverse-RDA experiment, a total of 38 clones were sequenced. The transcriptional profile did not display any similarity to the one previously observed in the cDNA RDA of *P. aeruginosa* exposed to antimicrobials (data not shown).

**Gene expression quantification.** From the differentially detected transcripts in RDA1, RDA2, and RDA3, eight with a redundancy value higher than 10 were quantified. The genes that encoded OprF and a hypothetical protein were those most highly induced (4.961-fold and 2.112-fold, respectively) in the presence of meropenem. Alanine racemase (2.106-fold) was the most induced by ciprofloxacin exposure. Significantly lower expression was observed for most of the differentially expressed genes in MCC-treated 1071-MRPA (Fig. 4) compared with meropenem and ciprofloxacin exposure alone. Of
the genes related to antimicrobial resistance, no significant difference in the levels of mexA expression was observed with the three treatments. However, ampC and oprD were upregulated when exposed to meropenem and significantly less expressed with MCC exposure (Fig. 4). The same was observed with the lexA SOS response repressor gene.

**DISCUSSION**

The use of antimicrobial combinations has been a strategy to clinically control MDR P. aeruginosa infection (28) and suppress the amplification of resistant subpopulations (10). However, the mechanism of action of the compounds in combination has been little investigated.
In the present study, important synergism of the MCC against MDR P. aeruginosa clinical isolates was observed, mainly reflected by the time-kill curves (40.6%). Some studies investigated this combination for the treatment of P. aeruginosa infection. However, comparisons of these results are hampered because of methodological differences, with differences in the percentages of synergism of 74.5% in the United States (14), 22.0% and 61.0% in Turkey (12), and 8.0% in Greece (8). Also, no synergism of the MCC was observed with American isolates (29) and Italian isolates (30). The checkerboard and time-kill curve assays used in the present study were justified because the best technique for assessing synergism in bacteria is unclear (31,32). A low correlation was found between the checkerboard and time-kill curve results in the present study. The data regarding correlation between the MIC and synergism results were inconclusive. The MCC time-kill curve assay showed synergism mainly in isolates resistant to ciprofloxacin, whereas checkerboard synergism was more evident among isolates that were resistant to both antimicrobials.

The 1071-MRPA cell changes observed by SEM and TEM increased with meropenem, ciprofloxacin, and MCC exposure time. In the present study, the morphological and structural changes observed in MCC-treated cells suggested a summation of the effects observed with meropenem and ciprofloxacin treatment alone. Nonetheless, the number of OMVs detected in MCC-treated cells was significantly lower than the number detected in meropenem- and mainly ciprofloxacin-exposed cells. Outer membrane vesicles are constantly and naturally discharged from the cell wall during Gram-negative bacterial growth (33). These vesicles play an important role in bacterial virulence, transporting enzymes and other virulence factors for the host cell (34, 35). Apparently, these structures can increase the survival of bacteria under conditions of stress, such as the stress produced by antimicrobials (36, 37). The antimicrobial ciprofloxacin targets DNA gyrase and topoisomerase IV, leading to breaks in the DNA strand and thus triggering the SOS response, stimulating bacterial outer membrane vesiculation, and contributing to cytotoxicity (36). Thus, a speculation could be made about the contribution of the MCC in reducing cytotoxicity in the host cell caused by bacterial OMVs.

Waishbren et al. (38) tested several antimicrobial combinations other than the MCC against P. aeruginosa. They observed some cellular changes by SEM that were not observed in cells exposed to the antimicrobials alone. According to those authors, the changes observed involved the protective mechanisms of which the organisms were deprived when exposed to synergistic combinations. Hayami et al. (39) studied MCCs using SEM and found different cellular changes with the MCC compared with the cells exposed to the antimicrobials alone.

In the present study, differences in the gene expression of 1071-MRPA exposed to subinhibitory concentrations of meropenem,

| Antimicrobial treatment | 3 h  | 12 h |
|-------------------------|-----|-----|
| No antimicrobial (control) | 6.8 (0.3) | 7.4 (1.1) |
| Meropenem | 22.0<sup>b</sup> (6.9<sup>b</sup>) | 68.5<sup>b</sup> (11.8<sup>b</sup>) |
| Ciprofloxacin | 23.5<sup>b</sup> (19.2<sup>b</sup>) | 46.9<sup>b</sup> (14.8<sup>b</sup>) |
| MCC | 32.0<sup>d</sup> (4.2<sup>d</sup>) | 92.7<sup>d</sup> (2.6<sup>c</sup>) |

<sup>a</sup> MCC, meropenem-ciprofloxacin combination; OMV, outer membrane vesicles; TEM, transmission electron microscopy.

<sup>b</sup> P < 0.01 compared to control.

<sup>c</sup> P < 0.01 compared to meropenem and ciprofloxacin alone.

<sup>d</sup> P < 0.01 compared to control and to meropenem and ciprofloxacin alone.

FIG 3 Transmission electron micrograph of multidrug-resistant Pseudomonas aeruginosa clinical isolate (1071-MRPA) incubated at 35 ± 2°C in CAMHB for 3 h (a to d) and 12 h (e to h) with a subinhibitory concentration (0.5× MIC) of meropenem (b and f), ciprofloxacin (c [and inset] and g [and inset]), and MCC (d and h). 1071-MRPA in CAMHB without antibiotic was incubated for 3 h (a) and 12 h (e) at 35 ± 2°C. The arrows in panels c and g (including the inset) indicate outer membrane vesicles, while the arrows in panel f indicate cell wall disintegration. Bar, 1 μm (a to h), 200 nm (c inset), or 50 nm (g inset).
Critical considerations when studying bacterial gene expression are the exposure time and antimicrobial concentrations to be used for analysis. The literature on this subject has been quite variable (40–42). A long exposure time and different concentrations may be related to toxic and side effects and may not represent the primary target of the antimicrobial (23). In the present study, we used 60 min of exposure to 0.5×MIC (meropenem, ciprofloxacin, and the MCC), which represents the beginning of the reduction of viable cells, and antimicrobial concentrations with better synergistic action, assessed by time-kill assays. Considering the evidence of polyadenylated mRNA in *P. aeruginosa* (43), the cDNA was obtained with random oligonucleotide hexamers plus oligo(dT) primers. This strategy was used in an attempt to have the highest fidelity in the analysis of gene expression by constructing cDNA.

By RDA analyses, transcripts related to cell wall repair or synthesis, DNA repair, and bacterial metabolism as well as several hypothetical proteins of unknown function were observed. The ciprofloxacin, and the MCC were identified compared with 1071-MRPA not exposed to antimicrobial agents.

### TABLE 4 ESTs of multidrug-resistant *Pseudomonas aeruginosa* clinical isolate (1071-MRPA) cultured in the presence of 0.5×MIC of meropenem and ciprofloxacin alone and in combination

| Antimicrobial treatment | Gene product | Function | Organism best hit/accession no. | E-value | Redundancy |
|-------------------------|--------------|----------|--------------------------------|---------|------------|
| Meropenem (RDA1)        | Serine acetyltransferase | L-cysteine biosynthesis | *Vibrio cholerae* /ZP_15787637.1 | 3e-10   | 26         |
| Meropenem (RDA1)        | OprF         | outer membrane protein | *Pseudomonas aeruginosa* /NP_250468.1 | 3e-10   | 22         |
| Hypothetical protein (Hyp I) | Unknown | Unknown | *Escherichia coli* /ZP_22178892.1 | 8e-11   | 18         |
| Hypothetical protein (Hyp I) | Unknown | Unknown | Uncultured *Chromatiales* bacterium /AD118284.1 | 2e-11   | 10         |
| Hypothetical protein (Hyp I) | Unknown | Unknown | Uncultured *gammaproteobacterium* /AD116715.1 | 3e-13   | 9          |
| Hypothetical protein (Hyp I) | Unknown | Unknown | — | — | 6         |
| Hypothetical protein (Hyp I) | Unknown | Unknown | — | — | 4          |

Ciprofloxacin (RDA2)

| Antimicrobial treatment | Gene product | Function | Organism best hit/accession no. | E-value | Redundancy |
|-------------------------|--------------|----------|--------------------------------|---------|------------|
| Ciprofloxacin (RDA2)    | Cell wall-associated hydrolase | Cell envelope biogenesis, outer membrane | *Enterobacter radioresistens* /ZP_10486838.1 | 7e-08   | 19         |
| Ciprofloxacin (RDA2)    | Alanine racemase | Cell wall biosynthesis | *Vibrio cholerae* /ZP_01680398.1 | 4e-06   | 16         |
| Ciprofloxacin (RDA2)    | Uracil-DNA glycosylase | DNA repair | *Vibrio cholerae* /ZP_01682025.1 | 2e-06   | 12         |
| Ciprofloxacin (RDA2)    | Hypothetical protein (Hyp III) | Unknown | *Burkholderia multivorans* /YP_001949471.1 | 3e-08   | 11         |
| Ciprofloxacin (RDA2)    | Hypothetical protein (Hyp III) | Unknown | *Escherichia coli* /ZP_22178892.1 | 2e-15   | 10         |
| Ciprofloxacin (RDA2)    | Hypothetical protein (Hyp III) | Unknown | *Escherichia sp.* /ZP_04532941.1 | 3e-06   | 8          |
| Ciprofloxacin (RDA2)    | Hypothetical protein (Hyp III) | Unknown | — | — | 6         |
| Ciprofloxacin (RDA2)    | Hypothetical protein (Hyp III) | Unknown | — | — | 4          |

MCC (RDA3)

| Antimicrobial treatment | Gene product | Function | Organism best hit/accession no. | E-value | Redundancy |
|-------------------------|--------------|----------|--------------------------------|---------|------------|
| MCC (RDA3)              | Conserved hypothetical protein (Hyp II) | Unknown | *Vibrio parahaemolyticus* /ZP_07663463.1 | 4e-36   | 19         |
| MCC (RDA3)              | Alulne racemase | Cell wall biosynthesis | *Vibrio cholerae* /ZP_01680398.1 | 2e-23   | 15         |
| MCC (RDA3)              | Conserved hypothetical protein | Unknown | *Escherichia sp.* /ZP_04532941.1 | 1e-05   | 12         |
| MCC (RDA3)              | Glycosyl transferase | Cell envelope biogenesis, outer membrane | *Rhodospirillum photometricum* /YP_005415586.1 | 1e-26   | 10         |
| MCC (RDA3)              | Uracil-DNA glycosylase | DNA repair | *Vibrio cholerae* /ZP_01682025.1 | 3e-35   | 8          |
| MCC (RDA3)              | Cell wall-associated hydrolase | Cell envelope biogenesis, outer membrane | *Vibrio cholerae* /EHH98088.1 | 1e-06   | 8          |
| MCC (RDA3)              | Hypothetical protein | Unknown | *Salmonella enterica subsp. enterica serovar Choleraesuis* /AAX64155.1 | 1e-22   | 8          |
| MCC (RDA3)              | Hypothetical protein | Unknown | *Clostridium bartlettii* /ZP_02210961.1 | 6e-21   | 6          |
| MCC (RDA3)              | Hypothetical protein | Unknown | *Vibrio hofmannii* /ZP_08745892 | 5e-13   | 3          |

*ESTs, expressed sequence tags; MCC, meropenem-ciprofloxacin combination. ^a^ Accession numbers at GenBank (http://www.ncbi.nlm.nih.gov). ^b^ —, no match.

**FIG 4** Transcript levels of differentially expressed genes from multidrug-resistant *Pseudomonas aeruginosa* clinical isolate (1071-MRPA) exposed to 0.5×MIC of meropenem and ciprofloxacin alone and in combination (MCC) normalized to 1071-MRPA grown in antimicrobial-free culture medium. *, P < 0.01 compared to the other two treatments. AlaRac, alanine racemase gene; SerAt, serine acetyltransferase gene; UDG, uracil-DNA glycosylase gene; HypI, HypIII, and HypIII, genes encoding hypothetical proteins.
most redundant contig detected by the RDA in meropenem-exposed 1071-MRPA (RDA1) was a transcript related to serine acetyltransferase protein. Additionally, similar and reduced expression levels of this protein were detected by qPCR in 1071-MRPA exposed to ciprofloxacin and the MCC (P > 0.01), respectively. Serine acetyltransferase catalyzes the first step of a two-step reaction pathway for \(\text{L}\)-cysteine biosynthesis from \(\text{L}\)-serine in bacteria, fungi, and plants (44), but the relationship between this enzyme and the antimicrobials studied here remains to be clarified. The redundancy of OprF observed in RDA1 was confirmed by qPCR, which demonstrated the (4.9-fold) overexpression of oprF. This protein is the most abundant outer membrane porin of \(P. \text{aeruginosa}\) (45), and its role in transporting antimicrobials is not well understood (46). OprF also plays an important role in maintaining cell shape, particularly in adverse environments (47).

The overexpression of oprF observed in 1071-MRPA cells exposed to meropenem could be an attempt to maintain cell shape, which is quite altered by the action of meropenem, as demonstrated by SEM and TEM. Previous RDA and qPCR studies conducted in our laboratory with \(P. \text{aeruginosa}\) ATCC 27853 (susceptible strain) showed that OprF was also differentially overexpressed in cells exposed to meropenem (data not shown). Recent studies suggest that OprF is related to virulence factor production, including the formation of OMVs, through the modulation of the levels of a quorum sensing signal, the \(Pseudomonas\) quinolone signal (PQS) (48, 49). These findings reinforce the idea of a relationship between OprF and meropenem. However, the relationship between OprF and MCC needs additional studies.

Cell wall hydrolase levels differentially detected in ciprofloxacin-exposed 1071-MRPA showed similar levels of expression with meropenem exposure, too. This enzyme, which appears to be involved in cell wall biosynthesis and remodeling by promoting peptidoglycan hydrolysis (50), exhibited lower expression in MCC-exposed cells (\(P < 0.01\)). A decrease in cell wall repair levels and hence in the bacterial defensive response could explain the higher activity of the MCC against 1071-MRPA.

The gene that encodes the alanine racemase, differentially expressed in 1071-MRPA under conditions of exposure to ciprofloxacin and MCC, was overexpressed under conditions of ciprofloxacin exposure. Alanine racemase is a bacterial enzyme that catalyzes the interconversion of \(L\) and \(D\)-alanine, which is an amino acid that represents an important component of peptidoglycan in most bacteria (51). Low concentrations of ciprofloxacin alter the structure of peptidoglycan (52). Thus, we may infer that the bacteria also overexpressed alanine racemase to repair the peptidoglycan as a defensive mechanism. Uracil-DNA glycosylase proteins were also differentially expressed in 1071-MRPA exposed to ciprofloxacin and the MCC. However, they were expressed more intensely (\(P < 0.01\)) in meropenem-exposed 1071-MRPA. This enzyme plays an important role in preventing mutagenesis, eliminating uracil from DNA molecules by cleaving the \(N\)-glycosylic bond, and initiating the base excision repair pathway (53).

Further studies are needed to better understand the relationship between serine acetyltransferase, alanine racemase, and uracil-DNA glycosylase in the response of \(P. \text{aeruginosa}\) to the tested antimicrobials.

1071-MRPA is resistant to ciprofloxacin and carbapenems (i.e., imipenem and meropenem), but metallo-\(\beta\)-lactamase was not detected by genotypic and phenotypic analysis (data not shown). The loss or decreased expression of the outer membrane porin OprD, coupled with the overexpression of the efflux pump and AmpC, is another important mechanism of resistance in \(P. \text{aeruginosa}\) (17) and could be a mechanism involved in the resistance of 1071-MRPA to meropenem and ciprofloxacin. Based on this, the \(\text{ampC, mexA, and oprD}\) genes were tested by qPCR to gain additional knowledge about the effect of the MCC on 1071-MRPA. A significant difference in the levels of \(\text{ampC}\) and \(\text{oprD}\) expression in 1071-MRPA was observed with meropenem, ciprofloxacin, and MCC exposure. As expected, 1071-MRPA exposed to meropenem, a \(\beta\)-lactam, showed \(\text{ampC}\) and \(\text{oprD}\) overexpression. An intriguing observation was the significantly lower expression of \(\text{ampC}\) and \(\text{oprD}\) with MCC exposure, which will require further study.

The use of certain antibiotics, including ciprofloxacin, triggers the SOS response, allowing the bacteria to adapt to the environment or host, in addition to contributing to the development of resistance (37, 54). The SOS response in \(P. \text{aeruginosa}\) involves the controlled derepression of 15 genes by the LexA repressor (41). In the present study, \(\text{lexA}\) was overexpressed with meropenem and ciprofloxacin exposure, indicating that the transcription of the SOS genes was activated. These data are consistent with the SEM and TEM OMV findings, which corroborate the results of Maredia et al. (36). Thus, as suggested by Cirz et al. (37), an inhibitor of LexA cleavage might have a favorable effect in \(P. \text{aeruginosa}\) therapy. Nonetheless, with MCC treatment, lower \(\text{lexA}\) expression and OMV production was found. Many questions remain unanswered about LexA-regulated expression in \(P. \text{aeruginosa}\), and further studies could clarify the MCC results obtained in the present study.

Although the present results need to be corroborated by additional studies, they suggest that the MCC could be an alternative for the treatment of infections caused by MDR \(P. \text{aeruginosa}\). The effects of this antimicrobial combination may result not only from the summation of the effects of meropenem and ciprofloxacin but also from differential actions that likely involve the protective mechanisms of the bacteria.

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