

# Bactericidal Effect of Tomatidine-Tobramycin Combination against Methicillin-Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* Is Enhanced by Interspecific Small-Molecule Interactions

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This study investigated the antibacterial activity of the plant alkaloid tomatidine (TO) against *Staphylococcus aureus* grown in the presence of *Pseudomonas aeruginosa*. Since the *P. aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) is known to cause a respiratory deficiency in *S. aureus* and respiratory-deficient *S. aureus* are known to be hypersensitive to TO, we assessed kill kinetics of TO (8 µg/ml) against *S. aureus* in coculture with *P. aeruginosa*. Kill kinetics were also assessed using *P. aeruginosa* mutants deficient in the production of different exoproducts and quorum sensing-related compounds. After 24 h in coculture, TO increased the killing of *S. aureus* by 3.4 log<sub>10</sub> CFU/ml in comparison to that observed in a coculture without TO. The effect of TO was abolished when *S. aureus* was in coculture with the *lasR rhIR*, *pqsA*, *pqsL*, or *lasA* mutant of *P. aeruginosa*. The bactericidal effect of TO against *S. aureus* in coculture with the *pqsL* mutant was restored by supplemental HQNO. In an *S. aureus* monoculture, the combination of HQNO and TO was bacteriostatic, indicating that the *pqsL* mutant produced an additional factor required for the bactericidal effect. The bactericidal activity of TO was also observed against a tobramycin-resistant methicillin-resistant *S. aureus* (MRSA) in coculture with *P. aeruginosa*, and the addition of tobramycin significantly suppressed the growth of both microorganisms. TO shows a strong bactericidal effect against *S. aureus* when cocultured with *P. aeruginosa*. The combination of TO and tobramycin may represent a new treatment approach for cystic fibrosis patients frequently colonized by MRSA and *P. aeruginosa*.

Cystic fibrosis (CF) is a common life-shortening autosomal recessive disorder. The disease is linked to a mutation of the CF transmembrane conductance regulator (CFTR) gene that leads to intestinal and pulmonary disorders (1). Although CFTR functions mainly by transporting Cl<sup>-</sup>, bacterial pulmonary infections are the primary causes of mortality in CF patients (2). The two most prevalent bacterial species recovered from the lungs of CF patients are *Staphylococcus aureus* and *Pseudomonas aeruginosa* (3), which can individually cause difficult-to-treat infections due to their ability to produce biofilms and a plethora of toxins affecting the integrity of host tissues and the immune system (4, 5). These pathogens are also well known for their resistance to antibiotics (5, 6). Notably, the prevalence of methicillin-resistant *S. aureus* (MRSA) in CF has steadily increased in recent years (3), and Dassenbrook et al. have demonstrated a decline in health conditions in patients carrying MRSA compared to those infected by methicillin-sensitive *S. aureus* (7, 8).

The lungs of CF patients are colonized by numerous bacterial species that are likely to interact (9, 10). A reported antagonistic interaction of *P. aeruginosa* with *S. aureus* is due to the production of electron transport chain inhibitors such as 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) and pyocyanin and also to the production of the staphylolytic LasA protease that can act as an endopeptidase targeting *S. aureus* peptidoglycan (11, 12). On the other hand, the presence of *P. aeruginosa* HQNO may select for *S. aureus* small-colony variants (SCVs) (13–15), which are often associated with persistent infections (16). *S. aureus* SCVs are respiratory-deficient bacteria and, consequently, have a lower growth rate and are less susceptible to aminoglycoside antibiotics, which

need the proton motive force (PMF) for efficient penetration into bacterial cells (16). The action of *P. aeruginosa* HQNO also stimulates biofilm production by prototypical *S. aureus* (17) and may thus affect the bacterial susceptibility to drugs (18, 19). Cocolonization of *P. aeruginosa* and *S. aureus* is frequent, and their combined presence in adult CF patients can often be underestimated (17). The deleterious effects of MRSA and *P. aeruginosa* coinfections on CF adults have been reported previously (20).

Tomatine is a steroidal glycoalkaloid acting as an important antimicrobial defense metabolite for many solanaceous plants like the tomato (21). Tomatine presents some antimicrobial action against yeast and fungi, and many fungal plant pathogens produce tomatinases that are able to detoxify the tomatine into its aglycon

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TABLE 1 *P. aeruginosa* strains and mutants used in this study

Strain or mutant	Relevant characteristics	Altered biosynthesis	Reference or source
PA14	Clinical isolate UCBPP-PA14	None	31
<i>lasR rhIR</i> mutant	PA14 <i>lasR rhIR</i> ; Gen <sup>r</sup> Tet <sup>r</sup>	Altered in QS circuitry and all AQ production	32
<i>pqsA</i> mutant	PA14 <i>pqsA</i> ::TnphoA; Kan <sup>r</sup>	HHQ, <sup>a</sup> PQS, <sup>b</sup> HQNO <sup>b</sup>	33
<i>pqsL</i> mutant	PA14 <i>pqsL</i>	HQNO	34
<i>rhIA</i> mutant	PA14 <i>rhIA</i> ::TnMrT7; Gen <sup>r</sup>	Rhamnolipids	35
<i>lasA</i> mutant	PA14 <i>lasA</i> ::TnMrT7; Gen <sup>r</sup>	Endopeptidase LasA	35
<i>hcnA</i> mutant	PA14 <i>hcnA</i> ::TnMrT7; Gen <sup>r</sup>	Hydrogen cyanide	35
<i>phzC1C2</i> mutant	PA14 <i>phzC1 phzC2</i> double deletion	Phenazines	Our unpublished results
<i>phzM</i> mutant	PA14 with a Gen <sup>r</sup> cassette inserted into PA4209 ( <i>phzM</i> ); Gen <sup>r</sup>	Pyocyanin	P. Cornelis <sup>c</sup>

<sup>a</sup> HHQ, 2-heptyl-4(1H)-quinolone.

<sup>b</sup> Altered biosynthesis of the precursor molecule.

<sup>c</sup> The strain was a generous gift from P. Cornelis (Free University of Brussels, Department of Microbiology, Brussels, Belgium).

form tomatidine (TO), which has little to no antifungal activity against pathogens (22). Some studies have shown a variety of anticancer and anti-inflammatory effects for tomatine and to a lesser degree for TO. Such activities require concentrations in the micromolar range. For example, TO had little or no cytotoxic effects on normal liver cells (Chang), normal lung cells (Hel299), prostate cancer cells (PC3), breast cancer cells (MDA-MB-231), gastric adenocarcinoma (KATO-III), or human myeloid leukemia HL-60 cells compared to those of tomatine, showing once again that the lycotetraose substitution of  $\alpha$ -tomatine is essential for activity against eukaryotic cells (23, 24). On the other hand, our laboratory recently reported the very potent and selective antibacterial activity of TO (25, 26, 27). TO has strong inhibitory activity against *S. aureus*, *Listeria*, and *Bacillus* SCVs (activities in the nanomolar range with MICs of 0.015 to 0.06  $\mu$ g/ml) but not against the prototypical strains of such *Bacillales* or any other bacterial species (MICs of >64  $\mu$ g/ml). Such selectivity also allows no cytotoxicity in cell culture at concentrations showing intracellular killing of SCVs (26). More recently, we have identified for the first time the putative target of TO in *S. aureus*: the bacterial ATP synthase subunit c (28). The ATP synthase uses the PMF generated by the respiratory chain to produce energy for the bacterial cell. The bacterial ATP synthase is now considered a validated antibiotic target following the recent approval of the antituberculosis agent bedaquiline (29, 30). Bedaquiline targets the ATP synthase subunit c and is a very selective antibacterial agent for *Mycobacterium*, which is reminiscent of the high selectivity of TO for *Bacillales*.

Since TO-hypersensitive SCVs are respiratory-deficient strains and HQNO was shown to sensitize prototypical *S. aureus* to TO (26) and to select for the SCV phenotype (15), we hypothesized that TO might strongly inhibit prototypical *S. aureus* cocultured with bacterial species producing electron transport inhibitors. This study characterized the antibacterial effect of TO on prototypical *S. aureus* in coculture with *P. aeruginosa* strains producing the electron transport inhibitor HQNO and also other exoproducts that might antagonize *S. aureus*. In addition, since tobramycin (TOB) is regularly used for prophylaxis against *P. aeruginosa* infections in CF patients, we examined the effects of the TO and TOB combination on both pathogens in cocultures. The resulting synergy between TO and *P. aeruginosa* exoproducts or TOB that we report here provides additional support for the possible usefulness of TO or related drug scaffolds in CF.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The antibiotic susceptibility test strain *S. aureus* ATCC 29213 and the methicillin-resistant *S. aureus* (MRSA) strain CF27A-L were used in this study. MRSA CF27A-L is a clinical isolate from an adult CF patient and is resistant to oxacillin, tobramycin (TOB), ciprofloxacin, and erythromycin with MICs of >128, >32, >64, and >64  $\mu$ g/ml, respectively. *P. aeruginosa* PA14 was the prototypical reference strain used in the present study (31). The relevant properties of the *P. aeruginosa* PA14-derived *pqsA*, *pqsL*, *rhIA*, *lasA*, *hcnA*, *phzC*, *phzM*, and *lasR rhIR* mutants are listed in Table 1. Bacteria were grown in cation-adjusted Mueller-Hinton broth (CAMHB) (BD, Mississauga, ON, Canada).

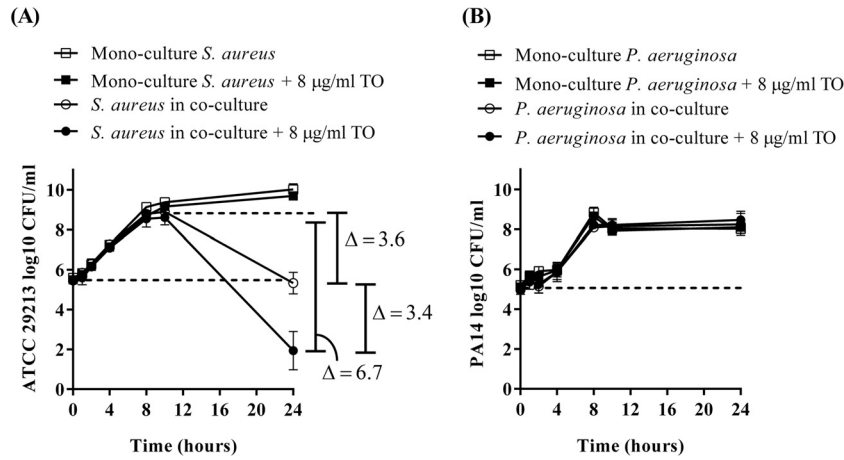
**Reagents.** TOB and TO were purchased from Sigma-Aldrich (Oakville, ON, Canada). TO was solubilized at 2 mg/ml in dimethyl sulfoxide (DMSO). HQNO (Axxora, San Diego, CA, USA) was solubilized in DMSO at a concentration of 5 mg/ml and used at 20  $\mu$ g/ml.

**Antibiotic susceptibility testing.** Antibiotic MICs were determined by a broth microdilution technique in CAMHB, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (36).

**Kill kinetics experiments.** The bactericidal action of TO on *S. aureus* strains was investigated in kill kinetics experiments. *S. aureus* was grown alone (monoculture) or in the presence of *P. aeruginosa* (coculture) in order to investigate the combined effect of TO and *Pseudomonas* exoproducts. The individual bacterial strains, inoculated at  $\sim 10^5$  to  $10^6$  CFU/ml, were grown in 100 ml of CAMHB. The cultures were incubated for 24 h at 35°C with shaking (225 rpm). The samples were collected at several points in time, serially diluted, and plated on mannitol salt agar (MSA) (BD) and cetrinide agar plates (Sigma) for determination of viable *S. aureus* and *P. aeruginosa* counts, respectively. Plates were incubated for 24 h at 35°C. In some experiments, the combined effect of TO and TOB on both *S. aureus* and *P. aeruginosa* was determined. For the cocultures of MRSA CF27A-L and *P. aeruginosa* PA14, 8  $\mu$ g/ml of TO and 0.5  $\mu$ g/ml of TOB were added after 0 and 10 h of incubation, respectively. Control cultures without TO were supplemented with the equivalent concentration of DMSO. Data were collected from at least three independent experiments and averaged.

## RESULTS

**TO is bactericidal to *S. aureus* ATCC 29213 in the presence of *P. aeruginosa* PA14.** Although TO has not shown any significant antibacterial activity on its own against prototypical *S. aureus* in monocultures (25, 26, 27), we evaluated the therapeutic potential of TO in a polymicrobial context by following time-kill kinetics of *S. aureus* ATCC 29213 in coculture with *P. aeruginosa* PA14. First, Fig. 1A confirms that 8  $\mu$ g/ml of TO has no inhibitory activity on a *S. aureus* ATCC 29213 monoculture (TO showed a MIC of >64  $\mu$ g/ml against this strain [25, 26, 27]). In addition, a coculture

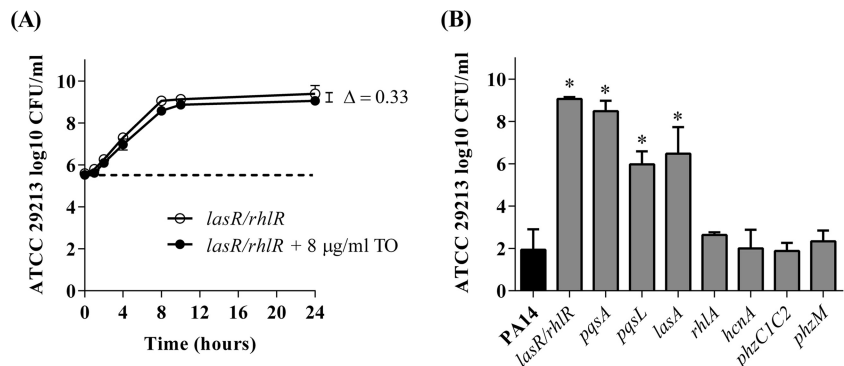


**FIG 1** Kill kinetics of *S. aureus* ATCC 29213 and *P. aeruginosa* PA14 in the absence or presence of TO. Viability of *S. aureus* (A) and *P. aeruginosa* (B) expressed in  $\log_{10}$  CFU/ml as determined by plating sample dilutions on MSA and cetrимide agar, respectively.  $\square$ , monocultures exposed to the antibiotic diluent (DMSO);  $\blacksquare$ , monocultures exposed to 8  $\mu\text{g/ml}$  of TO;  $\circ$ , cocultures exposed to the antibiotic diluent;  $\bullet$ , cocultures exposed to 8  $\mu\text{g/ml}$  of TO. The dotted lines represent reference points at time 0 h (starting inoculum) and 10 h.

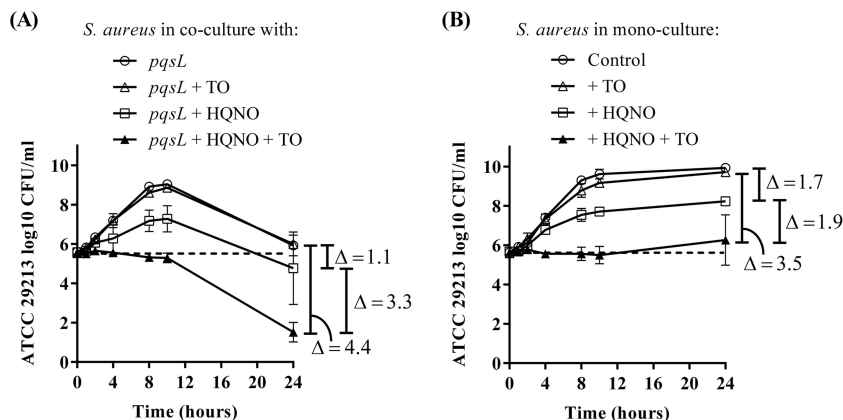
with *P. aeruginosa* PA14 provoked a 3.6- $\log_{10}$  decrease in *S. aureus* CFU/ml between the 10- and 24-h time points. Remarkably, the addition of TO to the coculture further enhanced the antagonistic effect of PA14 on ATCC 29213 by an additional 3.4  $\log_{10}$  CFU/ml. Overall, a difference of 6.7  $\log_{10}$  *S. aureus* CFU/ml was observed for the coculture with TO between the 10- and 24-h time points. On the other hand, the viability of *P. aeruginosa* PA14 was not affected by the presence of *S. aureus* (monoculture versus coculture) with or without the addition of TO (Fig. 1B).

***P. aeruginosa* PA14 QS-associated components and signaling molecules contribute to the bactericidal effect of TO on *S. aureus*.** In order to determine how *P. aeruginosa* enhances the bactericidal effect of TO against *S. aureus*, we performed coculture experiments using PA14 mutants defective in the production of a variety of quorum-sensing (QS)-associated components and signaling molecules (Table 1). Figure 2A shows that the *P. aeruginosa lasR rhIR* double mutant, which does not produce any *N*-acylhomoserine lactones (AHLs) (37), completely lost its ability to produce bactericidal synergy with TO against *S. aureus*. AHLs are

implicated in the regulation of the 2-alkyl-4-(1H)-quinolone (AQ) molecules (37), such as the *Pseudomonas* quinolone signal (PQS) and 4-hydroxy 2-heptyl-quinoline *N*-oxide (HQNO). AHLs are required for the production of rhamnolipids, hydrogen cyanide, and phenazines. Figure 2B shows further results with other mutants presenting specific defects in the production of AHL-regulated factors. The bactericidal activity of TO against *S. aureus* was not influenced by *P. aeruginosa* mutants altered in the production of rhamnolipids (*rhlA*), hydrogen cyanide (*hcnA*), or phenazines (*phzC1C2* and *phzM*). On the other hand, the bactericidal effect of TO was strongly attenuated for the *P. aeruginosa* mutants *pqsA*, which is impaired in the synthesis of AQS (precursors of HQNO) (25), and *pqsL*, a mutant unable to produce HQNO (35). Hence, the altered production of HQNO is shared among the *lasR rhIR*, *pqsA*, and *pqsL* mutants, which are unable to create a synergy with TO (Fig. 2B). Interestingly, the ability of the *lasA* mutant, a mutant strain deficient in the production of the staphylolytic LasA protease (12), to provide a synergy with TO against *S. aureus* was also impaired (Fig. 2B).



**FIG 2** Kill kinetics of *S. aureus* ATCC 29213 in coculture with *P. aeruginosa* PA14 or PA14 mutants. (A) Viability of *S. aureus* in coculture with the PA14 *lasR rhIR* double mutant, expressed in  $\log_{10}$  CFU/ml as determined by plating sample dilutions on MSA agar.  $\circ$ , coculture exposed to the antibiotic diluent;  $\bullet$ , coculture exposed to 8  $\mu\text{g/ml}$  of TO. The dotted line represents the inoculum size at 0 h. (B) Residual *S. aureus* in coculture with PA14 or a variety of PA14 mutants, all in the presence of TO (8  $\mu\text{g/ml}$ ), expressed in  $\log_{10}$  CFU/ml as determined by plating sample dilutions on MSA agar. The bars represent the means  $\log_{10}$  CFU/ml after 24 h of coculture for at least three independent replicates with standard deviation. One-way analysis of variance was performed with Dunnett's posttest using the PA14 coculture as the reference experiment (\*,  $P < 0.0001$ ).



**FIG 3** Effect of HQNO on the kill kinetics of TO against *S. aureus* ATCC 29213 in a coculture with mutant *P. aeruginosa pqsL* (A) or in a *S. aureus* monoculture (B). Viability of *S. aureus* is expressed in log<sub>10</sub> CFU/ml as determined by plating sample dilutions on MSA agar. ○, control cultures supplemented with the antibiotic diluent (DMSO); △, cultures with 8 μg/ml of TO; □, cultures supplemented with 20 μg/ml of HQNO; ▲, cultures supplemented with both TO and HQNO at the concentrations mentioned above. The dotted line represents the inoculum size at 0 h.

***P. aeruginosa* HQNO production contributes to the bactericidal effect of TO on *S. aureus*.** In order to confirm the implication of HQNO in the bactericidal action of TO against *S. aureus*, we chemically complemented a coculture of *S. aureus* and the *P. aeruginosa pqsL* mutant with 20 μg/ml of HQNO. Figure 3A shows the growth kinetics of *S. aureus* in coculture with the *P. aeruginosa pqsL* mutant supplemented or not with HQNO and/or TO. There was no difference in the antistaphylococcal activity of the *P. aeruginosa pqsL* mutant against *S. aureus* whether or not TO was added to the coculture. However, the addition of HQNO to the coculture improved the killing of *S. aureus* by the *pqsL* mutant by 1.1 log<sub>10</sub> CFU/ml at 24 h. TO further enhanced the antagonist effect of the HQNO-complemented *pqsL* mutant on *S. aureus* by an additional 3.3 log<sub>10</sub> CFU/ml after 24 h of incubation (Fig. 3A). Overall, a difference of 4.4 log<sub>10</sub> CFU/ml was observed between the *S. aureus* coculture with the *pqsL* mutant and the coculture supplemented with both HQNO and TO at 24 h. In addition, we performed a monoculture of *S. aureus* exposed to 8 μg/ml of TO and/or 20 μg/ml of HQNO (Fig. 3B). As seen before, the addition of TO did not affect the viability of *S. aureus* in the monoculture. The presence of HQNO alone led to a reduction of *S. aureus* counts by 1.7 log<sub>10</sub> CFU/ml compared to that in the control culture after 24 h. Overall, a difference of 3.5 log<sub>10</sub> CFU/ml was observed between the *S. aureus* monoculture with TO and the monoculture supplemented with both HQNO and TO at 24 h, showing that HQNO can enhance the antibacterial effect of TO (or vice versa) against *S. aureus*. However, at the concentration used, the combination of HQNO and TO only induced a bacteriostatic effect, considering that the CFU counts at 24 h did not differ from that of the initial inoculum. This indicates that at least one other important factor is contributing to the strong bactericidal effect of the HQNO-complemented *pqsL* mutant in the presence of TO (Fig. 3A). This factor might be LasA, which is still produced by the *pqsL* mutant. This explains the complete lack of bactericidal activity of the *lasR rhIR* double mutant against *S. aureus* even in the absence or presence of TO since this mutant is altered in the production of both LasA and HQNO (Fig. 2A).

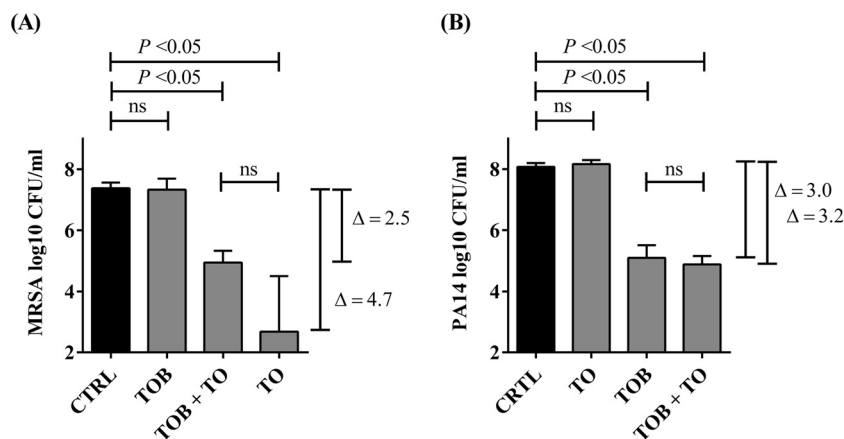
**Combination of TO and TOB kills both a multiresistant MRSA strain and *P. aeruginosa* PA14 in coculture.** Due to the frequent coisolation of *S. aureus* and *P. aeruginosa* from the spu-

tum of CF patients, we examined the possibility of taking advantage of the bactericidal synergy between TO and the presence of *P. aeruginosa* to efficiently kill a multiresistant MRSA strain in coculture with *P. aeruginosa*. Furthermore, since TOB is frequently used as a prophylactic agent against *P. aeruginosa*, we tested the efficacy of the combination of TO and TOB against both pathogens in coculture. Figure 4A reports the viability of the MRSA strain in coculture with PA14 and supplemented or not with TO and/or TOB. As expected, TOB did not affect the CFU counts of the TOB-resistant MRSA strain, but the addition of TO (8 μg/ml) or TO combined with TOB (0.5 μg/ml) significantly suppressed its growth at 24 h compared to that of the control culture. In the same cocultures, *P. aeruginosa* PA14 was not affected by the presence of TO, but the addition of TOB (or TOB combined with TO) also significantly suppressed its growth compared to the control at 24 h (Fig. 3B). Note that in these cocultures, TO was added at the time of inoculation, whereas TOB was added after 10 h to allow possible accumulation of some *P. aeruginosa* exoproducts such as HQNO and LasA.

## DISCUSSION

In CF, bacterial infections remain the most common reason for pulmonary exacerbation and hospitalization. *S. aureus* and *P. aeruginosa* are the most common pulmonary pathogens in CF. Cocolonization of CF lungs by *P. aeruginosa* and *S. aureus* is frequent (17) and may potentially affect the patient's health. One of the major issues in the control of bacterial infections in CF is the rising prevalence of multiresistant bacteria. In the United States, the prevalence of MRSA within the population of CF patients increased to 26.5% in 2012 (3). A recent study observed that MRSA in association with *P. aeruginosa* greatly impacts the health status of CF patients (20). The use of inhaled tobramycin for prophylaxis and control of *P. aeruginosa* infection is now widespread (38, 39), but new strategies to tackle both *P. aeruginosa* and *S. aureus*/MRSA together seem to be eminently needed.

In this study, we have investigated the bactericidal action of TO against *S. aureus* in a coculture with *P. aeruginosa*. Biswas et al. have previously observed a bactericidal effect of *P. aeruginosa* on *S. aureus* in planktonic growth (14), although, as seen here, killing is incomplete and *S. aureus* viability in coculture remains high (10<sup>6</sup>



**FIG 4** Residual viable counts of MRSA CF27A-L (A) or *P. aeruginosa* PA14 (B) after 24 h of coculture in the absence or presence of TO (8  $\mu$ g/ml), TOB (0.5  $\mu$ g/ml), or the TO and TOB combination. TO was added at 0 h, and TOB was added at 10 h. Residual bacterial counts are expressed in log<sub>10</sub> CFU/ml as determined by plating sample dilutions on MSA agar for *S. aureus* and on cetrimide agar for *P. aeruginosa*. The bars represent the means log<sub>10</sub> CFU/ml after 24 h of coculture for at least three independent replicates with standard deviation. One-way analysis of variance was performed with Dunnett's posttest. The *P* values are indicated (ns, not significant; *P* > 0.05). The control coculture in which only the antibiotic diluent was added is represented by the black bar (CTRL).

to 10<sup>7</sup> CFU/ml after 24 h) (Fig. 1A, open circles, and Fig. 4A, black bar). However, our results demonstrate a bactericidal synergy between TO and the antistaphylococcal activity of *P. aeruginosa*. Evidence suggests that this synergy depends on the activation of the QS system of *P. aeruginosa* and the production of at least two exoproducts (HQNO and LasA).

The antistaphylococcal activity of LasA involves the degradation of pentaglycine cross-links in the peptidoglycan of *S. aureus* (12), and LasA has been used as an antibiotic agent in experimental therapy against this bacterium (40). The reason that TO enhances the activity of LasA against *S. aureus* or vice versa remains elusive, and direct evidence for the contribution of this protease in the synergy between *P. aeruginosa* and TO is still needed. Furthermore, other products such as the rhamnolipids produced by *P. aeruginosa* can permeabilize the cell membranes of Gram-positive and Gram-negative bacteria (41). The enhanced activity of TO against *S. aureus* was not, however, associated with the presence of *P. aeruginosa* rhamnolipids. Similarly, the bactericidal synergy between TO and *P. aeruginosa* could not be associated with the production of hydrogen cyanide or pyocyanin. Naturally, the use of genetically defective *P. aeruginosa* mutants only provides indirect evidence that these exoproducts cannot help the bactericidal activity of TO against *S. aureus*. It is possible that these exoproducts might contribute to the killing of *S. aureus* under other cultivation conditions, especially because HQNO is also, like hydrogen cyanide or pyocyanin, an electron transport chain inhibitor (11, 42). In addition, the siderophores produced by *P. aeruginosa*, such as pyoverdine and pyochelin, may also contribute to reducing *S. aureus* viability in cocultures (43).

HQNO stimulates the emergence of *S. aureus* SCVs, possibly due to its activity as an electron transport chain inhibitor (13–15). SCVs are recognized as persistent forms of bacteria associated with chronic infections (16, 44) and show reduced susceptibility to aminoglycoside antibiotics such as TOB due to their dysfunctional respiratory chain and altered proton motive force. Conversely, SCVs are extremely susceptible to TO (25, 26, 27). It is therefore tempting to speculate that HQNO sensitizes *S. aureus* to TO by impairing its electron transport chain. Hydrogen cyanide

and pyocyanin have been proposed to interfere with electron transfer in a manner different from that known for quinolone *N*-oxides on the electron transport chain of *S. aureus* (11), and this difference may perhaps explain the specific synergy of HQNO with TO. The initial mode-of-action studies showed that TO ultimately inhibits bacterial protein and other macromolecular biosyntheses and that the difference in the biological effects on prototypical *S. aureus* and SCVs was linked to the respiratory chain (26). More recently, our efforts with whole-genome sequencing of high-level TO-resistant mutants identified the putative molecular target of TO as the ATP synthase subunit c, the activity of which is directly linked to the respiratory chain and proton motive force (28). Of note, the target of TO (bacterial ATP synthase) is a validated anti-infective target; a novel antibiotic targeting ATP synthase (bedaquiline) was recently approved for treatment of tuberculosis (29, 30). Interestingly, the mutations we found in TO-resistant mutants map in a stretch of amino acid sequence only present in *Bacillales* (it is not present in other bacteria or in the mitochondrial ATP synthase), hence explaining the selectivity of TO. These results warrant further investigation of the TO drug scaffold. We have been able to produce several analogs with various properties, and the biological activity of this class of compound may thus be adjusted by medicinal chemistry (45).

Hence, in the context of a cocolonization by *P. aeruginosa* and *S. aureus*, it is possible that production of HQNO stimulates emergence of SCVs, which in turn may favor a persistent infection. Genetically related pairs of prototypical and SCV isolates have frequently been recovered from CF patients (44, 46, 47), and, in principle, treatment of *S. aureus* prototypical and SCV isolates with a combination of TO and an aminoglycoside can efficiently kill both, as we experimentally demonstrated previously (48). In the present study, we extended the challenge to include *P. aeruginosa*. Interestingly, *P. aeruginosa* contributed to the bactericidal effect of TO against *S. aureus* and efficiently suppressed the growth of a multiresistant MRSA strain. Moreover, the combination of TO and TOB suppressed the growth of both the MRSA strain and *P. aeruginosa*. Therefore, one might ask whether this proposed therapy is kinetically feasible in the host. In a therapeutic context,

*P. aeruginosa* can be present for extended periods of time, and HQNO can be detected in 80% of CF sputum samples in amounts correlating with the *P. aeruginosa* cell density at the onset of pulmonary exacerbation (49). Hoffman et al. (15) reported amounts ranging from 0.39 to 1.24  $\mu\text{g/g}$  of dry sputum, which are effective amounts of HQNO for synergy with TO against prototypical *S. aureus*. Indeed, such HQNO amounts ( $\sim >1 \mu\text{M}$ ) are able to provoke phenotypic changes in prototypical *S. aureus*, such as an increase in biofilm formation, consistent with SCV formation (17). Most importantly, one should not forget that the proposed therapeutic strategy combines both TOB and TO and that this combination should act on *P. aeruginosa*, *S. aureus*, and *S. aureus* SCVs. Thus, in this context, if HQNO is also present, it should increase further the potency of the TOB and TO synergy for therapy of multiresistant *S. aureus* (i.e., those strains having high resistance to TOB). The effective amounts of TOB and TO needed in sputum should also be attainable. Indeed, typical inhaled TOB (300 mg per dose, twice a day) can rapidly lead to high concentrations of TOB ( $\sim 1,200 \mu\text{g/g}$  of sputum) in the lungs (50). As for TO, the fractional inhibitory concentration (FIC) indices calculated in checkerboard assays *in vitro* for the combination of an aminoglycoside and TO clearly revealed synergy using as little as 0.12  $\mu\text{g/ml}$  of TO (25). At this time, we do not know the entire pharmacokinetics of TO, but the intratracheal instillation of 100  $\mu\text{g}$  of TO (the highest dose tested at this time) yielded no adverse effect in mice (data not shown). Hence, TOB and TO combination therapy appears kinetically feasible, and the presence of HQNO in *P. aeruginosa*-colonized patients is likely to further improve efficiency against multiresistant MRSA.

Overall, this study demonstrated the strong bactericidal effect of TO against *S. aureus* when cocultured with *P. aeruginosa*, a phenomenon that is dependent on the presence of at least HQNO and LasA. To take advantage of this bactericidal effect, the combination of TO with an aminoglycoside such as TOB represents a new conceptual approach for treatment of CF patients who are frequently colonized by MRSA, *S. aureus* SCVs, and *P. aeruginosa*.

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