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PHARMACOLOGY

In Vitro Time-Kill Studies of Trimethoprim/Sulfamethoxazole against *Stenotrophomonas maltophilia* versus *Escherichia coli* Using Cation-Adjusted Mueller-Hinton Broth and ISO-Sensitest Broth

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ABSTRACT Trimethoprim/sulfamethoxazole (TMP/SMZ) is considered the treatment of choice for infections caused by *Stenotrophomonas maltophilia*, but limited pharmacodynamic data are available to support current susceptibility breakpoints or guide optimal dosing. Time-kill studies using a TMP/SMZ concentration of 4/40 μg/mL were conducted to compare 4 *S. maltophilia* with 4 *Escherichia coli* isolates having the same MICs (0.25/4.75 to 4/76 μg/mL) in cation-adjusted Mueller-Hinton broth (CAMHB) and ISO-Sensitest broth (ISO broth). With the exception of the resistant isolates (4/76 μg/mL), which resulted in regrowth approaching the growth of the control, TMP/SMZ displayed significantly greater killing for *E. coli* than for *S. maltophilia* at each MIC. Against *E. coli*, the mean changes at 24 h were −4.49, −1.73, −1.59, and +1.83 $\log_{10}$ CFU for isolates with MICs of 0.25/4.75, 1/19, 2/39, and 4/74 μg/mL, respectively. The area under the concentration-time curve for the free, unbound fraction of the drug (*f*AUC)/MIC ratio required for stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions were 40.7, 59.5, and 86.3, respectively. In contrast, TMP/SMZ displayed no stasis or CFU reductions against any *S. maltophilia* isolate regardless of the MIC, and no pharmacodynamic thresholds were quantifiable. Observations were consistent in both CAMHB and ISO broth. These data add increasing evidence that current TMP/SMZ susceptibility breakpoints against *S. maltophilia* should be reassessed.

KEYWORDS Gram negative, *in vitro*, pharmacodynamics, susceptibility breakpoint

Stenotrophomonas maltophilia is a multidrug-resistant Gram-negative bacterium that is increasing in prevalence, particularly among critically ill and immunocompromised patients (1, 2). This pathogen can cause severe infections in the respiratory tract, bloodstream, and skin and skin structures, among various other body sites. Unfortunately, there are few antibiotic regimens that retain predictable microbiological activity against *S. maltophilia*, making treatment challenging. Among these antibiotics, trimethoprim/sulfamethoxazole (TMP/SMZ) is widely considered the drug of choice largely due to its high susceptibility rates (3, 4). The Clinical and Laboratory Standards Institute (CLSI) defines *S. maltophilia* as susceptible when TMP/SMZ MICs are ≤2/38 μg/mL (5). At this breakpoint, approximately 95% of *S. maltophilia* isolates worldwide are susceptible to TMP/SMZ (6). In contrast, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines susceptibility at MICs of ≤0.001 μg/mL, thereby making most *S. maltophilia* isolates, by this definition, fall into the intermediate category and require a higher dosage for treatment (7).

Clinical studies supporting the utilization of TMP/SMZ for the treatment of *S. maltophilia* infections are generally small, retrospective, single-center assessments and offer mixed results (8–13). Notably, no studies have evaluated outcome by TMP/SMZ MIC, and pharmacodynamic

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TABLE 1 Modal broth microdilution MICs for TMP/SMZ in cation-adjusted Mueller-Hinton broth and ISO-Sensitest broth against the 4 *S. maltophilia* and 4 *E. coli* isolates[a]

		TMP/SMZ MIC (μg/mL)	
CAIRD ID	External ID	CAMHB	ISO broth
S. maltophilia isolates			
STM29		0.25/4.75	0.5/9.5
STMC43-11		1/19	1/19
STM106	IHMA 2097696	2/38	1/19
STMC42-15		4/76	4/76
E. coli isolates			
EC25922	ATCC 25922	0.25/4.75	0.25/4.75
EC762	CDC0019	1/19	1/19
EC778	IHMA 2249758	2/38	1/19
EC780	IHMA 2259927	4/76	4/76

[a]TMP/SMZ, trimethoprim/sulfamethoxazole; CAMHB, cation-adjusted Mueller-Hinton broth; ISO broth, ISO-Sensitest broth.

studies that characterize the exposure-response relationship for TMP/SMZ against *S. maltophilia* are not available. Such data could prove useful when reassessing susceptibility breakpoints and help to guide dosing regimen selection. Unfortunately, *in vivo* animal infection models are poorly translational for dihydrofolate reductase inhibitors since rodents have considerably high concentrations of thymidine in plasma compared with humans (14). This surplus permits the uptake of exogenous thymidine by bacteria and conversion into thymidylate by thiamine kinase, a known salvage pathway for DNA synthesis that antagonizes the *in vitro* activity of trimethoprim (15, 16). Notably, broth used for *in vitro* studies may also contain exogenous thymidine, which could influence the activity of TMP/SMZ against both Gram-positive and Gram-negative organisms (16, 17). Here, we employed time-kill studies to evaluate the exposure-response relationship of TMP/SMZ against 4 *S. maltophilia* clinical isolates compared with *Escherichia coli* isolates harboring the same MICs. *E. coli* was selected as a comparator since it is known to use exogenous thymidine to counteract TMP/SMZ activity (16), and resistance has been linked with clinical failure (18–20). This opportunity was also used to evaluate activity in standard cation-adjusted Mueller-Hinton broth (CAMHB) versus ISO-Sensitest broth (ISO broth), which may have differing thymidine concentrations.

RESULTS

Broth microdilution. Modal TMP/SMZ broth microdilution MIC results ranged from 0.25/4.75 to 4/76 μg/mL (Table 1). MICs for each isolate were the same or within 1 MIC dilution between CAMHB and ISO broth. Two isolates (one *S. maltophilia* and one *E. coli*) tested 1 dilution lower in ISO broth than in CAMHB, while a single *S. maltophilia* isolate tested 1 dilution higher in ISO broth than in CAMHB. Since CAMHB is frequently used during *in vitro* pharmacodynamic studies, MICs derived from CAMHB were used as the reference for all comparisons.

Time-kill studies. The average starting bacterial densities for *S. maltophilia* in CAMHB and ISO broth were 6.60 $\pm$ 0.11 and 6.59 $\pm$ 0.10 $\log_{10}$ CFU/mL, respectively. The mean starting bacterial densities for *E. coli* were 6.48 $\pm$ 0.11 $\log_{10}$ CFU/mL in CAMHB and 6.49 $\pm$ 0.16 $\log_{10}$ CFU/mL in ISO broth. Twenty-four-hour control bacterial densities increased robustly for *S. maltophilia* (CAMHB, 8.08 $\pm$ 0.39 $\log_{10}$ CFU/mL; ISO broth, 8.40 $\pm$ 0.35 $\log_{10}$ CFU/mL) and *E. coli* (CAMHB, 8.45 $\pm$ 0.38 $\log_{10}$ CFU/mL; ISO broth, 8.51 $\pm$ 0.30 $\log_{10}$ CFU/mL). After exposure to a concentration of TMP/SMZ of 4/40 μg/mL for 24 h, which is consistent with the average free steady-state concentration for 20 mg/kg of body weight daily (trimethoprim component, $\sim$100 μg $\cdot$ h/mL) in humans (21), none of the *S. maltophilia* isolates demonstrated greater than a static CFU reduction in CAMHB (Fig. 1). In contrast, *E. coli* isolates with MICs of $<$4/76 μg/mL achieved $>$1-$\log_{10}$ CFU/mL reductions. The results were nearly identical in ISO broth (data not shown). The nonsusceptible *S. maltophilia* and *E. coli* isolates regrew to control CFU in both media. Aside from the isolates with an MIC of 4/76 μg/mL, significant differences were observed between the *S. maltophilia* and *E. coli* isolates at all other MICs in both

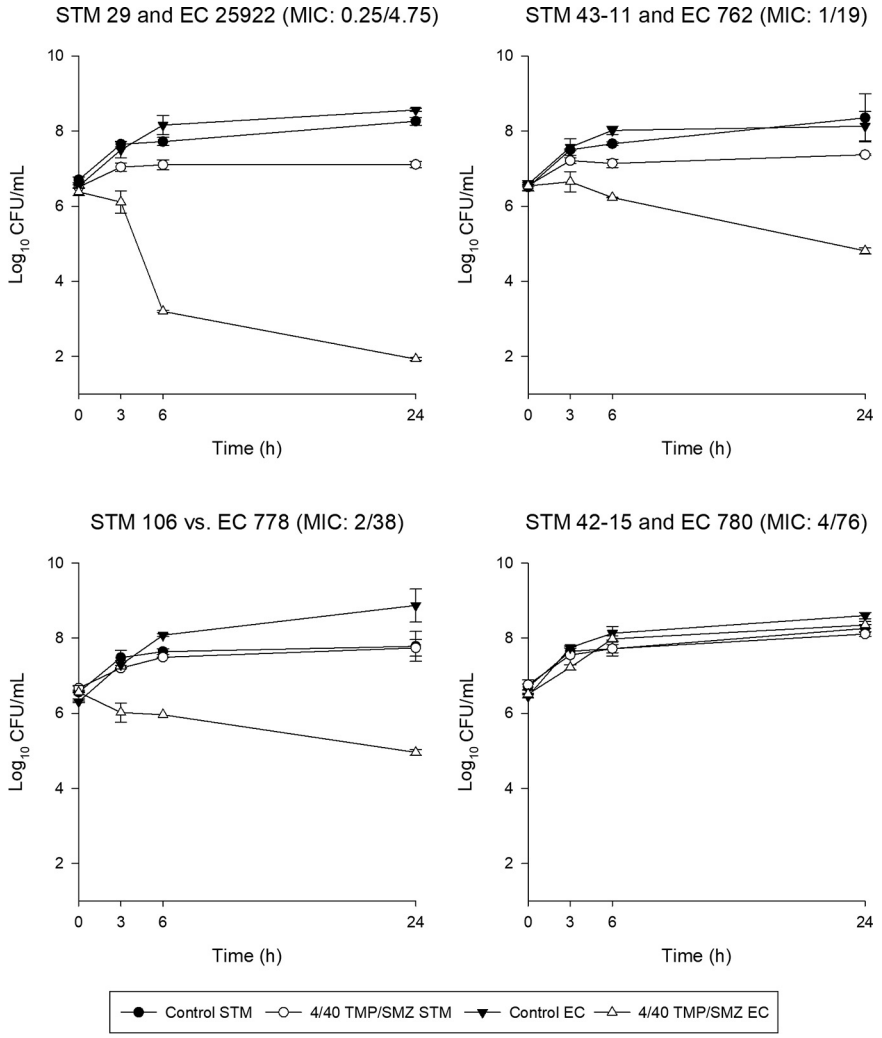

FIG 1 *In vitro* time-kill growth curves of *S. maltophilia* and *E. coli* in CAMHB after exposure to TMP/SMZ at 4/40 μg/mL. Isolates are referenced to CAMHB MICs for comparison.

media (Table 2). Finally, no significant differences were seen between CAMHB and ISO broth for individual isolates with the exception of two observations (EC762 and EC780).

Pharmacodynamic analyses. Final maximum-effect (E_{max}) model fits are shown in Fig. 2a for *S. maltophilia* and Fig. 2b for *E. coli*. Data from both CAMHB and ISO broth were analyzed together since no changes in parameters or thresholds were observed when analyzed separately (data not shown). Table 3 provides the E_{max} model parameters and area under the concentration-time curve for the free, unbound fraction of the drug (*f*AUC)/MIC exposures (of the trimethoprim component) required for stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions. Notably, values were quantifiable for only *E. coli*.

DISCUSSION

Attributed to its high susceptibility rate, TMP/SMZ is considered by many to be the antibiotic of choice for the treatment of infections caused by *S. maltophilia* (2). The CLSI and EUCAST define susceptibility breakpoints at $\leq$2/38 μg/mL and $\leq$0.001 μg/mL (trimethoprim component only), respectively, which results in fewer than ~5% of *S. maltophilia* isolates being defined as resistant. In the United States, TMP/SMZ is not approved for the treatment of any Gram-negative infections outside the urinary tract (approved dose of 8 to 10 mg/kg/day divided into 2 to 4 equal doses daily); however, dosing regimens of 15 to 20 mg/kg daily are indicated for *Pneumocystis jirovecii* pneumonia (PJP) (22). In retrospective studies of TMP/SMZ for the treatment of *S. maltophilia* infection, dosing regimens vary from

TABLE 2 Comparison of 24-h CFU reductions following TMP/SMZ exposure in time-kill studies between *S. maltophilia* and *E. coli* at the same MIC in CAMHB and by broth for individual isolates[a]

Comparison	Mean change in $\log_{10}$ CFU/mL at 24 h ± SD		*P* value[b]
By isolate at the same MIC (TMP/SMZ MIC [μg/mL])	*S. maltophilia*	*E. coli*	
CAMHB			
STM29 vs EC25922 (0.25/4.75)	0.60 ± 0.08	−4.49 ± 0.06	<0.001
STMC43-11 vs EC762 (1/19)	0.81 ± 0.16	−1.73 ± 0.10	0.007
STM106 vs EC778 (2/38)	1.07 ± 0.16	−1.59 ± 0.26	0.005
STMC42-15 vs EC780 (4/76)	1.36 ± 0.08	1.83 ± 0.07	0.016
ISO broth[c]			
STM29 vs EC25922 (0.25/4.75)	0.49 ± 0.06	−4.42 ± 0.65	<0.001
STMC43-11 vs EC762 (1/19)	1.15 ± 0.06	−2.93 ± 0.16	0.002
STM106 vs EC778 (2/38)	1.03 ± 0.18	−1.46 ± 0.24	0.003
STMC42-15 vs EC780 (4/76)	1.52 ± 0.15	1.20 ± 0.11	NS
By broth for individual isolates	CAMHB	ISO broth	
S. maltophilia			
STM29	0.60 ± 0.08	0.49 ± 0.06	NS
STMC43-11	0.81 ± 0.16	1.15 ± 0.06	NS
STM106	1.07 ± 0.16	1.03 ± 0.18	NS
STMC42-15	1.36 ± 0.08	1.52 ± 0.15	NS
E. coli			
EC25922	−4.49 ± 0.06	−4.42 ± 0.65	NS
EC762	−1.73 ± 0.10	−2.93 ± 0.16	0.010
EC778	−1.59 ± 0.26	−1.46 ± 0.24	NS
EC780	1.83 ± 0.07	1.20 ± 0.11	0.022

[a]TMP/SMZ, trimethoprim/sulfamethoxazole; CAMHB, cation-adjusted Mueller-Hinton broth; ISO broth, ISO-Sensitest broth; NS, not significant.
[b]Comparisons were done by one-way ANOVA and the Holm-Sidak pairwise test for multiple comparisons.
[c]Isolates referenced by their CAMHB MICs during comparison.

8 to 10 mg/kg/day up to 15 to 20 mg/kg/day in individual patients. Unfortunately, no pharmacodynamic studies for TMP/SMZ are available to establish stasis and 1-log CFU reduction thresholds to support current susceptibility breakpoints or optimal dosing against *S. maltophilia*. Here, we performed time-kill studies against 4 *S. maltophilia* clinical isolates with increasing MICs, which offered an opportunity to compare exposure-response relationships with *E. coli* isolates having the same MICs as well as to compare the effects of different broth media for these *in vitro* studies.

The effects of thymidine concentrations on TMP/SMZ *in vitro* and *in vivo* activities against certain Gram-positive and Gram-negative bacteria are well established (16, 17, 23, 24). Furthermore, the CLSI requires broth quality control (QC) with *Enterococcus faecalis* ATCC 29212, an organism very sensitive to the antagonistic effects of exogenous thymidine, to confirm the suitability of media used during MIC studies (25). To our knowledge, no data are available to demonstrate or refute that *S. maltophilia* uses these exogenous nucleosides to escape TMP/SMZ activity. Given that both media passed all QCs, it was not surprising that TMP/SMZ MICs in CAMHB and ISO media for these *S. maltophilia* and *E. coli* isolates were within 1 dilution of each other, if not the same. Further studies are needed to understand if exogenous thymidine can be used by *S. maltophilia*. Additionally, 24-h time-kill CFU reductions were consistent for these isolates compared between broths (Table 2), with only two exceptions among *E. coli* isolates. EC762 showed a 1-$\log_{10}$-greater reduction in CFU at 24 h in ISO broth (−2.93 $\log_{10}$ CFU) than in CAMHB (−1.73 $\log_{10}$ CFU); notably, this isolate demonstrated the same MIC (1/19 μg/mL) in both media. EC780 also showed a significant difference in CFU at 24 h, but regrowth that approached the growth of the control isolates was observed in both media. Since most MIC and *in vitro* pharmacodynamic studies within our laboratory are conducted with CAMHB, we referenced the isolates by their MICs in this medium for comparisons and used the broth-specific MICs during pharmacodynamic analyses (i.e., *f*AUC/MIC ratio).

In CAMHB, the kill curves of *S. maltophilia* when exposed to average free steady-state concentrations of an aggressive TMP/SMZ 20-mg/kg daily dosage demonstrated little activity. Regardless of the TMP/SMZ MIC (0.25/4.75 to 4/76 μg/mL), 24-h CFU counts

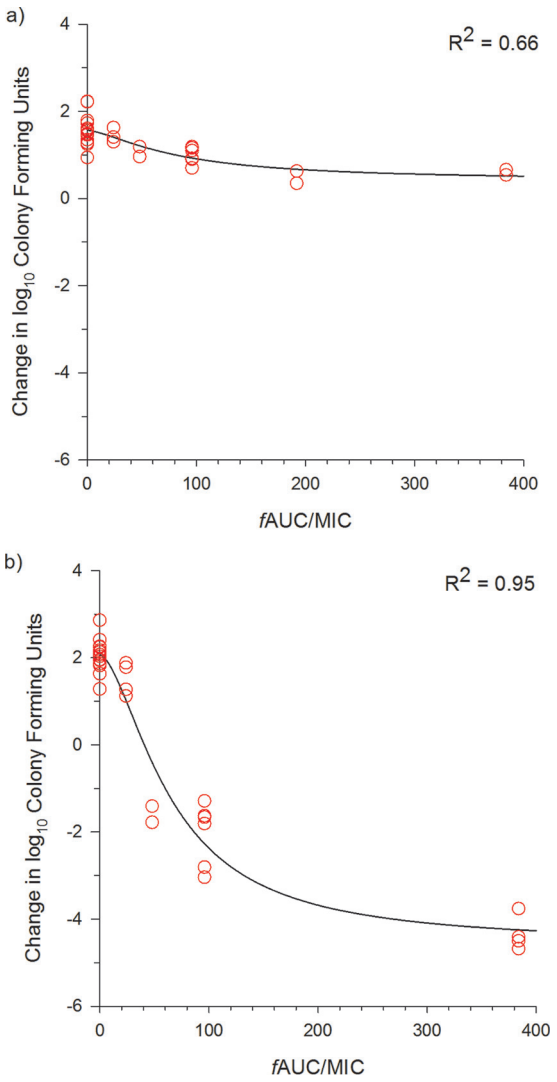

FIG 2 TMP/SMZ exposure-response E_{max} model fits based on the trimethoprim component for *S. maltophilia* (a) and *E. coli* (b). Individual data points are plotted by the modal MIC in the respective media.

were +0.6 to +1.36 log_{10} CFU (Fig. 1). In contrast, TMP/SMZ demonstrated substantial killing at 24 h against the *E. coli* isolates, which was in agreement with their MICs. Mean changes in 24-h CFU were −4.49, −1.73, −1.59, and +1.83 log_{10} CFU for isolates with MICs of 0.25/4.75, 1/19, 2/39, and 4/74 μg/mL, respectively. All changes were significantly different from *S. maltophilia* at the same MIC (Table 2). Similar observations were made in ISO broth.

Previous literature corroborates the lack of *in vitro* single-agent trimethoprim/sulfamethoxazole activity against *S. maltophilia* despite defined susceptibility; however, none of these studies attempted to characterize any exposure-response relationship. Biagi and colleagues evaluated TMP/SMZ as monotherapy and in combination with cefiderocol against *S. maltophilia* in a time-kill model using CAMHB (26). Exposures of 4× MIC demonstrated stasis or regrowth at 24 h against the three susceptible isolates (TMP/SMZ MICs of ≤0.5 μg/mL). Of note, the addition of cefiderocol at 1/2 the MIC increased 24-h kill by 1 to 2 logs for these isolates. Time-kill studies using a fixed concentration of 2/38 μg/mL were also unable to identify CFU reductions against 12 *S. maltophilia* isolates with TMP/SMZ MICs of between 0.25/4.75 and ≥8/152 μg/mL (27). Using an *in vitro* chemostat pharmacodynamic model, Zelenitsky and colleagues simulated free-drug exposures of TMP/SMZ at 5 mg/kg (trimethoprim component) every 12 h (q12h) against 4 *S. maltophilia* isolates with MICs of 1/19 to 2/38 μg/mL (28). Similar to our time-kill experiments, stasis or regrowth was observed for all isolates.

TABLE 3 Final E_{max} model parameters and *f*AUC/MIC ratios (of the trimethoprim component) required for stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions for *S. maltophilia* and *E. coli*[a]

Parameter	*S. maltophilia*	*E. coli*
Model parameters		
I_{max}	1.16	6.63
IC_{50}	83.5	65.8
E_0	1.57	2.05
Gamma	1.47	1.66
PD threshold (*f*AUC/MIC ratio [of the trimethoprim component])		
Stasis	NC	40.7
1-$\log_{10}$ CFU reduction	NC	59.9
2-$\log_{10}$ CFU reduction	NC	86.3

[a]I_{max}, maximum effect (difference between the minimum and maximum observed CFU); IC_{50}, inhibitory concentration required for 50% of the maximum effect; E_0, maximum observed CFU; Gamma, slope constant; PD, pharmacodynamic; NC, not able to be calculated.

Given the lack of any exposure-response analyses and the range of MICs for these *S. maltophilia* and *E. coli* isolates, E_{max} modeling using the composite curve of isolates was employed to determine the exposures required for stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions. For *E. coli* isolates, these thresholds were *f*AUC/MIC ratios (based on the trimethoprim component) of 40.7, 59.5, and 86.3, respectively. We assumed the *f*AUC/MIC ratio to be the pharmacodynamic parameter for TMP/SMZ, as have other studies targeting tuberculosis, melioidosis, *Neisseria meningitidis* infections, and enterococcal urinary tract infections (29–32). These studies reference an *f*AUC/MIC ratio of >25 as the threshold for the TMP component but admit that this target is arbitrary. Indeed, at least for *E. coli*, higher thresholds are required but should be achievable for most isolates currently defined as susceptible. In contrast, against these *S. maltophilia* isolates, we were unable to quantify the *f*AUC/MIC ratio required for any of these endpoints. Further pharmacodynamic studies are warranted to confirm that the *f*AUC/MIC ratio is the pharmacodynamic parameter best correlated with killing for TMP/SMZ as well as to conduct dose-ranging studies on individual isolates to determine if 1-$\log_{10}$ CFU reductions are achievable against *S. maltophilia*. For serious infections such as pneumonia and bloodstream infections, pharmacodynamic thresholds that achieve 1-$\log_{10}$ CFU reductions are preferred during dosage optimization and the selection of susceptibility breakpoints (33). It is therefore concerning that TMP/SMZ is unable to achieve such thresholds against this pathogen.

In summary, the exposure-response relationship was analyzed for TMP/SMZ against 4 *S. maltophilia* and *E. coli* isolates. Despite having the same MIC, these studies yielded discordant CFU reductions for susceptible *S. maltophilia* and *E. coli* isolates. TMP/SMZ *f*AUC/MIC thresholds for stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions were identified for *E. coli*. However, no such thresholds were observed against *S. maltophilia*, and further studies to define the TMP/SMZ pharmacodynamic target against this pathogen are needed. These data add increasing evidence that current TMP/SMZ susceptibility breakpoints against *S. maltophilia* should be reassessed.

MATERIALS AND METHODS

Antimicrobial agents and broth. Trimethoprim (lot number 019M4019V, expiration date of October 2022) and sulfamethoxazole (lot number BCCB6035, expiration date of April 2022) analytical powders were purchased separately from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in line with CLSI document M100 (5). Cation-adjusted Mueller-Hinton broth (lot number 0286591, expiration date of April 2024; Becton, Dickinson and Company, Sparks, MD, USA) and ISO broth (lot number 3143645, expiration date of September 2025; Oxoid Ltd., Cheshire, UK) were acquired from approved vendors.

Isolates. Eight clinical isolates (4 *S. maltophilia* and 4 *E. coli*) were included. Isolates were selected based on initial TMP/SMZ MICs in CAMHB and sourced from the CAIRD isolate repository, the American Type Culture Collection (ATCC), International Health Management Associates (IHMA), and the Centers for Disease Control and Prevention and Food and Drug Administration Antimicrobial Resistance Bank (CDC/FDA-ARB). Isolates were stored at −80°C and subcultured twice prior to experiments.

***In vitro* susceptibility.** MICs were determined in triplicate by broth microdilution to confirm previously reported TMP/SMZ MICs in CAMHB and to determine MICs in ISO broth. Briefly, trimethoprim/

sulfamethoxazole trays (1:19) were prepared in each broth medium according to CLSI guidance with quality control testing (5, 25). *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls in both broth media.

Simulated TMP/SMZ exposures. Time-kill studies were performed using the free-drug average steady-state concentration (fC_{ss}) for a 20-mg/kg daily TMP/SMZ dose (based on the trimethoprim component) from critically ill patients (21). The trimethoprim 24-h total drug area under the curve was ~177 μg · h/mL and, when corrected by 44% protein binding (22) and divided by 24 h, was equal to an fC_{ss} of 4 μg/mL. When administered in the clinically available 1:5 ratio of TMP/SMZ, sulfamethoxazole total drug concentrations are 19 times higher than those of trimethoprim, but when corrected for sulfamethoxazole protein binding of 70%, the concentration ratio is 1:10; therefore, the sulfamethoxazole fC_{ss} was 40 μg/mL (or 4/40 μg/mL for the TMP/SMZ combination).

Time-kill studies. Time-kill studies were conducted as described previously (34). All experiments were conducted in duplicate, and control experiments (no TMP/SMZ) were conducted simultaneously with treatment studies. CAMHB and ISO broth were inoculated with bacterial suspensions to achieve a starting inoculum of 10^6 CFU/mL. Final broth volumes were 10 mL. Broth was immediately incubated in a shaking water bath at 37°C. For each experiment, samples were taken at 0, 3, 6, and 24 h; serially diluted onto blood agar plates; and allowed to incubate overnight, and bacterial densities were enumerated (CFU per milliliter). The lowest accurately countable number was 5×10^1 CFU.

Pharmacodynamic and statistical analyses. Trimethoprim exposure (*f*AUC/MIC ratio) was modeled using an E_{max} model (Hill equation) in Phoenix WinNonlin version 8.3 (Pharsight Corp., Mountain View, CA, USA) for each organism separately. Exposures predictive of stasis and 1-$\log_{10}$ and 2-$\log_{10}$ CFU reductions were calculated. Comparisons of 24-h CFU changes between isolates (*S. maltophilia* versus *E. coli* at the same MIC) and broth (CAMHB versus ISO broth for individual isolates) were made by one-way analysis of variance (ANOVA) with the Holm-Sidak test for multiple comparisons (Sigma Plot version 14; Systat Software, Inc., San Jose, CA).

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