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Supporting Information

Disrupting Transcription and Folate Biosynthesis Leads to Synergistic Suppression of *Escherichia coli* Growth

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Materials and Methods:

Materials and consumables. Antibiotics were purchased from the following venders: ChemScene (amikacin, ceftazidime, clarithromycin, D-cycloserine, fosfomycin, fusidic acid, minocycline, novobiocin, ofloxacin, polymyxin B, puromycin, sulfamonomethoxine, tobramycin, trimethoprim), Sigma-Aldrich (cerulenin), BioShop Canada (ampicillin, chloramphenicol, kanamycin, rifampicin), PanReac AppliChem (5-fuorouracil). Organic solvents and other chemicals were purchased from Sigma-Aldrich and Tokyo Chemical Industry. Consumables were purchased from Thermo Fisher Scientific and Bioman Scientific.

Strains, plasmid, and growth medium. Strains and plasmids used in this study are listed in **Table S1**. *Escherichia coli* MG1655 was provided by the Nai-Chun Lin laboratory at National Taiwan University. The pTUC202 plasmid was provided by Professor Mohamed A. Marahiel. Luria-Bertani (LB) broth was prepared from premixed powder purchased from BioShop Canada. All microbial strains were cultured in LB broth; cultures of *E. coli* carrying pTUC202 was supplemented with 25 µg/mL of chloramphenicol.

MccJ25 production and purification. MccJ25 was produced and purified based on published protocols with minor modifications. Briefly, *E. coli* BL21(DE3) carrying pTUC202 was cultivated in M9 medium supplemented with 0.4% (w/v) glucose and 2 mM $MgSO_4$ at 37 °C for 4 days. Cells were removed by centrifugation. The supernatant was heated in boiling water for 10 min and extracted with 2 volumes of 1-butanol. The organic layer was collected and dried *in vacuo*. The resulting residue was redissolved in a minimum amount of aqueous acetonitrile (5%, v/v) and loaded onto a solid-phase extraction cartridge (Sep-Pak C18 Vac, Waters) that had been pre-washed with acetonitrile and water, successively. Water and acetonitrile supplemented with 0.1% (v/v) formic acid were used as the mobile phase, denoted as solvent A and B, respectively. MccJ25 typically elutes at 25 to 35%B when the cartridge was washed with 5% (v/v) stepwise increments of solvent B. Fractions that contained MccJ25 were pooled and subjected to a second round of cartridge purification to yield materials for various antibiosis assays. A DMSO stock solution (16 mg/mL) was prepared from lyophilized MccJ25 powder and stored as aliquots at –20 °C. Quality assessment was performed by reversed-phase HPLC (Waters) with an analytical SHARPSIL-U C18 column (250 × 4.6 mm ID, 100 Å, 5 µm) (***Figure S3***). The identity of MccJ25

was confirmed by mass spectrometry (microTOF-QII, Bruker); HRMS (ESI-TOF) calculated for $C_{101}H_{141}N_{23}O_{27}$ $[M+2H]^{2+}$: 1054.0178, found: 1054.0184 (***Figure S4***).

MIC determination. Susceptibility of *E. coli* MG1655 to individual antibiotics was performed in 96-well microtiter plates using the broth dilution method. First, LB medium was added to all wells (50 μL). Antibiotic solutions in LB broth (50 μL, 128 μg/mL) were added to the first wells of each row and diluted serially (1/2×) across the plate. The last two wells were reserved for positive (no drug) and negative (no bacteria) controls. Overnight cultures in LB medium grown from a single-colony (200 rpm, 37 °C) were diluted 5,000-fold and added to each well (50 μL). MIC values were determined by visual inspection after static incubation at 30 °C for 22 h. All assays were performed at least in triplicate.

Checkerboard assay. Serial dilutions of MccJ25 and a select antibiotic were prepared separately. They were then combined at equal volumes in a 96-well microtiter plate to generate a 12 × 8 grid of two serially diluted antibiotics; the results were presented as a 6 × 6 grid starting at the respective MIC of the two antibiotics. Overnight *E. coli* MG1655 cultures in LB medium grown from a single-colony (200 rpm, 37 °C) were diluted 5,000-fold and added to each well. The plate was incubated statically at 30 °C for 22 h and bacterial growth was quantified by optical density recorded at 600 nm (OD_{600}). All assays were done at least in triplicate. The type of interaction between two antibiotics was categorized based on the minimum Fractional Inhibitory Concentration Index ($FICI_m$),

$$FICI = \frac{A}{MIC_A} + \frac{B}{MIC_B}$$

where MIC_A and MIC_B denote the individual MIC of each antibiotic, and A and B denote their respective MIC in the presence of the other antibiotic. Antibiotic pairs with $FICI_m < 0.5$ and $FICI_m = 0.5$ are categorized as strongly and weakly synergistic, respectively. Those with $0.5 < FICI_m \leq 4.0$ were categorized as indifferent to the presence of each other, and $FICI \geq 4.0$ are antagonistic (not observed in our studies).

Time-kill assay. An overnight culture was grown from a single *E. coli* MG1655 colony in Müller-Hinton broth (MHB). The culture was used to start fresh MHB cultures at 10^6 CFU/mL supplemented with MccJ25 (1 μg/mL), SMM (0.512 mg/mL), or both antibiotics (MccJ25 at 0.5

µg/mL and SMM at 0.256 mg/mL). These cultures were incubated at 37 °C with agitation, serially diluted, and spotted on MHB agar plates after 0, 1, 2, 4, and 8 h. The plates were incubated at 37 °C until the formation of visible colonies. The number of colonies at each timepoint was counted and plotted into a time-kill trace. This assay was performed in triplicate.

Resistance development assay. An overnight culture was grown from a single *E. coli* MG1655 colony in MHB. Overnight cultures were washed with fresh MHB, serially diluted, and spotted on MHB agar plates supplemented with 1 µg/mL MccJ25 and varying concentrations of SMM. The plates were incubated at 37 °C until the formation of visible colonies. The number of colonies at each timepoint was counted, and the number of CFU relative to that of the antibiotic-free control was used as a proxy for overall resistance development.

Table S1. Strains and Plasmids

Designation	Relevant Characteristic	Reference
Escherichia coli Strains		
K12 MG1655	F^-, λ^-, $ilvG^-$, *rfb-50*, *rph-1*	N. C., Lin, National Taiwan University
BL21(DE3)	*E. coli* B, F^-, *dcm*, *ompT*, *hsdS*(rB^-mB^-), gal (DE3)	RBCBioscience, Taiwan
Plasmids		
pTUC202	MccJ25 producing plasmids, Cm^R	[1]

[1] J. O. Solbiati, M. Ciaccio, R. N. Farias, R. A. Salomon, Genetic analysis of plasmid determinants for microcin J25 production and immunity. *J. Bacteriol.* **1996**, *178*, 3661-3663.

Figure S1. Raw data of **Figure 2**

Figure 1 raw data; values in each cell refers to OD_{600}

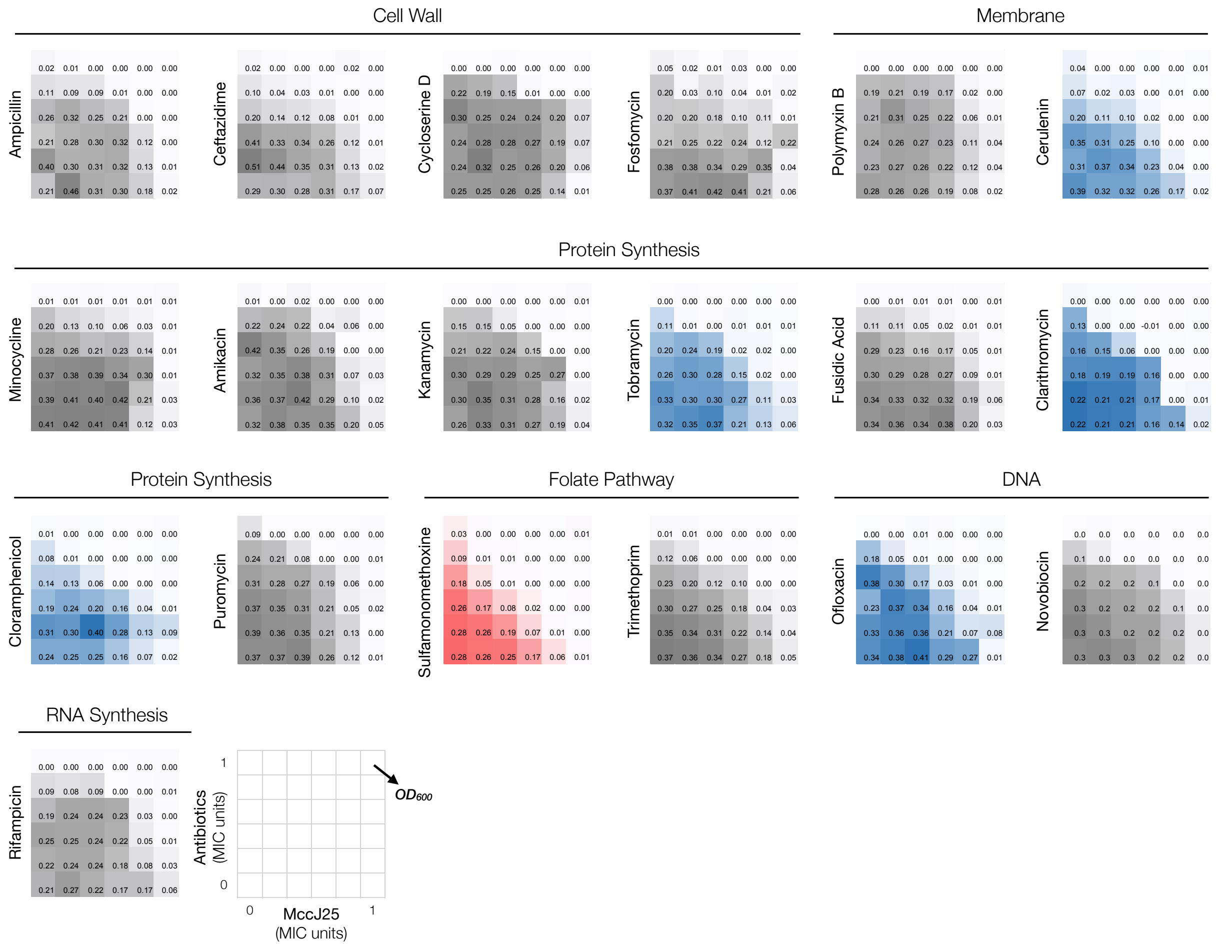

Figure S2. Additional sulfonamide antibacterial agents were tested. Both sulfamethoxazole and sulfadiazine show moderate synergy with MccJ25 (FICI = 0.5) against *E. coli* MG1655. MIC for sulfamethoxazole: 128 μg/mL; sulfadiazine: 128 μg/mL.

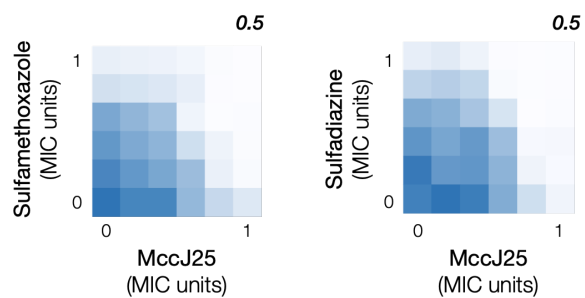

Figure S3. GTP and dTMP supplementation results. **a.** The addition of GTP (2 mM) in the growth medium did not lead to an apparent change in MccJ25-SMM synergy. **b.** The addition of dTMP (50 μM) in the growth medium results in no synergy between MccJ25 and SMM.

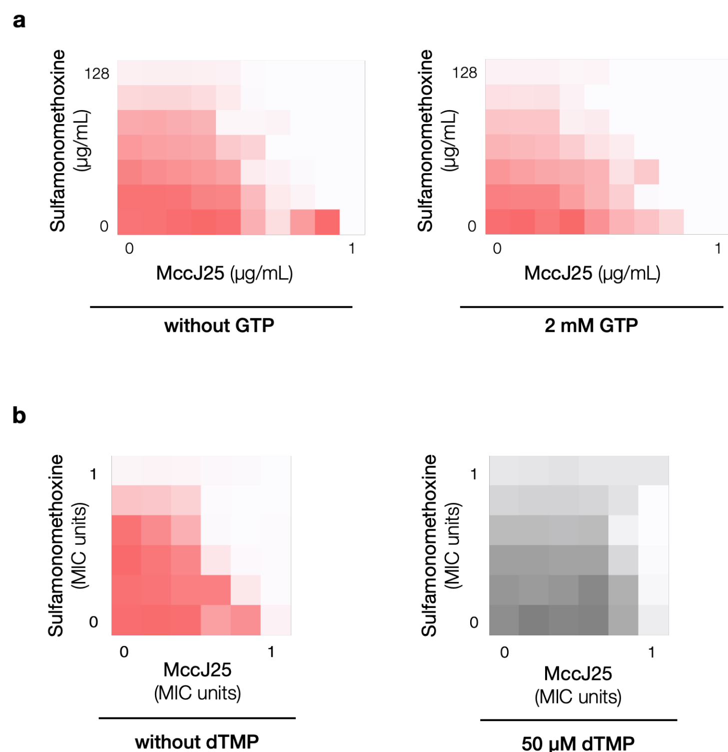

Figure S4. HPLC trace of purified McCJ25

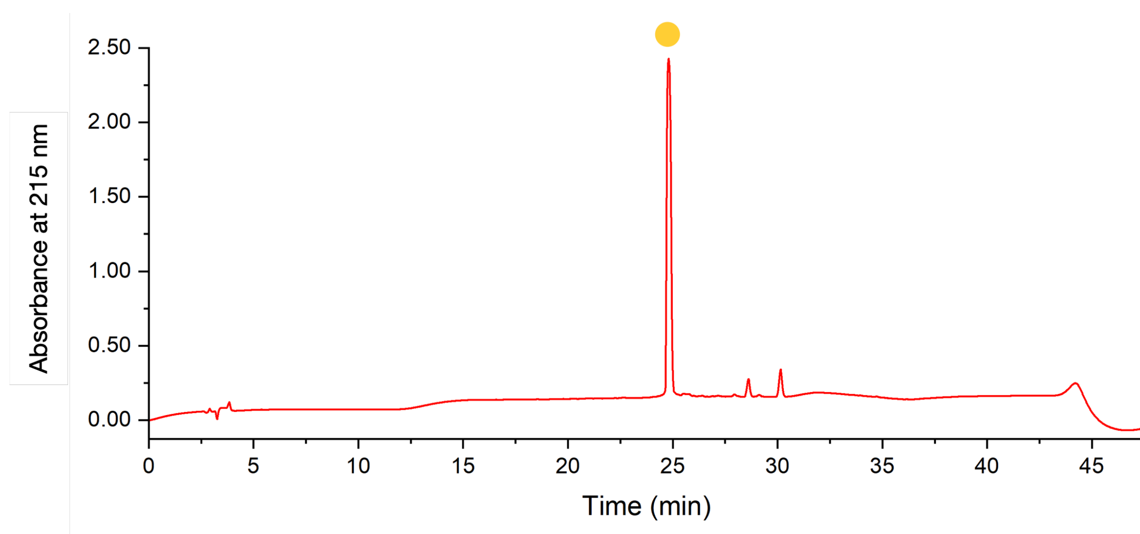

Figure S5. High-resolution mass spectrometry data of MccJ25

Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	mSigma	Score	rdb	e⁻ Conf	N-Rule
1054.0184	1	C101H141N23O27	1054.0178	0.6	0.5	21.3	100.00	43.0	even	ok

+MS, 0.1-0.3min #6-15