Supplementary Information

Supplementary Methods

Tricine-SDS-PAGE. To investigate if YdcY is a protein, a tricine-SDS-PAGE was performed. BW25113 wild-type strains carrying pCA24N, pCA24N-*ydcY*, and pCA24N-*ghoS* (positive control) were grown to an optical density at 600 nm of 0.25 in LB with chloramphenicol, then 1 mM ITPG was added to induce *ydcY* and *ghoS* expression for 4 h. One mL cell samples were harvested by centrifugation and lysed with 10% SDS to a final concentration of 0.4% SDS. The protein concentration was measured using a Pierce™ BCA Protein assay kit (Thermo Scientific™, Rockford, IL, USA). Ten μg of total cell protein for each sample was denatured at 95 ℃ for 10 min and loaded for SDS-PAGE analysis. The tricine-SDS-PAGE was performed as described earlier [70].

Western blot analysis. To detect OrtT F38R, BW25113 wild-type strains carrying pCA24N, pCA24N-*ortT*F38R, and pCA24N-*ghoS* (positive control) were grown to a turbidity at 600 nm of 0.15 in LB with chloramphenicol, then 0.2 mM ITPG was added to induce *ydcY* and *ghoS* expression for 3 h. Cell samples (5 mL) were harvested by centrifugation and prepared for tricine-SDS-PAGE as described above. Western blot analysis was performed as described previously [18] with 20 μg of total cell protein.

Table S1. Summary of qRT-PCR results. The cycle number (C_t) for each sample is indicated for target gene, *ortT* as well as the housekeeping gene, *gyrA*, which was used to normalize the data. Fold changes in transcription were calculated using [66]: $2^{-}(C_{t\ ortT,\ treatment} - C_{t\ gyrA,\ treatment})/2^{-}(C_{t\ ortT,\ control} - C_{t\ gyrA,\ control})$. The specificity of the qRT-PCR products were verified by melting curve analysis [66].

Stringent Condition	Strain	Condition	Biological Replicate	C_t (*ortT*)	C_t (*gyrA*)	Fold Change
TMP treatment	BW25113	Control	#1	23.73 ± 0.09	21.04 ± 0.25	4.53 ± 0.01
		TMP		24.91 ± 0.05	24.40 ± 0.01	
		Control	#2	24.45 ± 0.08	26.60 ± 0.37	1.70 ± 0.30
		TMP		24.83 ± 0.11	27.72 ± 0.39	
RelA production	BW25113/pCA24N	pCA24N	#1	23.58 ± 0.22	24.29 ± 0.05	2.94 ± 0.19
	BW25113/pCA24N-*relA*	pCA24N-*relA*		25.36 ± 0.02	27.63 ± 0.22	
	BW25113/pCA24N	pCA24N	#2	22.86 ± 0.04	19.38 ± 0.01	1.74 ± 0.66
	BW25113/pCA24N-*relA*	pCA24N-*relA*		26.36 ± 0.62	23.68 ± 0.02	
MUP treatment	BW25113	Control	#1	23.43 ± 0.19	26.05 ± 0.43	4.14 ± 0.12
		MUP		23.96 ± 0.02	28.73 ± 0.09	
		Control	#2	24.26 ± 0.14	24.92 ± 0.07	5.08 ± 0.26
		MUP		24.84 ± 0.18	27.84 ± 0.14	
SHX treatment	BW25113	Control	#1	22.18 ± 0.16	22.96 ± 0.00	9.13 ± 0.60
		SHX		21.58 ± 0.01	25.55 ± 0.43	
		Control	#2	24.23 ± 0.51	20.08 ± 0.01	168 ± 0.34
		SHX		34.06 ± 0.28	37.30 ± 0.25	
Heat shock	BW25113	Control	#1	22.94 ± 0.15	22.79 ± 0.03	2.1 ± 0.02
		Heat		22.98 ± 0.04	23.93 ± 0.03	
		Control	#2	22.95 ± 0.44	22.14 ± 0.07	1.8 ± 0.40
		Heat		22.34 ± 0.08	22.42 ± 0.46	

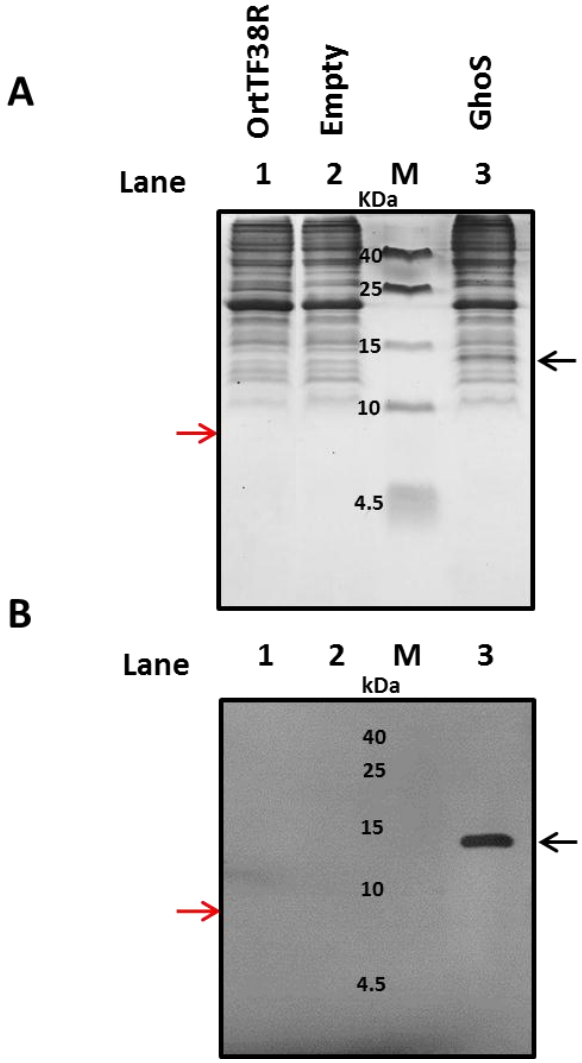

Figure S1. Western blot analysis of OrtTF38R. His-tagged OrtTF38R and His-tagged GhoS (positive control) were visualized by tricine-SDS-PAGE (**A**) and a Western blot (**B**). Empty plasmid pCA24N was used as the negative control (lane 2). Twenty µg of total cell proteins were loaded on the gel and the expected sizes of OrtTF38R (8.89 kDa) and GhoS (13.8 kDa) are indicated by a red and a black arrow, respectively. Plasmid abbreviations: OrtTF38R, pCA24N-*ortT*F38R; Empty, pCA24N; GhoS, pCA24N-*ghoS*.

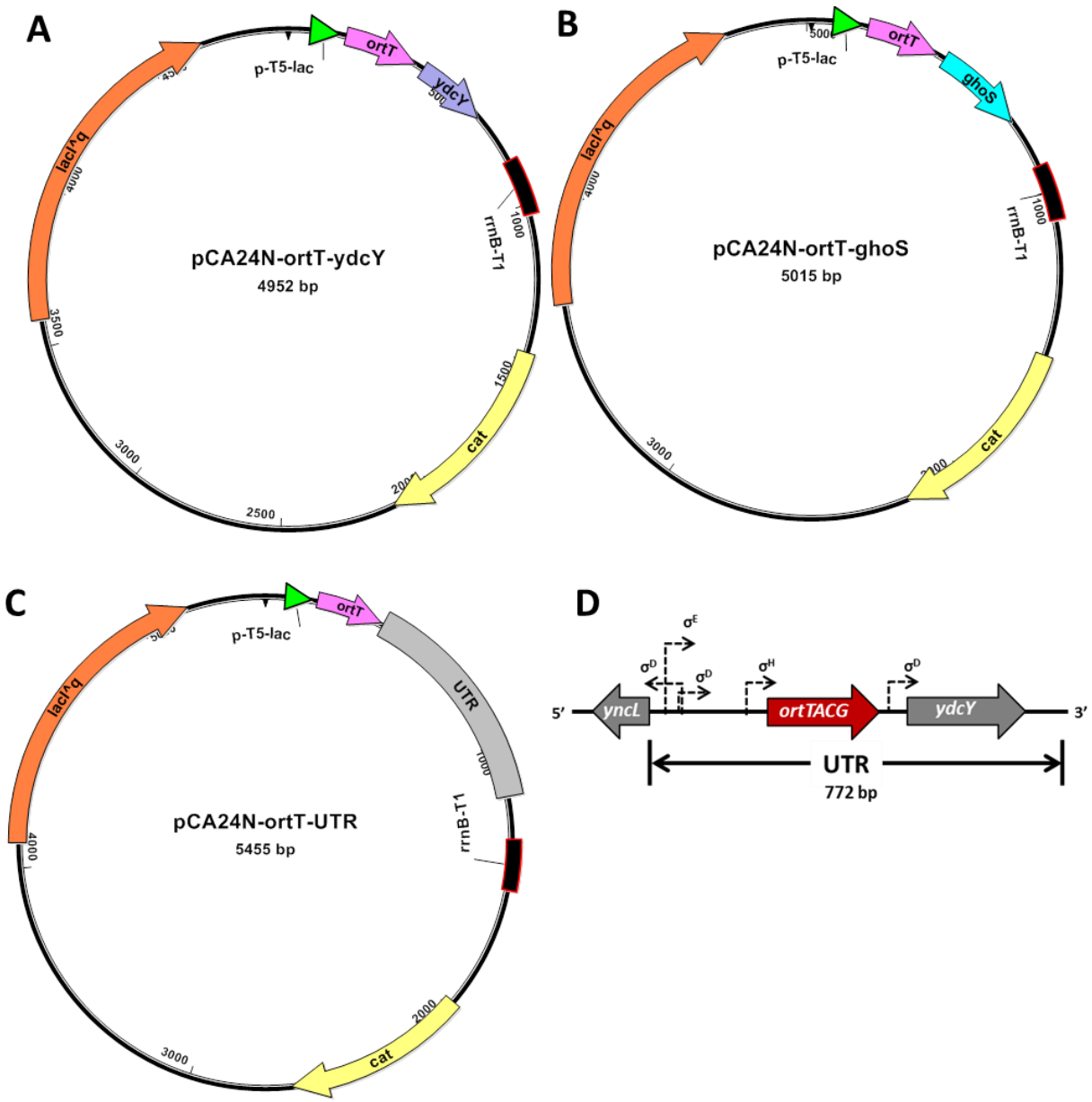

Figure S2. Plasmid maps for finding antitoxin for OrtT. (**A**) pCA24N-*ortT-ydcY* with both *ortT* and *ydcY* under control of the $P_{T5\text{-}lac}$ promoter; (**B**) pCA24N-*ortT-ghoS* with both *ortT* and *ghoS* under control of the $P_{T5\text{-}lac}$ promoter; (**C**) pCA24N-*ortT*-UTR with both *ortT* and the UTR (*ortT* flanking region from the chromosome) under control of the $P_{T5\text{-}lac}$ promoter; (**D**) UTR construction using the upstream intergenic region (between *yncL* and *ortT*), an inactivated *ortT* (*ortT*ACG), and the downstream intergenic region including *ydcY* for investigating the presence of a small RNA antitoxin partner for OrtT.

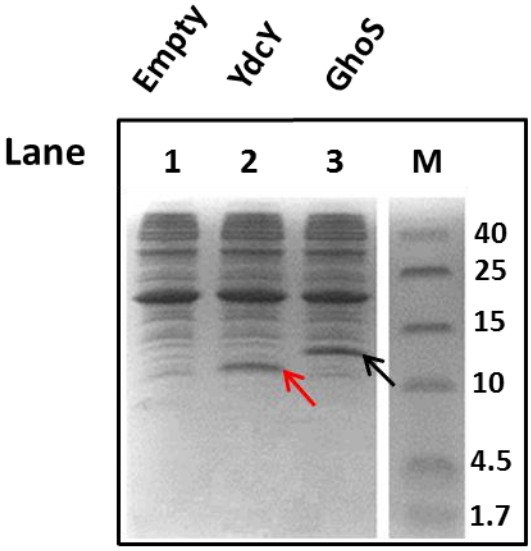

Figure S3. YdcY is a protein. His-tagged YdcY and his-tagged GhoS were produced from pCA24N-*ydcY* and pCA24N-*ghoS* and visualized by tricine-SDS-PAGE. Empty plasmid pCA24N was used as the negative control. Ten μg of total cell proteins were loaded on the gel and the expected sizes of YdcY (11.14 KDa) and GhoS (13.8 KDa) are indicated by a red and a black arrow, respectively. Plasmid abbreviations: Empty, pCA24N; YcdY, pCA24N-*ydcY*; GhoS, pCA24N-*ghoS*.

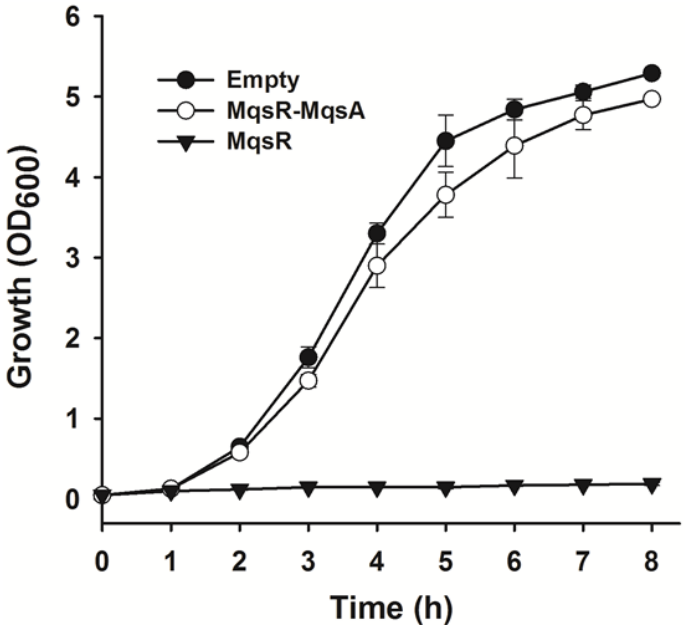

Figure S4. Co-expression of *mqsR* and *mqsA* from the same promoter. Growth of BW25113 Δ*mqsR* Δ*mqsA* Δ*Kan* cells harboring pBS(Kan) (empty plasmid), pBS(Kan)-*mqsR*, and pBS(Kan)-*mqsR*-*mqsA* in LB medium with kanamycin (50 μg/mL) to retain the plasmids along with 1 mM IPTG to induce protein production after 1 h.

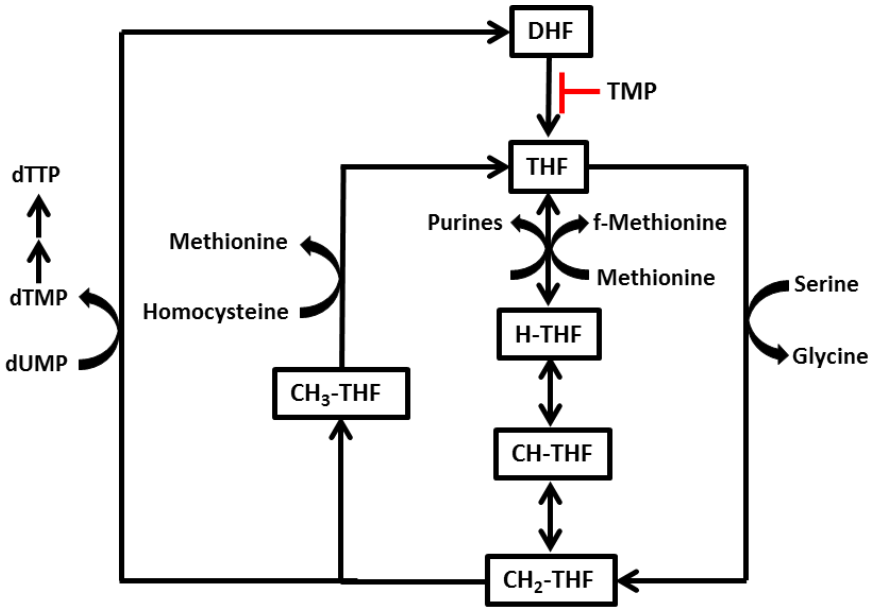

Figure S5. Tetrahydrofolate-related pathways. Abbreviations: TMP, trimethoprim; DHF, dihydrofolate; THF, tetrahydrofolate; H-THF, formyltetrahydrofolate; CH-THF, 5,10-methenyltetrahydrofolate; CH_2-THF, 5,10-methylenetetrahydrofolate; CH_3-THF, 5-methyltetrahydrofolate; Met, methionyl-$tRNA^{fMet}$; f-Met, formylmethionyl-$tRNA^{fMet}$.

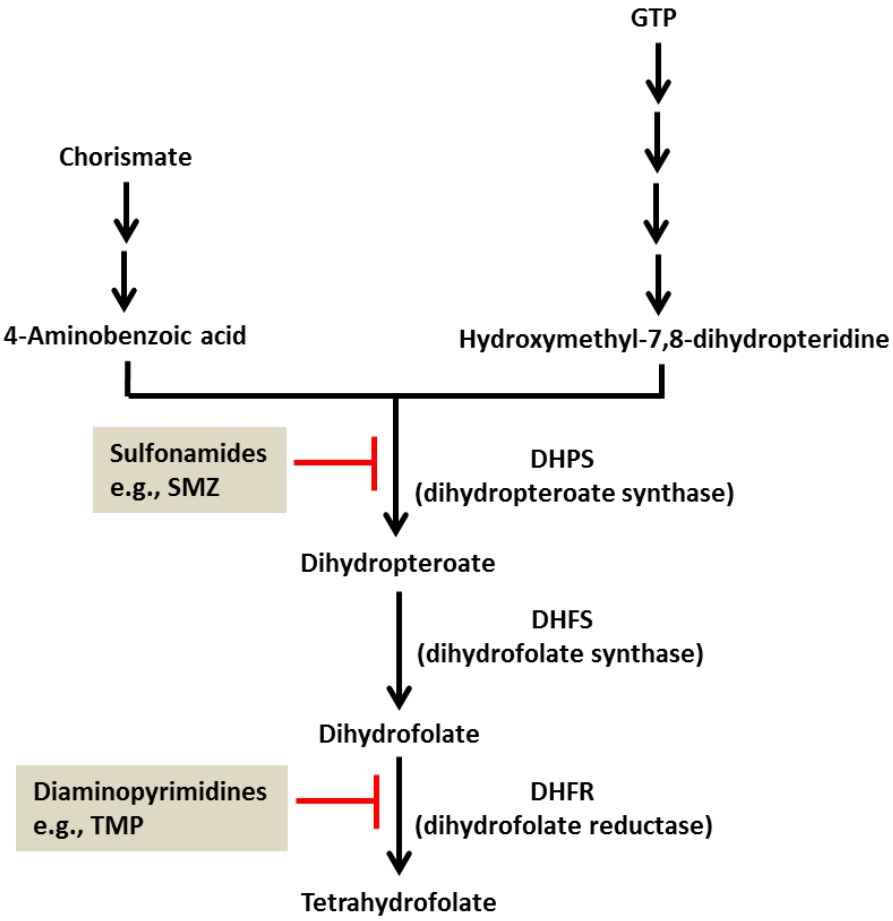

Figure S6. Tetrahydrofolate biosynthesis synthesis pathway.