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#### **Supplemental Information**

#### Inhibiting the Evolution of Antibiotic Resistance

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Figure S1. Cells lacking Mfd are not significantly sensitive to DNA damaging agents, Related to Figure 1 Survival assays to (A) UV damage and (B) 4NQO for WT,  $\Delta mfd$ , and  $\Delta uvrA$  strains of *B. subtilis* HM1 (Bs) and *S. typhimurium* ST19 (St). (*uvrA* knockouts, known to be sensitive to DNA damage, were included for comparison to *mfd* knockouts). Data represents at least two independent experiments with duplicates for each experiment. Errors bars indicate s.e.m. Statistical significance was determined using two-tailed Student's t-test (\*p-value <0.05, \*\*p-value <0.01, \*\*\*p-value <0.001).

A			-	-												
Γ			No mutat	tions					Γ			No m	utations			*
			No mutat	tions											L511P D516G	
					1572S	1572S								1572F	1572F	*
WT replicates		G536V S574F	G536V S574F	G536V S574F	G536V S574F	G536V S574F	* *	∆ mfd					L538P	L538P	L538P	*
	1572F	1572F	1572F	1572F	1572F	1572F							H526Y	H526Y	H526Y	
	1572F	1572F	1572F	1572F	1572F	1572F							1572N	1572N Q148L	1572N Q148L	
	S512F	S512F	S512F H526Y	S512F H526Y	S512F H526Y	S512F H526Y		replicates					Q148L	Q148L	Q148L H526Y	
	1572F	1572F	1572F	1572F	1572F	1572F						S509R	S509R	S509R	S509R	
	S512P	S512P	S512P	S512P	S512P	S512P						D516G	D516G	D516G	D516G	*
	Q148L	Q148L	Q148L	Q148L	Q148L	Q148L						Q148L	Q148L	Q148L	Q148L	*
	L511Q	L511Q	L511Q	L511Q	L511Q	L511Q	*				Q148L	Q148L	Q148L	Q148L	Q148L	
	Q513H D516G	Q513H	*			1572N	572N 1572N 1572N 1572N	1572N	1572N	1572N	*					
L	24	48	72	96	120	144				24	48	72	96	120	144	
			Tin	ne (h)								Tim	ie (h)			
Вг			Tin Trin	ne (h) nethoj	orim							Tim Tri	ne (h) meth	oprim		
В			Tin Trim	ne (h) nethoj	orim		D27E		Γ			Tim Tri	ne (h) metho No mutation	oprim s		
в	-		Tin	ne (h) nethor	orim		D27E F153S M201	*				Tim Tri	ne (h) metho No mutation	oprim s		
В			Tin	ne (h) nethor <sup>M201</sup>	Drim M201	M201	D27E F153S M20I D27E F153C	*				Tim Tri	ie (h) metho No mutation No mutation	oprim s s		
B			Tin Trin w30G	ne (h) nethor M201 W30G	M20I W30G	M201 W30G	D27E F153S M20i D27E F153S W30G	*				Tim Tri	ie (h) metho No mutation No mutation	oprim s s		P21L
В			Tin Trim w30G M20I	ne (h) nethor M201 W30G M201	Drim M201 W30G M201	M201 W30G M201 F153S	D27E F153S M201 D27E F153S W30G M201 F153S	*				Tim Tri	ie (h) methe to mutation to mutation	oprim s s		P21L M20I
B			Tin Trim W30G M20I F153S	me (h) methop M201 W30G M201 F153S	M201 W30G M201 F153S M201	M20I W30G M20I F153S M20I M20I	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I	* * ronlicatos				Tim Tri	ie (h) methe No mutation No mutation	oprim s s	W30C	P21L M201 W300
B WT replicates			Tin Trim W30G M20I F153S M20I	ne (h) nethor M201 W30G M201 F153S M201	M201 W30G M201 F153S M201 F153S	M20I W30G M20I F153S M20I M20I F153S	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I F153S D27E M20I F153S W30G	* * ^ ^ ^ ^ ^ mfd replicates				Tim Tri	ie (h) metho No mutation No mutation	oprim s s L28R	W30C L28R	P21L M20I W300 L28R
B WT replicates		W30G	Tin Trim W30G M20I F153S M20I W30G	M201 M201 M201 F153S M201 W30G M201 M201	M201 M201 W30G M201 F153S M201 F153S W30G M201	M20I W30G M20I F153S M20I F153S W20I F153S W30G M20I	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I F153S D27E M20I F153S D27E M20I F153S	* * ∆ <i>mfd</i> replicates				Tim Tri	ie (h) methe	oprim s s L28R F153S	W30C L28R F153S	P21L M20I W300 L28R F1533
B WT replicates		W30G M201	Tin Trim W30G M20I F153S M20I W30G M20I E1550	ne (h) nethor M201 W30G M201 F153S M201 D27E	M20I           W30G           M20I           F153S           M20I           F153S           W30G           M20I           F153S           W30G           M20I           F153S           W30G           M20I           F153S           W30G           M20I           D27E           F4555	M20I W30G M20I F153S M20I F153S W30G M20I D27E F153S	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I F153S W30G D27E M20I F153S W30G D27E F153S	* * ∆ <i>mfd</i> replicates				Tim Tri	Ie (h) Impethential Internation Internatio	oprim s s L28R F153S D27E	W30C L28R F153S D27E	P21L M201 W3000 L28R F1533 D27E
B WT replicates	F153S	W30G M20I F153S	Tin Trim W30G M20I F153S M20I W30G M20I F153S	ne (h) nethop M20i W30G M20i F153S M20i D27E F153S	M20I           W30G           M20I           F153S           M20I           F153S           W30G           M20I           F153S           W30G           F153S           W30G           F153S           W30G           F153S           W30G           M20I           D27E           F153S           M20I	M20I W30G M20I F153S M20I F153S W30G M20I D27E F153S D27E F153S D27E M20I	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I D27E M20I D27E F153S D27E M20I D27E F153S D27E	* * ∆ <i>mfd</i> replicates				Tim Tri	Ie (h) Impethence In the impet	oprim s s s L28R F153S D27E F153S	W30C L28R F153S D27E F153S	P21L M20I W3000 L28R F1533 D27E F1533
B WT replicates	F153S M20I	W30G M20I F153S M20I	Tin Trim W30G M20I F153S M20I W30G M20I F153S M20I F153S	ne (h) nethop M201 W30G M201 F153S M201 D27E F153S M201 F153S	M20I           W30G           M20I           F153S           M20I           F153S           W30G           M20I           F153S           W30G           M20I           F153S           W30G           M20I           F153S           M20I           F153S	M20I W30G M20I F153S M20I F153S M20I F153S D27E F153S D27E M20I F153S D27E M20I F153S S F153S F153S	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I F153S D27E M20I D27E F153S D27E M20I D27E F153S D27E M20I D27E F153S D27E	* * ∆ <i>mfd</i> replicates				Tim Tri	Ie (h) Impethence Io mutation	DPrim s s s L28R F153S D27E F153S W30G	W30C L28R F153S D27E F153S W30G D27E	P21L M201 W300 L28R F1533 D27E F1533 W300 D27E
B WT replicates	F153S M20I F153S	W30G M20I F153S M20I F153S	Tin Trim W30G M20I F153S M20I F153S M20I F153S M20I F153S	M20I M20I W30G M20I F153S M20I D27E F153S M20I F153S P21L CC	M20I           W30G           M20I           F153S           M20I           F153S           W30G           M20I           F153S           W30G           M20I           F153S           M20I           F153S           M20I           F153S           M20I           F153S           M20I           F153S           P21E           P21E           P21C           P220	M20I W30G F153S M20I F153S W30G M20I F153S D27E F153S D27E M20I F153S D27E M20I F153S P21L D27E	D27E F153S M20I D27E F153S W30G M20I F153S D27E M20I F153S D27E M20I F153S D27E M20I D27E F153S D27E M20I F153S D27E M20I D27E F153S D27E	* * ∆ <i>mfd</i> replicates * *					IE (h) Impethential Internation Internatio	oprim s s s L28R F153S D27E F153S W30G D27E	W30C L28R F153S D27E F153S W30G D27E F153S	P21L M20I W300 L28R F1533 D27E F1533 W300 D27E F1533

## Figure S2. Cells lacking Mfd show fewer and delayed resistance-conferring mutations, Related to Figure 2, Figure S3, and Table S1

(A) Sequencing of *rpoB* was performed at each time point from rifampicin evolution assays to identify mutations that confer resistance in WT and  $\Delta mfd$  strains of *S. typhimurium* (12 replicates per strain). (B) Sequencing of *folA* was performed at each time point from trimethoprim evolution assays to identify mutations that confer resistance in WT and  $\Delta mfd$  strains of *S. typhimurium* (12 replicates per strain). Shown are the position and corresponding amino acid changes. \*Indicates replicates sequenced by whole genome sequencing.



# Figure S3. Development of hypermutation in evolved WT strains of *S. typhimurium*, Related to Figure 2, Figure S2, and Table S1

Mutation rate analysis of *S. typhimurium* strains evolved to trimethoprim. Assays were performed on rifampicin plates as described in Figure 1. The individual ancestor and evolved WT (containing a *dnaQ*133N mutation) isolates used in this experiment are indicated in Table S1. The number of replicates per isolate is 12.



## Figure S4. Mfd requires interaction with RNAP and UvrA to promote evolution to antibiotics, Related to Figure 4

Evolution of indicated *S. typhimurium* ST19 strains to phosphomycin (A) and trimethoprim (B). Plots and statistical testing for evolution assays were performed as described in Figure 2. \*\*\*p-value <0.001 between WT and  $\Delta mfd$ , between WT and  $\Delta mfd$ ::mfd(L499R), and between WT and  $\Delta mfd$ ::mfd(R165A) strains for evolution to phosphomycin. \*\*p-value <0.01 between WT and  $\Delta mfd$  and between WT and  $\Delta mfd$ ::mfd(L499R) strains for evolution to trimethoprim. n = 12-24 replicates per strain.



## Figure S5. Strains lacking Mfd show no survival defects in bone marrow macrophages, Related to Figure 1

Murine-derived bone marrow macrophages (BMMs) were infected with WT and  $\Delta mfd$  strains of *S. typhimurium* ST19 and harvested for CFU enumeration at indicated times points. Data represents two independent experiments with triplicate samples for each given experiment. Error bars indicate s.e.m.