Extreme Acid Modulates Fitness Tradeoffs of Multidrug Efflux Pumps MdtEF-TolC and AcrAB-TolC in *Escherichia coli* K-12

By

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ABSTRACT

Bacterial genomes encode various multidrug efflux pumps (MDR) whose specific conditions for fitness advantage are unknown. We show that the efflux pump MdtEF-TolC, in *Escherichia coli*, confers a fitness advantage during exposure to extreme acid (pH 2). Our flow cytometry method revealed pH-dependent fitness tradeoffs between bile acids (a major pump substrate) and salicylic acid, a membrane-permeant aromatic acid that induces a drug-resistance regulon but depletes proton motive force (PMF). The PMF drives MdtEF-TolC and related pumps such as AcrAB-TolC. Deletion of *mdtE* (with loss of pump MdtEF-TolC) increased the strain's relative fitness during growth with or without salicylate or bile acids. However, when the growth cycle included a 2-h incubation at pH 2 (below the pH growth range), MdtEF-TolC conferred a fitness advantage. The fitness advantage required bile salts but was decreased by the presence of salicylate, whose uptake is amplified by acid. For comparison, AcrAB-TolC, the primary efflux pump for bile acids, conferred a PMF-dependent fitness advantage with or without acid exposure in the growth cycle. A different MDR pump, EmrAB-TolC, confered no selective benefit during growth in the presence of bile acids. Without bile acids, all three MDR pumps incurred a large fitness cost with salicylate when exposed at pH 2. These results are consistent with the increased uptake of salicylate at low pH. Overall, we showed that MdtEF-TolC is an MDR pump adapted for transient extreme-acid exposure; and that low pH amplifies the salicylate-dependent fitness cost for drug pumps.

IMPORTANCE

Antibiotics and other drugs that reach the gut must pass through stomach acid. Yet little is known of how extreme acid modulates the effect of drugs on gut bacteria. We find that extreme-acid exposure leads to a fitness advantage for a multidrug pump that otherwise incurs a

fitness cost. At the same time, extreme acid amplifies the effect of salicylate selection against multidrug pumps. Thus, organic acids and stomach acid could play important roles in regulating multidrug resistance in the gut microbiome. Our flow cytometry assay provides a way to measure the fitness effects of extreme-acid exposure to various membrane-soluble organic acids including plant-derived nutrients and pharmaceutical agents. Therapeutic acids might be devised to control the prevalence of multidrug pumps in environmental and host-associated habitats.

INTRODUCTION

Bacterial multidrug resistance (MDR) efflux systems export diverse antibiotics, metals and harmful products of metabolism (1–3). These MDR pumps also remove environmental or host-derived antimicrobials like bile acids (4) as well as toxic products of the bacterium's own metabolism (5,6). For pathogens, MDR pumps serve as first-line defense against multiple antibiotics at low levels (7). Thus, MDR pumps in pathogens pose a major threat to human health (8–11). Yet the metabolic and biochemical conditions in which they provide a fitness benefit are poorly understood.

The *Escherichia coli* K-12 genome contains genes for at least 36 multidrug efflux systems (5,12). Only a few of these are understood in detail. The pump best known is AcrAB-TolC, a member of the Resistance-Nodulation-Cell Division (RND) superfamily (13). AcrAB-TolC exports bile acids (4,14) as well as antimicrobial drugs, dyes, organic solvents, essential oils, and hormones (15,16). AcrAB expression is upregulated by many proteins including the global regulator MarA, which is activated by aspirin derivatives such as salicylate and benzoate (17,18) (**Fig. 1**). The salicylate-induced Mar regulon intersects with the Gad acid fitness island, which includes *mdtEF* genes encoding components of MdtEF-TolC, an RND pump structurally similar to AcrAB-TolC (19,20). MdtEF-TolC contributes to biofilm formation (21) and nitrate respiration (22). Other tripartite pumps similar to AcrAB-TolC include members of the Major Facilitator Superfamily (MFS) such as EmrAB-TolC (23).

Clinical and environmental management of MDR-associated antibiotic resistance requires understanding the physiological tradeoffs of such pumps, most of which spend substantial amounts of energy (12,13). Our laboratory uses experimental evolution to explore conditions that could reverse the fitness benefit of drug efflux pumps and thus decrease their prevalence in

microbial communities (24–26). Surprisingly, such conditions include the presence of salicylate or benzoate that induce drug resistance regulons. Under serial culture, these MDR inducers actually select against resistance to antibiotics, favoring mutants that have lost pumps such as MdtEF and EmrAB (24,26,27). Transcription profiles support a model in which genes responding to transient aromatic-acid stress actually decrease fitness over periods of chronic exposure (26).

Little is known regarding the effects of salicylates on the gut microbiome, and their interactions with substances such as bile acids. The antimicrobial activity of bile acids plays an important role in structuring the gut microbiome (28–30); and bile acids induce AcrAB-TolC (31).

Most drug pumps power their efflux by spending proton motive force (PMF). For example, PMF drives pumps of the RND superfamily such as AcrAB-TolC (15,16) and the MFS superfamily pump EmrD (32). However, these pumps cannot function when exposed to PMF uncouplers such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (12,25). Full uncouplers penetrate the membrane in the protonated and unprotonated forms, whereas partial uncouplers cross mainly in the protonated form. Partial uncouplers include membrane-soluble aromatic acids such as aspirin, salicylic acid, and ibuprofen, which belong to the class of nonsteroidal anti-inflammatory drugs (NSAIDs) (33,34). Membrane-soluble acids and their derivatives are of interest for human diet, as many are phytochemicals produced by plants, which control their microbiomes in ways that are poorly understood.

An important aspect of proton-driven drug pumps is their association with extreme-acid resistance (35). Extreme-acid resistance enables *E. coli* and other enteric bacteria to survive transient exposure at a range of pH 1-3, as found in the human stomach (36,37). Moderate acid

(pH 5.0-6.8) upregulates systems for extreme-acid survival such as those of the Gad acid fitness island (38,39). The acid fitness island includes genes *mdtEF* for the MdtEF-TolC pump (19,20) and for Gad transcriptional regulators such as GadE (40). Gad-dependent acid resistance allows stationary-phase survival under extreme acid where *E. coli* cannot grow. The system raises intracellular pH by consuming protons through the decarboxylation of glutamate and glutamine by GadA and GadB, regulated by GadE. Despite the importance of this system, few studies of experimental evolution incorporate extreme-acid exposure. In one report, twenty cycles at pH 2.5 selected for mutations in the acid-resistance regulator EvgS (41). Such an experiment, in effect, tests evolving mutants for relative death rates under an adverse condition.

We investigated the tradeoffs between PMF-driven MDR pumps and the presence of salicylate and bile acids (24–26). We developed a method using flow cytometry (42) to measure the relative fitness contributions of MDR genes versus null alleles under various conditions, including the presence of uncouplers and of exportable substrates such as bile acids (cholic acid and deoxycholic acid). We modified the method to include cycles of extreme acid exposure. Using this approach, we showed that the MdtEF-TolC pump requires pH 2 exposure to confer positive relative fitness. This finding represents a novel case of an extreme acid-dependent drug efflux pump. We also observed distinct patterns of relative fitness for the AcrAB-TolC and EmrAB-TolC efflux pumps.

RESULTS

Relative fitness measurement by flow cytometry of YFP and CFP. We sought to measure the relative fitness contributions of MDR genes in the presence of various organic acids. For this purpose we modified the flow cytometry assay of Gullberg (42) which uses yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) markers (**Fig. 2**). In our assay, each strain had a gene of interest knocked out by replacement with *kanR* from alleles of the KEIO collection with the exception of the Δ*slp-gadX* strain (43). The *kanR* gene constitutively expresses an aminoglycoside 3'-phosphotransferase (43,44). In early competition trials, we found that the presence of *kanR* incurs a small fitness cost relative to control strain, *E. coli* W3110. Therefore, in all assays involving knock-out strains that expressed *kanR*, we used a control strain with the *yhdN*::*kanR* allele. For the single gene deletions used in this experiment (*ΔacrA*::*kanR, ΔmdtE*::*kanR, ΔgadE*::*kanR*, and *ΔemrA*::*kanR*) each allele was transduced into a strain of *E. coli* K-12 W3110 containing the fluorophore allele *galK*::*yfp* or *galK*::*cfp* inducible via a *lac* promoter (42).

The mixture of two strains was serially diluted 1000-fold each day, and observed over a total of 30 generations (doublings) from day zero to day 3. This period was sufficient to permit accurate measurement of relative fitness, but not long enough to make the rise of new mutations likely (42). All culture media were buffered at pH 6.8, a level that allows cytoplasmic pH depression by membrane-permeant acids of lower pK_a. Each day, a parallel dilution with IPTG inducer was performed using LBK-PIPES pH 6.8 buffered medium without stressors. The IPTG-induced populations express YFP or CFP for flow cytometry, whereas the overnight 1000-fold dilutions avoid energy-expensive fluorophore expression. This procedure enabled us to minimize the fitness effects of fluorophore expression during stress selection.

Fig. 2A shows the appearance of a distinctive population of YFP-expressing cells showing high fluorescence intensity with 488-nm excitation, and low-intensity fluorescence at 405 nm, versus a second population expressing CFP with low intensity at 488-nm excitation and high intensity at 405 nm. **Fig. 2B** show a typical experiment in which the $\log_2$ ratios of YFP and CFP populations were reported for the tested mutant (W3110 *acrA*::*kanR*) and the control (W3110 *yhdN*::*kanR*). For each competition experiment, an equal number of trials were performed with the gene knock-out strain expressing YFP and the control strain expressing CFP, and vice versa.

Relative fitness was measured as the selection rate, defined as the daily change in $\log_2$ ratio of cocultured strain populations (45). A selection rate of 1 unit per day indicates a two-fold increase in population of one strain compared to the competing strain's population. This measure indicates the relative fitness of two strains cultured under a given stress condition.

The fitness benefit of MdtE requires extreme acid exposure (pH 2). In a strain lacking AcrAB-TolC, overexpression of MdtEF-TolC confers resistance to antibiotics and bile acids (46). The range of substrates transported by an RND pump depends on its distal pocket, the periplasmic portion of its substrate-binding pocket (47). MdtE shares 55% amino-acid sequence identity with AcrA (13) but its periplasmic substrate-binding pocket includes amino-acid residues with a lower isoelectric point (pI=3.1 for MdtEF, pI=4.0 for AcrAB) (20). For this reason, we investigated roles for acids and acidic conditions in pump function.

We first tested the relative fitness contribution of *mdtE* by competition of an *mdtE*$^+$ strain (W3110 *ΔyhdN*::*kanR*) against W3110 *ΔmdtE*::*kanR* in the presence of 6 mM salicylate and 0.15% bile acids (**Fig. 3A**). The *mdtE* deletion allele increased the relative fitness under all

158 conditions—the pH 6.8 control medium, as well as in 0.15% bile acids, or in 6 mM salicylate
159 ($p<0.001$). Selection against the *mdtE*$^+$ strain was further increased by the addition of 10 μM
160 CCCP ($p<0.001$) which depletes the PMF needed to drive efflux (**Fig. 3A**). The expenditure of
161 limited PMF by the MdtEF-TolC could be one possible explanation for the pump's fitness cost.
162 All conditions tested led to a fitness cost or neutral selection for MdtEF-TolC.
163 Under what growth conditions does MdtEF-TolC confer advantage? We tried including a
164 period of pH 2 exposure, a condition for which the Gad regulon maintains extreme-acid survival
165 dependent on stationary phase (35). This condition of stationary phase in extreme acid mimics
166 the passage of bacteria through an acidic stomach, an adaptation that helps enteric *E. coli* gain
167 access to the intestinal tract and may activate drug efflux pumps (19). Our serial cultures enter
168 stationary phase for at least 12 h, so they are fully acid resistant before each dilution. Starting on
169 Day zero, the CFP/YFP strain mixture was diluted 100-fold in unbuffered LBK at pH 2.0. The
170 acidified cell suspension, approximately pH 2, was incubated for 2 hours. Under this condition,
171 survival of *E. coli* K-12 strains is typically 50-100% (35,48,49). The acidified suspension was
172 then diluted ten-fold in media buffered with PIPES at pH 7.0, leading to a final pH of 6.8. The
173 total dilution overall was 1,000-fold per day, comparable to our original relative fitness assay.
174 This period of pH 2 exposure was repeated for each of Days 1-3 of the assay.
175 The 2-h exposure to extreme acid eliminated the fitness cost of *mdtE* during coculture at
176 pH 6.8. The *mdtE* allele conferred a fitness benefit in the presence of bile acids, with or without
177 2 mM salicylate (**Fig. 3B)**. A higher concentration of 4 mM salicylate, however, incurred a large
178 fitness cost for MdtE ($p<0.001$). The highest salicylate concentration tested (6 mM) did not
179 permit growth over three days. Note that after 100-fold dilution in LBK pH 2, there would still
180 be 0.06 mM concentration of salicylate. At external pH 2, where the *E. coli* internal pH is about

pH 5 (49) the uptake of salicylic acid (with a $pK_a = 2.8$) would be greatly increased by the transmembrane pH difference, a ΔpH of approximately three units. So the low pH could amplify the fitness effect of a small salicylate concentration.

AcrA incurs fitness tradeoffs. AcrAB-TolC is the best known of *E. coli* RND-type efflux pumps, and is the most important for efflux of bile acids and other deleterious molecules (13). Nevertheless, we found that the $acrA^+$ strain incurs a measurable fitness cost during culture at pH 6.8 ($p<0.01$, **Fig. 4A**). The fitness cost was seen with or without salicylate. The $acrA^+$ allele conferred fitness benefit only in the presence of bile acids ($p<0.001$; **Fig. 4A**). This result might show that the AcrAB-TolC efflux pump is worth the energy expenditure only when it is needed to export a deleterious substrate. The advantage conferred by bile acids was eliminated by CCCP, which would be consistent with pump dependence on PMF.

The daily growth cycle was adjusted to include a 2-h period in stationary phase at pH 2 (**Fig. 4B**). Under this adjusted cycle, the $acrA^+$ strain showed a small fitness cost ($p<0.01$) and lost its fitness benefit in the presence of bile acids. A large fitness cost was incurred with 4 mM salicylate ($p<0.001$), whereas the fitness advantage was restored with bile acids and a small amount of salicylate ($p<0.001$, **Fig. 4B**). The smaller amount of salicylate may tip the balance by inducing AcrAB expression during the pH 6.8 portion of the growth cycle (31). So the bacteria's ability to make AcrAB-TolC leads to very different tradeoffs in the presence of bile acids and salicylate, dependent on pH 2 exposure.

EmrA carries a fitness cost. The EmrAB-TolC efflux pump structurally resembles AcrAB-TolC efflux pump, with EmrA providing a link between the outer membrane efflux pump

component, TolC and inner membrane component, EmrB (50). The EmrAB-TolC efflux pump is activated by salicylate, and confers resistance to some antibiotics as well as CCCP (51). As seen for *mdtE*, *emrA* incurred a fitness cost in the pH 6.8 under all conditions tested, with the exception of bile acids plus salicylate (**Fig. 5A**). The fitness cost was increased by the presence of CCCP ($p<0.01$, **Fig. 5A**). This is surprising since the EmrAB-TolC efflux pump confers resistance to CCCP, but CCCP resistance may require *emrA* overexpression through mutations to the transcriptional repressor *emrR* (*mprA*) (25).

Inclusion of pH 2 exposure during the growth cycle did not increase relative fitness for $emrA^+$ (**Fig. 5B**). The $emrA^+$ strain was less fit in the presence of salicylate, with or without bile acids ($p<0.001$). Unlike MdtEF-TolC or AcrAB-TolC, EmrAB-TolC did not show evidence of functional bile acid export under the conditions tested.

The Gad island shows fitness tradeoffs with bile acids and salicylate. In addition to the multidrug efflux pump components *mdtEF* (46), the Gad acid fitness island (**Fig. 1**) includes genes whose products counteract acid stress, such as periplasmic acid chaperones *hdeA* and *hdeB* and acid-resistance regulators *gadE* and *gadX* (38). Nonetheless, this region of the genome often undergoes deletion during experimental evolution with acid stress (26,52).

We investigated how the *mdtE* fitness effects compares to the fitness effects of the entire Gad island. Competition assays were conducted using a strain with most of the Gad island deleted by recombineering (*Δslp-gadX*) (26). In this assay, the parent strain W3110 had a small fitness advantage over the *Δslp-gadX* strain (**Fig. 6A**). This result differed from the $mdtE^+$ competition (**Fig. 3A**) in which the *mdtE* deletion had no effect on fitness. The advantage conferred by the Gad island was independent of the presence or absence of bile acids, but was

reversed by the presence of 6 mM sodium salicylate. Thus, the negative effect of salicylate overcomes whatever net fitness contribution is provided by components of the Gad island. The net effects on fitness are small compared to the fitness effects observed for individual knock-out strains (**Fig. 3, 4, 5**).

An important regulatory component of the glutamate-dependent acid resistance pathway is *gadE* (**Fig. 1**; (53,54)). GadE activates the decarboxylation of glutamate in the cell by the upregulation of *gadA* and *gadB* (48). We sought to determine if salicylate affects the fitness benefit of $gadE^{+}$ similarly to that of the overall Gad island (*slp-gadX*) . In pH 6.8 medium, the control strain had a fitness advantage over *ΔgadE*::*kanR*, similar to the fitness advantage conferred by the Gad island as a whole (**Fig. 6B)**. With bile acids, however, GadE conferred no selective benefit. The presence of 6 mM salicylate, in the absence of bile acids, incurred a fitness cost for *gadE* ($p<0.001$; **Fig. 6B**). These results suggest that some component regulated by GadE is sensitive to salicylate; and that some unidentified tradeoff exists with bile acids.

DISCUSSION

Previously, it was unknown why genes encoding the MdtEF pump reside in the Gad acid fitness island (**Fig. 1**) along with a complex set of regulators of acid resistance via glutamate decarboxylase and periplasmic chaperones (38). The MdtE fitness benefit with pH 2 exposure (**Fig. 3**) suggests that MdtEF-TolC effluxes bile acids under extreme acid, where the cell cannot grow but its death rate can be slowed by various acid-resistance components of the Gad island. This finding would be consistent with the previously unexplained requirement of pump component TolC for Gad-related survival at pH 2 (55).

Our modified flow cytometry assay enables us to explore the fitness effects of extreme-acid exposure, which enteric pathogens as well as commensal strains must survive to reach the intestine (35,48,49). Our results may provide clues as to the relative fitness of drug-exporting strains during human uptake of aromatic acid medications such as aspirin. These efflux pumps consume PMF and thus their fitness contribution may be neutralized or reversed by the presence of the uncoupler CCCP (Figures 3A, 4A, 5A). We show that the efflux pump fitness contribution may also be reversed by a membrane-permeant aromatic acid such salicylate during extreme-acid exposure (Figures 3B, 4B, 5B). The mechanisms of antimicrobial action of aromatic acids may involve depletion of PMF, as well as the ΔpH-amplified uptake of a molecule that disrupts the membrane (24,26,27,56,57).

Extreme-acid exposure had surprising impact on the fitness effects of bile acids. Bile acids occur at highest concentration in the lumen of the small intestine, where they enhance lipid absorption (58–60). While bile does not normally reach the stomach, many patients exhibit chronic bile reflux gastritis (61), a condition associated with disorders such as carcinogenesis.

Thus, pumps capable of extreme acid-dependent efflux of bile acids could be useful for bacteria that experience gastric transit.

The different MDR pumps we tested showed diverse effects of bile acids. AcrAB-TolC conferred a fitness benefit in the presence of bile acids under various conditions, whereas MdtEF-TolC confer an advantage only when the growth cycle included exposure at pH 2. By contrast, EmrAB-TolC conferred no fitness advantage under any of our conditions tested. Thus, conditions favoring this pump's action must involve substrates other than bile acids.

Although the intact *mdtE* gene decreased fitness under almost all conditions at pH 6.8, the overall Gad island (**Fig. 6A**) conferred a small selective benefit at pH 6.8. This advantage may derive from the net fitness contributions of Gad acid-resistance genes other than *mdtEF.* The Gad regulon includes various components whose functions in acid resistance are poorly understood (26). The *hdeAB* acid stress operon encodes two periplasmic proteins that prevent protein aggregation at pH 2.0 (62). It may be more advantageous for the cell to lose one energetically expensive pump rather than its entire acid defense system. We will explore further the effect of acid exposure on fitness of other Gad components, as well other MDR pumps.

Our interest in assessing fitness under acid exposure with salicylate has pharmaceutical implications. High levels of salicylate in the human stomach, whether through changes in diet or periodic exposure to drugs such as aspirin, can affect the relative survival of different bacteria to enter the stomach. Other pharmaceuticals likely interact in unexpected ways with the microbiome that influence clinical outcomes. Our relative fitness assay can reveal such interactions and explore their mechanisms.

MATERIALS AND METHODS

Strains and media. All strains used in our experiments are derived from *E. coli* K-12 W3110 (**Table 1**). Strains were constructed by phage P1 transduction, with confirmation by PCR amplification across the sequence joint (55). The main growth medium was LBK broth (10 g/l tryptone, 5 g/l yeast extract, 7.45 g/l potassium chloride) buffered at pH 6.8 with 100 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES, pK_a= 6.80) using NaOH to adjust pH (26). This medium is designated LBK-PIPES pH 6.8. Sodium salicylate, bile acids (50/50 mixture of sodium cholate and sodium deoxycholate) and CCCP were all obtained from Millipore Sigma.

FACS Competition Assays. Relative fitness of cocultured strains was measured by flow cytometry of strains expressing yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) (42). Strains were cultured in 2 mL of LBK-PIPES pH 6.8 incubated in separate tubes at 37°C with rotation for 16-h. On the next day (Day -1) 20 µl of each inoculated culture was pipetted into 2 ml of the appropriate competition medium for which the strains were competed and incubated for 24-h at 37°C with rotation. On Day 0, for each experimental condition 2 µl of a 1:1 mixture of the CFP and YFP strains to compete was added to 2 ml of the appropriate competition medium and incubated at 37°C with rotation for 24 h. From this culture, serial dilutions were repeated (2 µl into 2 ml) on Days 1 and 2. Thus each day leads to a 1000-fold dilution followed by approximately 10 doublings (generations), for a total of 30 doublings by Day 3. During each daily cycle the cells spend approximately 12-14 h in stationary phase (24,26).

To include a period of pH 2 exposure, overnight cultures were inoculated in unbuffered LBK pH 2 for the first 2-h of incubation each day (starting in the morning, Day 0 through Day

306 2). At pH 2, the bacteria cannot grow but they remain viable via the stationary phase-induced
307 Gad acid resistance (38,40). Each day, 2 µl of the mixture of competing CFP and YFP strains
308 was added to 200 µl of LBK pH 2 and incubated at 37°C for 2-h. After this incubation period,
309 1.8 ml of the competition medium buffered to pH 7 was added (restoring pH to approximately
310 6.8) and the tubes were incubated for 24 h at 37°C.

311 For daily flow cytometry: A separate dilution of each CFP and YFP coculture was
312 performed by adding 50 µl (1:40 dilution) of the 1:1 mixtures of labeled strains and 20 µl (1:100
313 dilution) of 100 mM IPTG to 2 ml of LBK-PIPES pH 6.8. This tube was incubated for 2 h at
314 37°C while rotating and then sampled using the BD FACSMelody Cell Sorter with a blue laser
315 (488 nm) and violet laser (405 nm). A 545/20 filter was used for YFP emission, and a 528/45
316 filter for CFP emission. Each competition mixture was diluted with 1X phosphate-buffered
317 saline (PBS) to obtain 50,000 total events. The dilution was set such that the processed events
318 were greater than 98% and the event rate was less than 10,000 events/second. The threshold
319 value of counts for each tested strain in a coculture was set at 0.01%. Two technical replicates of
320 each competition mixture were recorded and averaged. The percentages of cells with YFP or
321 CFP fluorescence was recorded. This process was repeated each day for days 0-3 of testing. For
322 each experimental condition, unless specified otherwise, 12 biological replicates were averaged,
323 always with equal numbers of YFP to CFP and CFP to YFP competitions.

324 For each gene tested, the selection rate s was calculated as:

325 $$s = \log_2 (R_t / R_0) / t$$

326 where R represents the ratio of cell numbers for the control strain (W3110 Δ*yhdN* or W3110) to
327 the knock-out strain of interest; and t represents time in days (with daily dilution 1:1,000,
328 approximately 10 generations per day) (45,63,64). This rate gives a biological indication of the

change in population distribution of cocultured genetic variants over time. For example, a selection rate of 1 unit per day (one doubling per 10 generations) means that each day, one of two cocultured strains increases its population advantage two-fold over the competing strain.

Statistical Analysis. ANOVA and post-hoc Tukey tests were performed to compare trials under different conditions. In the figures, brackets indicate the Tukey results (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Single sample t-tests were performed to compare the value of each selection rate to zero. (†$p<0.05$, ††$p<0.01$, †††$p<0.001$). Statistical results are presented in Supplemental File 1.

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530

TABLES

Table 1. *E. coli* strains used in this study

Strain	Genotype/Description	Source
W3110	*E. coli* K-12 $F^- \lambda^-$	Lab stock (65)
CH367	Δ*lac*::FRT *ΔgalK*::*cfp-bla* (AmpR)	(42)
CH372	Δ*lac*::FRT *ΔgalK*::*yfp-bla* (AmpR)	(42)
JLS1779	W3110 *ΔgalK*:: *cfp-bla*	This study
JLS1780	W3110 *ΔgalK*:: *yfp-bla*	This study
JLS1910	W3110 *ΔgalK*:: *cfp-bla*, *ΔyhdN*::*kanR*	This study
JLS1911	W3110 *ΔgalK*:: *yfp-bla* , *ΔyhdN*::*kanR*	This study
JLS1826	W3110 *ΔgalK*:: *cfp-bla*, *ΔacrA*::*kanR*	This study
JLS1832	W3110 *ΔgalK*:: *yfp-bla*, *ΔacrA*::*kanR*	This study
JLS1834	W3110 *ΔgalK*:: *cfp-bla*, *ΔmdtE*::*kanR*	This study
JLS1835	W3110 *ΔgalK*:: *yfp-bla*, *ΔmdtE*::*kanR*	This study
JLS1912	W3110 *ΔgalK*:: *cfp-bla*, *ΔemrA*::*kanR*	This study
JLS1913	W3110 *ΔgalK*:: *yfp-bla*, *ΔemrA*::*kanR*	This study
JLS1817	W3110 *ΔgalK*:: *cfp-bla*, *Δslp-gadX*	This study
JLS1818	W3110 *ΔgalK*:: *yfp-bla*, *Δslp-gadX*	This study
JLS1919	W3110 *ΔgalK*:: *cfp-bla*, *ΔgadE*::*kanR*	This study
JLS1920	W3110 *ΔgalK*:: *yfp-bla*, *ΔgadE*::*kanR*	This study

FIGURES

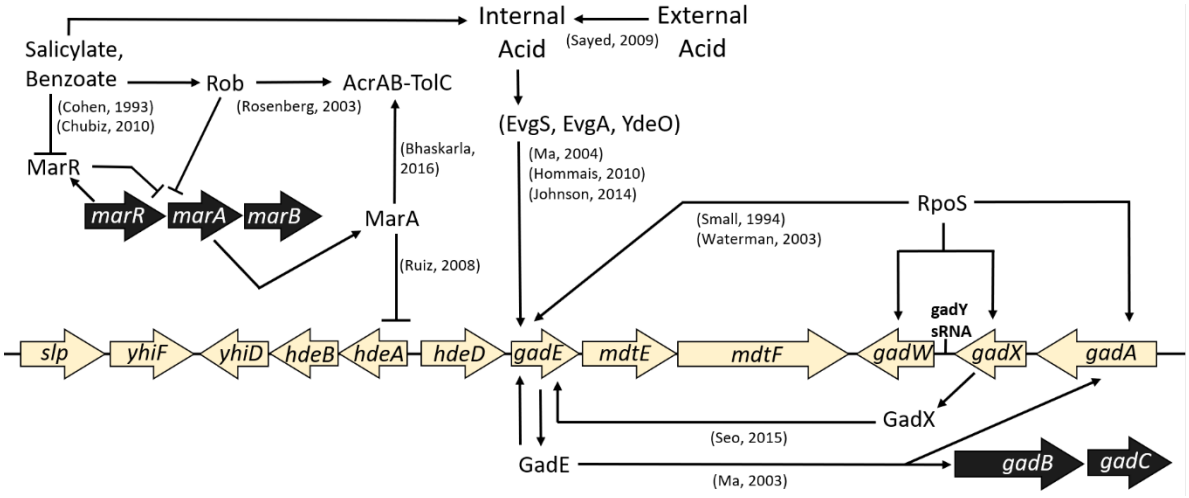


Figure 1. The salicylate-inducible Mar drug resistance regulon intersects with the Gad acid resistance regulon. Selected components relevant to this work are shown. Modified from Ref. (26). Cited references include (17,31,41,49,66–73).

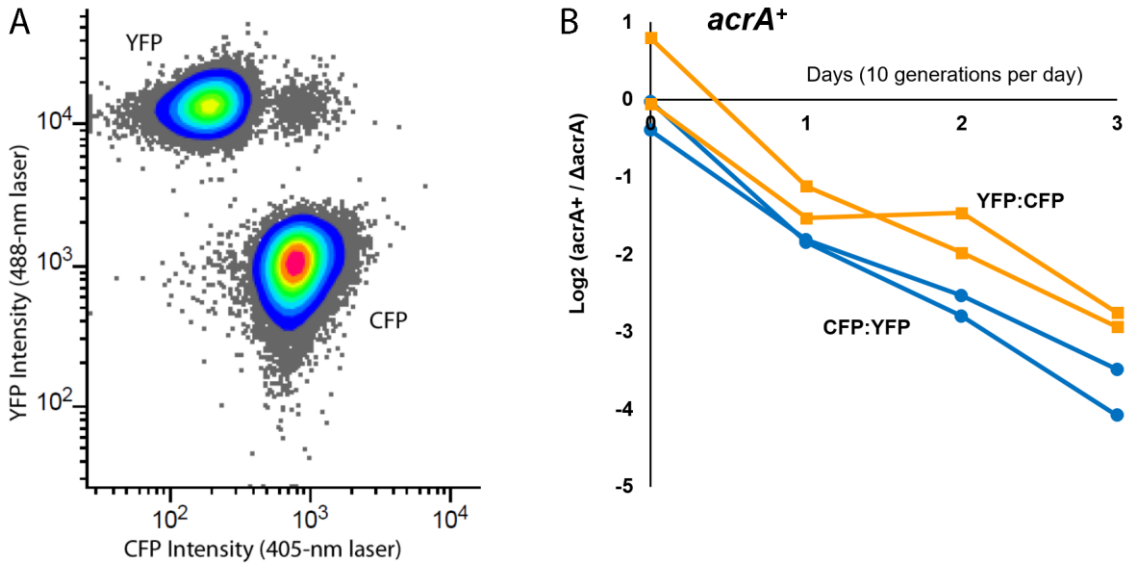
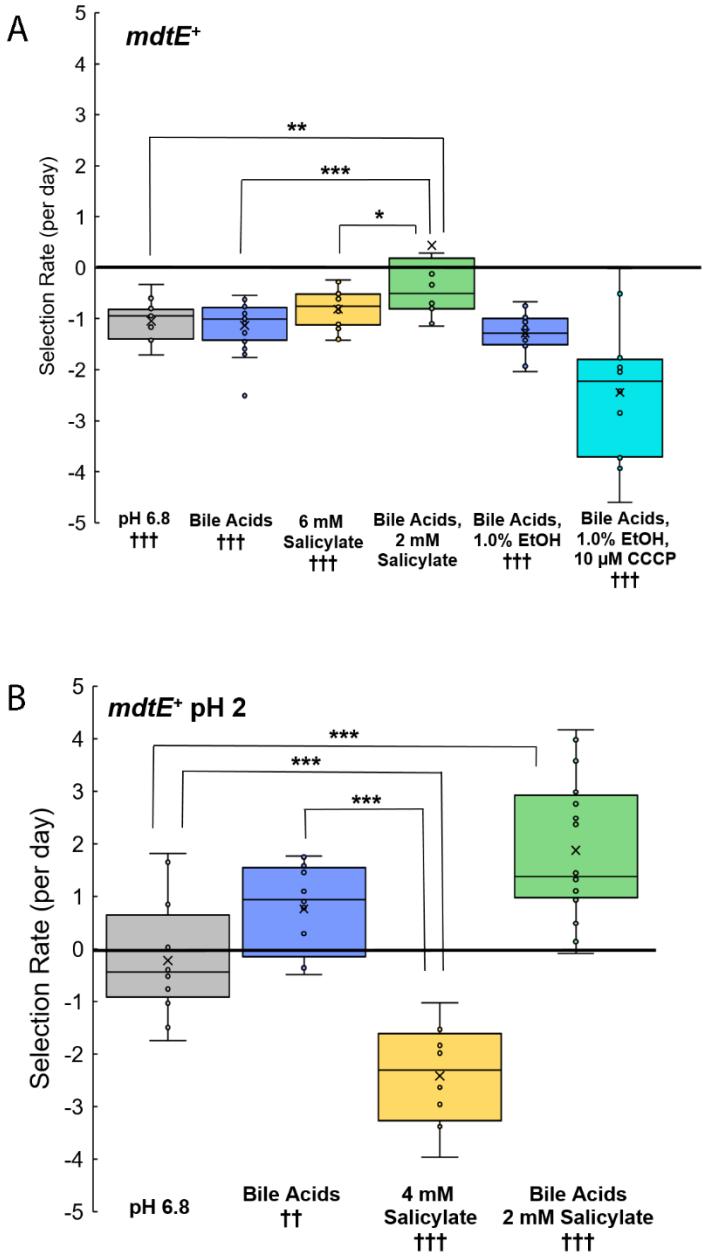


Figure 2. Flow cytometry competition assays of strains expressing YFP or CFP.

A. Emission intensity indicates cells expressing cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) for two strains in coculture. The combined culture is diluted and incubated for 2-h with IPTG to induce fluorophore expression.

B. The slope of the $\log_2$ ratios for LBK-PIPES pH 6.8 control media (n=12) is compiled for Day 0 through Day 3 for all weeks tested. The absolute values of these ratios are taken from cell counts for *ΔyhdN*::*kanR* (*acrA*$^+$) / *ΔacrA*::*kanR*. Each competition assay included an equal number of assays in which the *ΔyhdN*::*kanR* strain expressed YFP versus CFP. Cultures were diluted 1:1000 daily and assayed by flow cytometry as described under Methods. Slopes were calculated over days 0-3 of testing. The flow-cytometry threshold for percentage of each cell type was 0.01%.



553

554

555 **Figure 3. Selection for *mdtE*$^+$ with or without pH 2 exposure.** Selection rate is given by

556 $\log_2$(*ΔyhdN*::*kanR* /*ΔmdtE*::*kanR*)/day. The slopes were calculated over days 0-3 of testing.

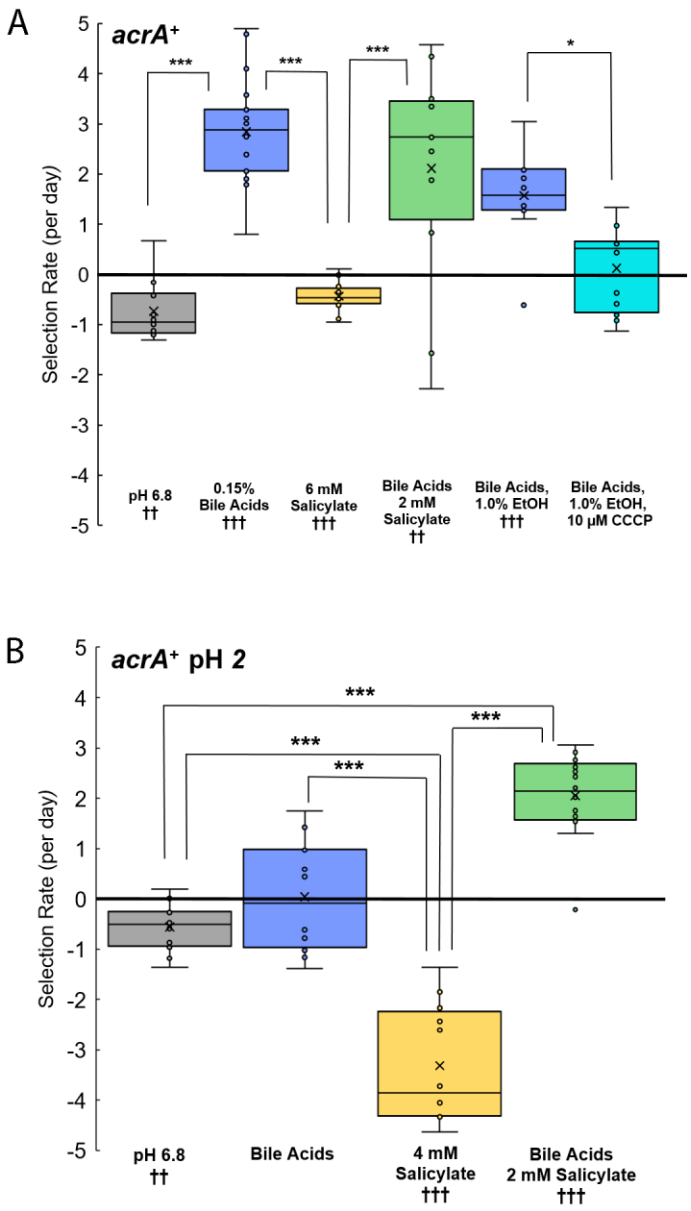
557 **A**. Conditions include LBK-PIPES pH 6.8 (n=16) with 0.15% bile acids (n=24), 6 mM salicylate

558 (n=16), 0.15% bile acids and 2 mM salicylate (n=12), 0.15% bile acids and 1.0% ethanol (n=12),

559 or 0.15% bile acids, 1.0% ethanol and 10 µM CCCP (n=12).

560 **B**. Conditions include LBK-PIPES pH 6.8 (n=16), with 0.15% bile acids (n=16), 4 mM salicylate
561 (n=8), or 0.15% bile acids and 2 mM salicylate (n=16). The daily growth cycle included 100-fold
562 dilution in unbuffered LBK pH 2, with incubation for 2 h. Then the cultures were diluted 10-fold
563 in the appropriate competition media adjusted to pH 7.0, yielding a final pH of 6.8.

564 For each condition, ANOVA and post-hoc Tukey tests were used to compare conditions to one
565 another using brackets (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Single sample t-tests were performed
566 to compare each selection rate to a value of zero (†$p<0.05$, ††$p<0.01$, †††$p<0.001$). Statistical
567 results are presented in Supplemental File 1.



568

569 **Figure 4. Selection for *acrA*$^+$ in the presence of salicylate, bile acids, and CCCP.** Selection

570 rate is given by $\log_2$(*ΔyhdN*::*kanR* / *ΔacrA*::*kanR*)/day. The slopes were calculated over days 0-3

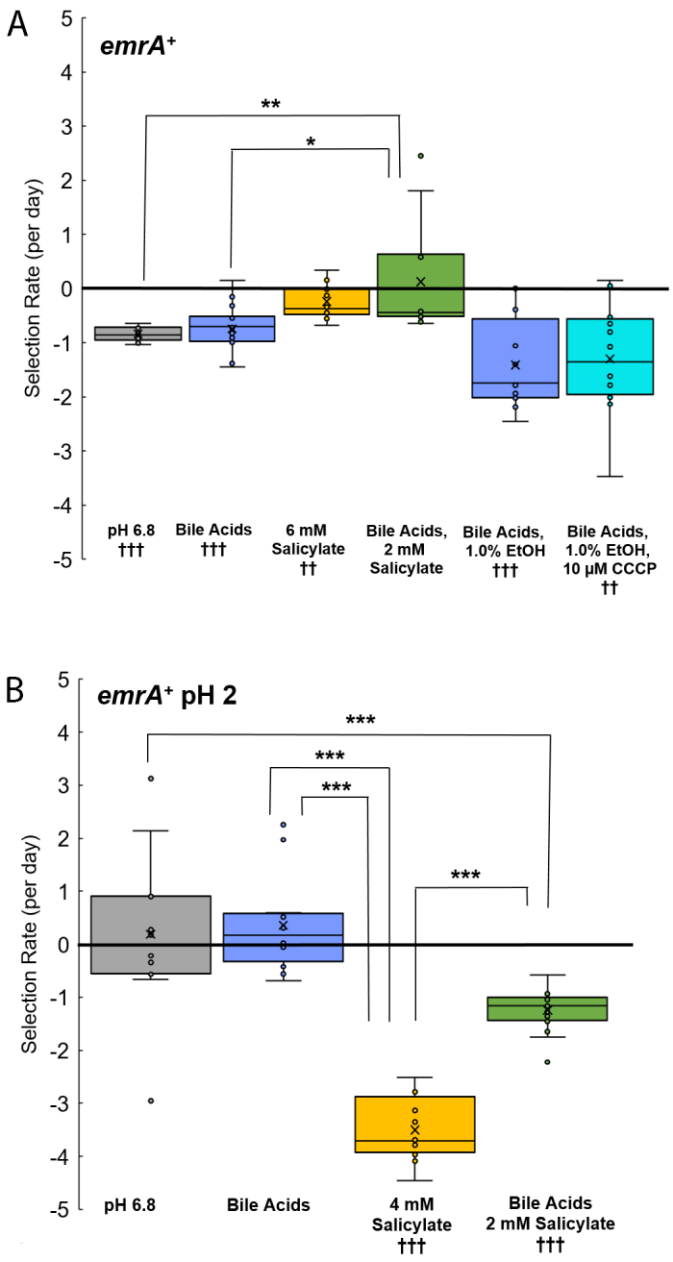
571 of testing. Significance was determined as for Figure 3.

572 **A.** Conditions include LBK-PIPES pH 6.8 (n=12), with 0.15% bile acids (n=20), 6 mM

573 salicylate (n=12), 0.15% bile acids and 2 mM salicylate (n=12), 0.15% bile acids and 1.0%

574 ethanol (n=12), or 0.15% bile acids, 1.0% ethanol (n=12), 10 µM CCCP (n=12).

575 **B.** Conditions include LBK-PIPES pH 6.8 (n=12), with 0.15% bile acids (n=12), 4 mM salicylate
576 (n=12), or 0.15% bile acids and 2 mM salicylate (n=16). The daily growth cycle included 100-
577 fold dilution in unbuffered LBK pH 2, with incubation for 2 h. Then the cultures were diluted
578 10-fold in the appropriate competition media adjusted to pH 7.0, yielding a final pH of 6.8.
579



580

581 **Figure 5. Selection for *emrA*$^+$.** Selection rate is given by $\log_2$(*ΔyhdN*::*kanR* /*ΔemrA*::*kanR*)/day.

582 The slopes were calculated over days 0-3 of testing. Significance was determined as for Figure 2.

583 **A.** Conditions include LBK-PIPES pH 6.8 (n=16), with 0.15% bile acids (n=16), 6 mM

584 salicylate (n=16), 0.15% bile acids and 2 mM salicylate (n=12), 0.15% bile acids and 1.0%

585 ethanol (n=12), or 0.15% bile acids, 1.0% ethanol, and 10 µM CCCP (n=12).

B. Conditions include LBK-PIPES pH 6.8 (n=12), with 0.15% bile acids (n=12), 4 mM salicylate (n=16), or 0.15% bile acids and 2 mM salicylate (n=16). The daily growth cycle included 100-fold dilution in unbuffered LBK pH 2, with incubation for 2 h before a 10-fold dilution in the appropriate competition media adjusted to pH 7.0, yielding a final pH of 6.8.

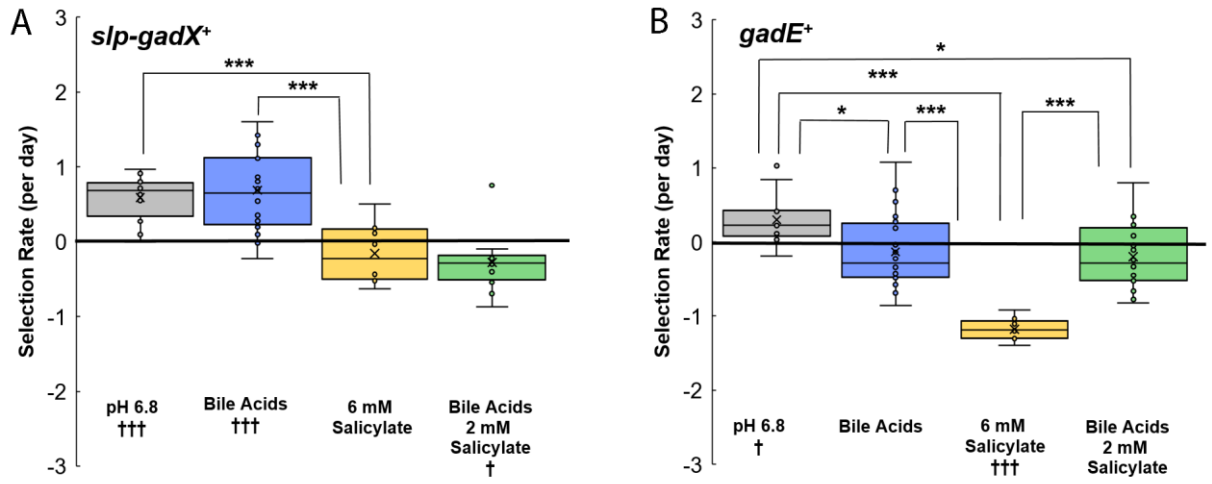


Figure 6. Selection for Gad island and for *gadE*$^+$. The slopes were calculated over days 0-3 of testing. Significance was determined as for Figure 2.

A. *slp-gadX*$^+$: Selection rate is given by $\log_2$(W3110/*Δslp-gadX*)/day. Conditions include LBK-PIPES pH 6.8 (n=12), with 0.15% bile acids (n=20), 6 mM salicylate (n=12), or 0.15% bile acids and 6 mM salicylate (n=12).

B. *gadE*$^+$: Selection rate is given by $\log_2$(*ΔyhdN::kanR /ΔacrA::kanR)*/day. Conditions include LBK-PIPES pH 6.8 (n=12), with 0.15% bile acids (n=20), 6 mM salicylate (n=12), or 0.15% bile acids and 6 mM salicylate (n=16).