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GENETICS AND MOLECULAR BIOLOGY

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

Samantha H. Schaffner,[a] Abigail V. Lee,[a] Minh T. N. Pham,[a] Beimnet B. Kassaye,[a] Haofan Li,[a] Sheetal Tallada,[a] Cassandra Lis,[a] Mark Lang,[a] Yangyang Liu,[a] Nafeez Ahmed,[a] Logan G. Galbraith,[a] Jeremy P. Moore,[a] Katarina M. Bischof,[a] Chelsea C. Menke,[a] Joan L. Slonczewski[a]

[a]Department of Biology, Kenyon College, Gambier, Ohio, USA

Samantha H. Schaffner and Abigail V. Lee contributed equally to this work. Samantha H. Schaffner devised the method of competition assays for MDR knockout strains. Abigail V. Lee modified the competition assays to include low pH exposure. Both first authors coordinated the team of undergraduate researchers and drafted the manuscript.

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 trade-offs 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 the 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, conferred 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. However, 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.

KEYWORDS AcrAB-TolC, Gad, MdtEF-TolC, bile acid, drug resistance evolution, extreme acid, flow cytometry, multidrug efflux pump, relative fitness, salicylate

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 a first-line defense against

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Address correspondence to Joan L. Slonczewski, slonczewski@kenyon.edu.

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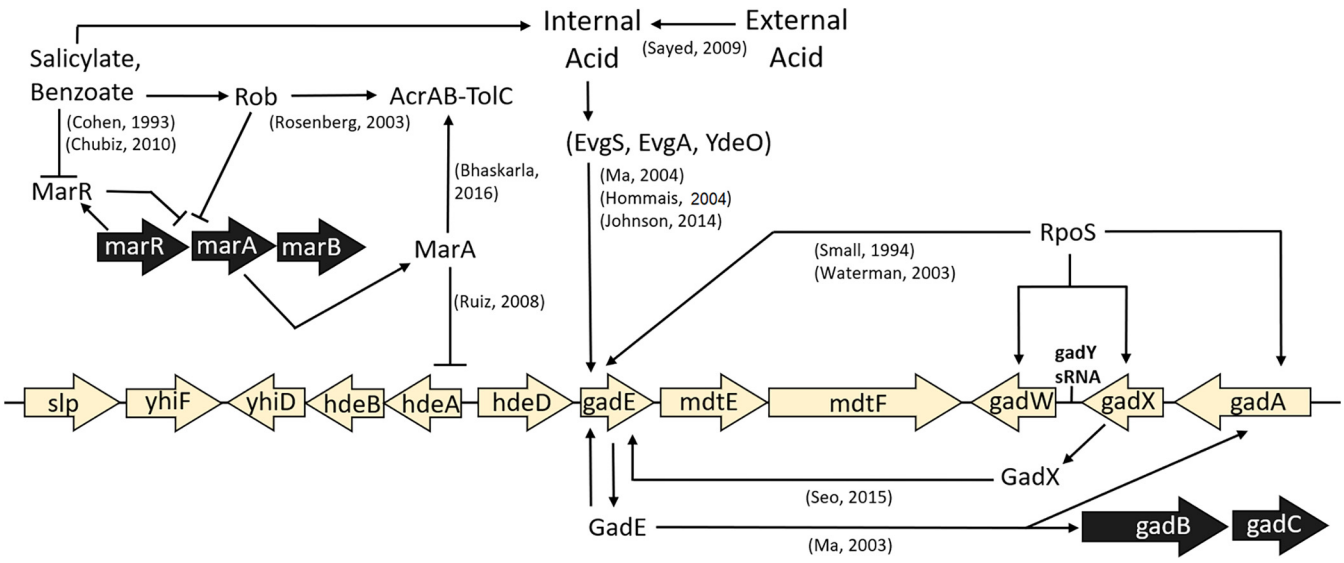

FIG 1 The salicylate-inducible Mar drug resistance regulon intersects with the Gad acid resistance regulon. Selected components relevant to this work are shown. Cited references include 17, 31, 41, 49, 52, and 64–71. (Modified from reference 26.)

multiple antibiotics at low levels (7). Thus, MDR pumps in pathogens pose a major threat to human health (8–11). However, 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 that is 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 trade-offs 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, which induce drug resistance regulons. In 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 the cell is 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 the 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 to 3, as found in the human stomach (36, 37). Moderate acid (pH 5.0 to 6.8) upregulates systems for extreme-acid survival such as those of the Gad acid fitness island (38, 39). The acid fitness island includes *mdtEF*, encoding the MdtEF-TolC pump (19, 20), and genes for Gad transcriptional regulators such as GadE (40). Gad-dependent acid resistance allows stationary-phase survival during exposure to 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, 20 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 trade-offs 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 et al. (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 the control strain, *E. coli* W3110. Therefore, in all assays involving knockout strains that expressed *kanR*, we used a control strain with the *yhdN*::*kanR* allele. For the single gene deletions used in this experiment (Δ*acrA*::*kanR*, Δ*mdtE*::*kanR*, Δ*gadE*::*kanR*, and Δ*emrA*::*kanR*), each allele was transduced into a strain of *E. coli* K-12 W3110 containing the fluorophore allele *galK*::*yfp* or *galK*::*cfp*, both of which are inducible via a *lac* promoter (42).

The mixture of two strains was serially diluted 1,000-fold each day and observed over a total of 30 generations (doublings) from day 0 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 (isopropyl-β-D-thiogalactopyranoside) inducer was performed using LBK-PIPES pH 6.8 (see Materials and Methods) buffered medium without stressors. The IPTG-induced populations express YFP or CFP for flow cytometry, whereas the overnight 1,000-fold dilutions avoid energy-expensive fluorophore expression. This procedure enabled us to minimize the fitness effects of fluorophore expression during stress selection.

Figure 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-

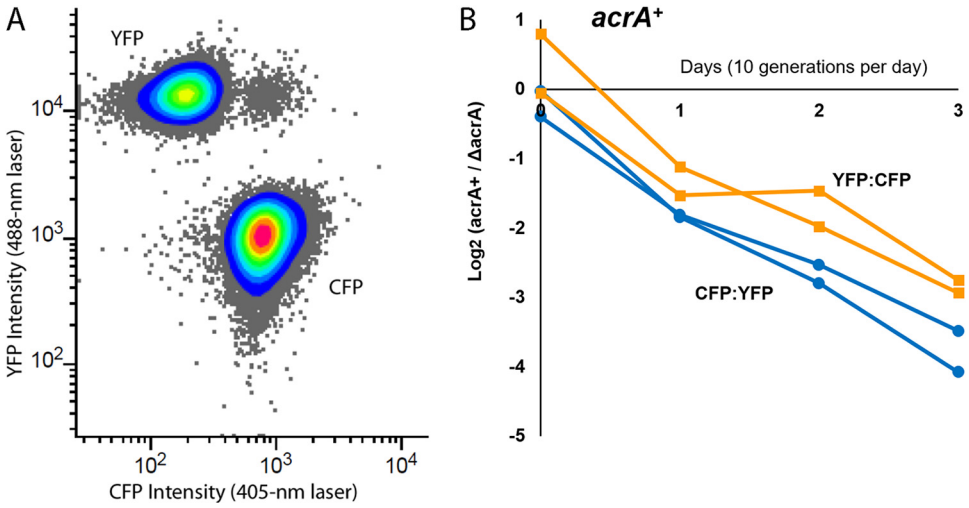

FIG 2 Flow cytometry competition assays of strains expressing YFP or CFP. (A) Emission intensity indicates cells expressing cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) for two strains in coculture. The combined culture was diluted and incubated for 2 h with IPTG to induce fluorophore expression. (B) The slope of the $\log_2$ ratios for LBK-PIPES pH 6.8 control medium ($n = 12$) was compiled for day 0 through day 3 for all weeks tested. The absolute values of these ratios were taken from cell counts for Δ*yhdN*::*kanR* (*acrA*$^+$)/Δ*acrA*::*kanR* strains. Each competition assay included an equal number of assays in which the Δ*yhdN*::*kanR* strain expressed YFP versus CFP. Cultures were diluted 1:1,000 daily and assayed by flow cytometry as described in Materials and Methods. Slopes were calculated over days 0 to 3 of testing. The flow cytometry threshold for percentage of each cell type was 0.01%.

nm excitation and high intensity at 405 nm. Figure 2B show results of 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, equal numbers of trials were performed with the gene knockout 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 2-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 and 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 Δ*yhdN*::*kanR*) against W3110 Δ*mdtE*::*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 conditions—in the pH 6.8 control medium, as well as in 0.15% bile acids or in 6 mM salicylate ($P < 0.001$). Selection against the *mdtE*$^+$ strain was further increased by the addition of 10 μM CCCP ($P < 0.001$), which depletes the PMF needed to drive efflux (Fig. 3A). The expenditure of limited PMF by the MdtEF-TolC could be one possible explanation for the pump's fitness cost. All conditions tested led to a fitness cost or neutral selection for MdtEF-TolC.

Under what growth conditions does MdtEF-TolC confer advantage? We tried including a period of pH 2 exposure, a condition for which the Gad regulon maintains extreme-acid survival dependent on stationary phase (35). This condition of stationary phase in extreme acid mimics the passage of bacteria through an acidic stomach, an adaptation that helps enteric *E. coli* gain access to the intestinal tract and may activate drug efflux pumps (19). Our serial cultures enter stationary phase for at least 12 h, so they are fully acid resistant before each

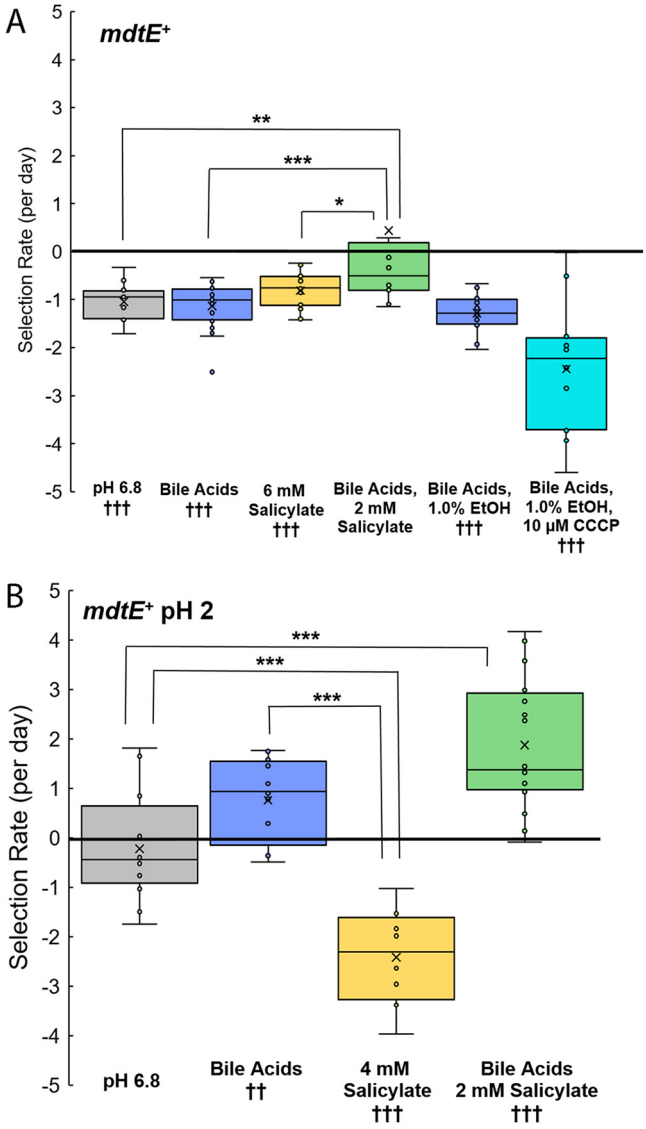

FIG 3 Selection for *mdtE*$^+$ with or without pH 2 exposure. The selection rate is given by $\log_2$(Δ*yhdN*::*kanR*/Δ*mdtE*::*kanR*)/day. The slopes were calculated over days 0 to 3 of testing. (A) Conditions include LBK-PIPES pH 6.8 alone ($n = 16$), with 0.15% bile acids ($n = 24$), with 6 mM salicylate ($n = 16$), with 0.15% bile acids and 2 mM salicylate ($n = 12$), with 0.15% bile acids and 1.0% ethanol ($n = 12$), and with 0.15% bile acids, 1.0% ethanol, and 10 μM CCCP ($n = 12$). (B) Conditions include LBK-PIPES pH 6.8 alone ($n = 16$), with 0.15% bile acids ($n = 16$), with 4 mM salicylate ($n = 8$), and with 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. Then the cultures were diluted 10-fold in the appropriate competition media adjusted to pH 7.0, yielding a final pH of 6.8. For each condition, ANOVA and *post hoc* Tukey tests were used to compare conditions to one another, shown with brackets (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Single-sample *t* tests were performed to compare each selection rate to a value of zero (††, $P < 0.01$; †††, $P < 0.001$). Statistical results are presented in File S1.

dilution. Starting on day 0, the CFP/YFP strain mixture was diluted 100-fold in unbuffered LBK at pH 2.0. The acidified cell suspension, approximately pH 2, was incubated for 2 h. Under this condition, survival of *E. coli* K-12 strains is typically 50 to 100% (35, 48, 49). The acidified suspension was then diluted 10-fold in medium buffered with PIPES at pH 7.0, leading to a final pH of 6.8. The total dilution overall was 1,000-fold per day, comparable to our original relative fitness assay. This period of pH 2 exposure was repeated for each of days 1 to 3 of the assay.

The 2-h exposure to extreme acid eliminated the fitness cost of *mdtE* during coculture at pH 6.8. The *mdtE* allele conferred a fitness benefit in the presence of bile acids, with or

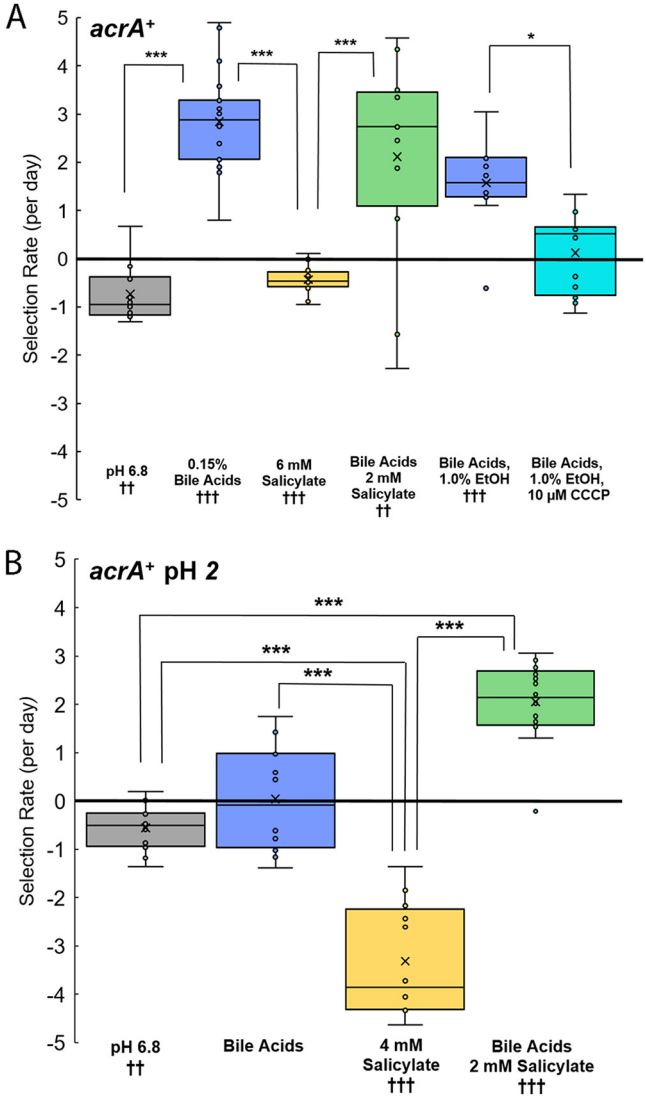

FIG 4 Selection for *acrA*$^+$ in the presence of salicylate, bile acids, and CCCP. The selection rate is given by $\log_2$(Δ*yhdN*::*kanR*/Δ*acrA*::*kanR*)/day. The slopes were calculated over days 0 to 3 of testing. Significance was determined as for Fig. 3. (A) Conditions include LBK-PIPES pH 6.8 alone ($n = 12$), with 0.15% bile acids ($n = 20$), with 6 mM salicylate ($n = 12$), with 0.15% bile acids and 2 mM salicylate ($n = 12$), with 0.15% bile acids and 1.0% ethanol ($n = 12$), and with 0.15% bile acids, 1.0% ethanol ($n = 12$), and 10 μM CCCP ($n = 12$). (B) Conditions include LBK-PIPES pH 6.8 alone ($n = 12$), with 0.15% bile acids ($n = 12$), with 4 mM salicylate ($n = 12$), and with 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. Then, the cultures were diluted 10-fold in the appropriate competition medium adjusted to pH 7.0, yielding a final pH of 6.8.

without 2 mM salicylate (Fig. 3B). A higher concentration of 4 mM salicylate, however, incurred a large fitness cost for MdtE ($P < 0.001$). The highest salicylate concentration tested (6 mM) did not permit growth over 3 days. Note that after 100-fold dilution in LBK pH 2, there would still be a 0.06 mM concentration of salicylate. At external pH 2, where the *E. coli* internal pH is about 5 (49), the uptake of salicylic acid (with a pK_a of 2.8) would be greatly increased by the transmembrane pH difference, a ΔpH of approximately 3 units. Thus, the low pH could amplify the fitness effect of a small salicylate concentration.

AcrA incurs fitness trade-offs. AcrAB-TolC is the best known of the *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 a 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). With 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). Thus, the bacteria's ability to make AcrAB-TolC leads to very different trade-offs 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 the AcrAB-TolC efflux pump, with EmrA providing a link between the outer membrane efflux pump component, TolC, and the inner membrane component, EmrB (50). The EmrAB-TolC efflux pump is activated by salicylate and confers resistance to some antibiotics as well as CCCP (23). As seen for *mdtE*, *emrA* incurred a fitness cost in the pH 6.8 medium 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 trade-offs 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 the periplasmic acid chaperone genes *hdeA* and *hdeB* and the acid resistance regulator genes *gadE* and *gadX* (38). Nonetheless, this region of the genome often undergoes deletion during experimental evolution with acid stress (26, 51).

We investigated how the *mdtE* fitness effects compare 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 (Δ*slp-gadX*) (26). In this assay, the parent strain W3110 had a small fitness advantage over the Δ*slp-gadX* strain (Fig. 6A). This result differed from the *mdtE*$^+$ competition (Fig. 3A) in which the *mdtE* deletion increased 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 knockout strains (Fig. 3 to 5).

An important regulatory component of the glutamate-dependent acid resistance pathway is *gadE* (Fig. 1) (52, 53). 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 the Δ*gadE*::*kanR* strain, 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 trade-off 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

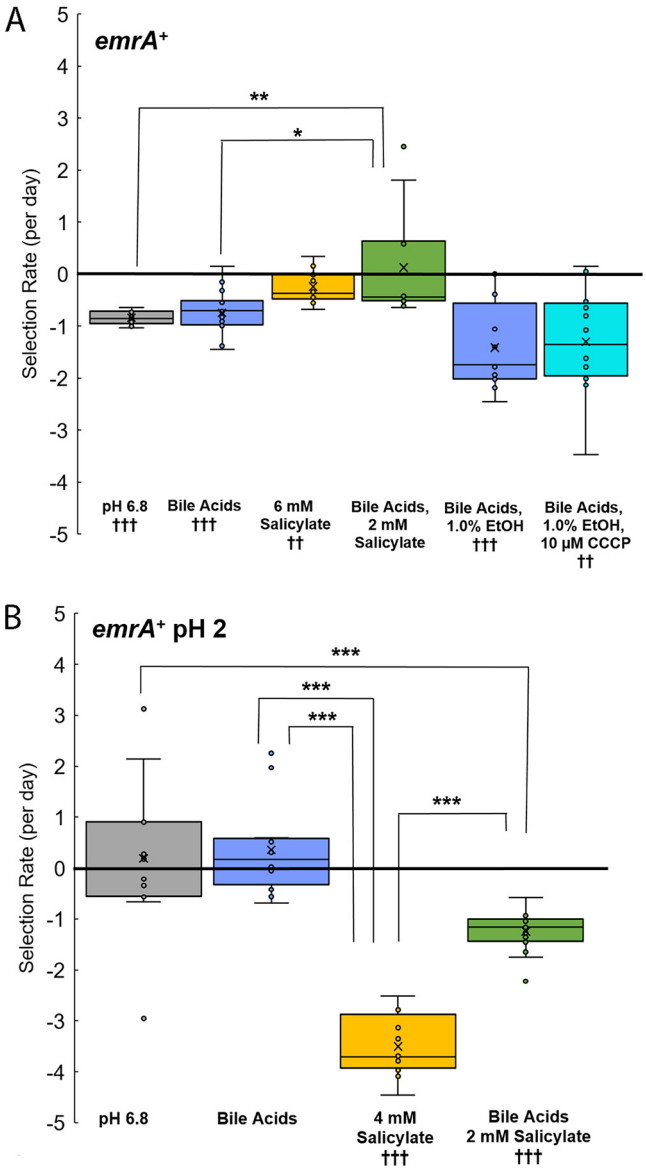

FIG 5 Selection for *emrA*$^+$. Selection rate is given by $\log_2$(Δ*yhdN*::*kanR*/Δ*emrA*::*kanR*)/day. The slopes were calculated over days 0 to 3 of testing. Significance was determined as for Fig. 3. (A) Conditions include LBK-PIPES pH 6.8 alone ($n = 16$), with 0.15% bile acids ($n = 16$), with 6 mM salicylate ($n = 16$), 0.15% bile acids and 2 mM salicylate ($n = 12$), with 0.15% bile acids and 1.0% ethanol ($n = 12$), and with 0.15% bile acids, 1.0% ethanol, and 10 μM CCCP ($n = 12$). (B) Conditions include LBK-PIPES pH 6.8 alone ($n = 12$), with 0.15% bile acids ($n = 12$), with 4 mM salicylate ($n = 16$), and with 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 medium adjusted to pH 7.0, yielding a final pH of 6.8.

with pH 2 exposure (Fig. 3) suggests that MdtEF-TolC exports bile acids during exposure to extreme acid, where the cell cannot grow but its death rate can be slowed by various acid resistance components of the Gad island. This would be consistent with the previously unexplained requirement of pump component TolC for Gad-related survival at pH 2 (54).

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

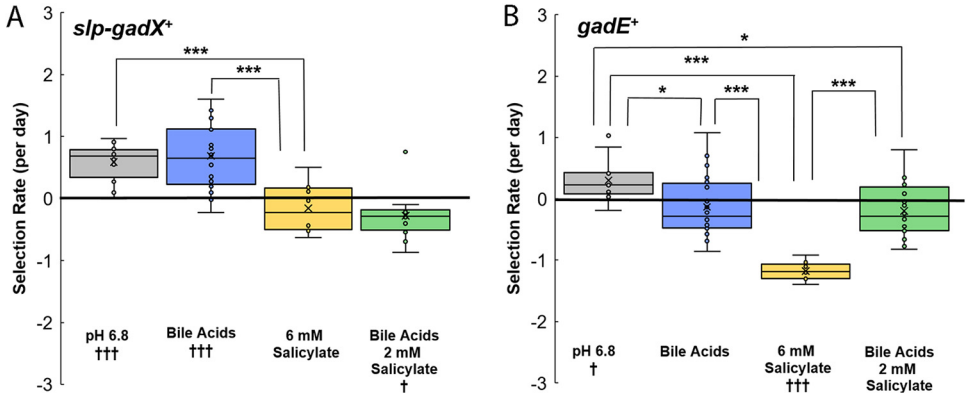

FIG 6 Selection for Gad island and for *gadE*$^+$. The slopes were calculated over days 0 to 3 of testing. Significance was determined as for Fig. 3 (†, $P < 0.05$). (A) *slp-gadX*$^+$. Selection rate is given by $\log_2$(W3110/Δ*slp-gadX*)/day. Conditions include LBK-PIPES pH 6.8 alone ($n = 12$), with 0.15% bile acids ($n = 20$), with 6 mM salicylate ($n = 12$), and with 0.15% bile acids and 6 mM salicylate ($n = 12$). (B) *gadE*$^+$. Selection rate is given by $\log_2$(Δ*yhdN*::*kanR*/Δ*gadE*::*kanR*)/day. Conditions include LBK-PIPES pH 6.8 alone ($n = 12$), with 0.15% bile acids ($n = 20$), with 6 mM salicylate ($n = 12$), and with 0.15% bile acids and 6 mM salicylate ($n = 16$).

reversed by the presence of the uncoupler CCCP (Fig. 3A, 4A, and 5A). We show that the efflux pump fitness contribution may also be reversed by a membrane-permeant aromatic acid such as salicylate during extreme-acid exposure (Fig. 3B, 4B, and 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, 55, 56).

Extreme-acid exposure had a 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 (57–59). While bile does not normally reach the stomach, many patients exhibit chronic bile reflux gastritis (60), 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 conferred an advantage only when the growth cycle included exposure at pH 2. In 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 (61). 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 as other MDR pumps.

Our interest in assessing fitness during 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 that enter the stomach. Other pharmaceuticals likely interact with the microbiome in unexpected ways 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

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

Strain	Genotype/description	Source
W3110	*E. coli* K-12 F$^-$ λ^-	Lab stock (72)
CH367	Δ*lac*::FRT Δ*galK*::*cfp-bla* (AmpR)	42
CH372	Δ*lac*::FRT Δ*galK*::*yfp-bla* (AmpR)	42
JLS1779	W3110 Δ*galK*::*cfp-bla*	This study
JLS1780	W3110 Δ*galK*::*yfp-bla*	This study
JLS1910	W3110 Δ*galK*::*cfp-bla* Δ*yhdN*::*kanR*	This study
JLS1911	W3110 Δ*galK*::*yfp-bla* Δ*yhdN*::*kanR*	This study
JLS1826	W3110 Δ*galK*::*cfp-bla* Δ*acrA*::*kanR*	This study
JLS1832	W3110 Δ*galK*::*yfp-bla* Δ*acrA*::*kanR*	This study
JLS1834	W3110 Δ*galK*::*cfp-bla* Δ*mdtE*::*kanR*	This study
JLS1835	W3110 Δ*galK*::*yfp-bla* Δ*mdtE*::*kanR*	This study
JLS1912	W3110 Δ*galK*::*cfp-bla* Δ*emrA*::*kanR*	This study
JLS1913	W3110 Δ*galK*::*yfp-bla* Δ*emrA*::*kanR*	This study
JLS1817	W3110 Δ*galK*::*cfp-bla* Δ*slp-gadX*	This study
JLS1818	W3110 Δ*galK*::*yfp-bla* Δ*slp-gadX*	This study
JLS1919	W3110 Δ*galK*::*cfp-bla* Δ*gadE*::*kanR*	This study
JLS1920	W3110 Δ*galK*::*yfp-bla* Δ*gadE*::*kanR*	This study

sequence joint (54). The main growth medium was LBK broth (10 g/liter tryptone, 5 g/liter yeast extract, 7.45 g/liter 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.

Fluorescence-activated cell sorting (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 led to a 1,000-fold dilution followed by approximately 10 doublings (generations), for a total of 30 doublings by day 3. During each daily cycle, the cells spent approximately 12 to 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 2). At pH 2, the bacteria cannot grow but they remain viable via stationary-phase-induced Gad acid resistance (38, 40). Each day, 2 μl of the mixture of competing CFP and YFP strains was added to 200 μl of LBK pH 2 and incubated at 37°C for 2 h. After this incubation period, 1.8 ml of the competition medium buffered to pH 7 was added (restoring the pH to approximately 6.8), and the tubes were incubated for 24 h at 37°C.

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

For each gene tested, the selection rate (*s*) was calculated as $\log_2(R_t/R_0)/t$, where *R* represents the ratio of cell numbers for the control strain (W3110 Δ*yhdN* or W3110) to the knockout strain of interest and *t* represents time in days (with a daily dilution of 1:1,000, approximately 10 generations per day) (45, 62, 63). 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 2-fold over the competing strain.

Statistical analysis. Analysis of variance (ANOVA) and *post hoc* Tukey tests were performed to compare trials under different conditions. Single sample t-tests were performed to compare the value of each selection rate to zero. Statistical results are presented in File S1 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.04 MB.

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