



Experimental Evolution of *Escherichia coli* K-12 in the Presence of Proton Motive Force (PMF) Uncoupler Carbonyl Cyanide *m*-Chlorophenylhydrazone Selects for Mutations Affecting PMF-Driven Drug Efflux Pumps

Jessie M. Griffith,^a Preston J. Basting,^a Katarina M. Bischof,^a Erintrude P. Wrona,^a Karina S. Kunka,^a Anna C. Tancredi,^a Jeremy P. Moore,^a Miriam R. L. Hyman,^a Joan L. Slonczewski^a

^aDepartment of Biology, Kenyon College, Gambier, Ohio, USA

ABSTRACT Experimental evolution of *Escherichia coli* K-12 with benzoate, a partial uncoupler of the proton motive force (PMF), selects for mutations that decrease antibiotic resistance. We conducted experimental evolution in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a strong uncoupler. Cultures were serially diluted daily 1:100 in LBK medium containing 20 to 150 μM CCCP buffered at pH 6.5 or at pH 8.0. After 1,000 generations, the populations tolerated up to 150 μM CCCP. Sequenced isolates had mutations in *mprA* (*emrR*), which downregulates the EmrAB-TolC pump that exports CCCP. A *mprA*:*kanR* deletion conferred growth at 60 μM CCCP, though not at the higher levels resisted by evolved strains (150 μM). Some *mprA* mutant strains also had point mutations affecting *emrA*, but deletion of *emrA* abolished the CCCP resistance. Thus, CCCP-evolved isolates contained additional adaptations. One isolate lacked *emrA* or *mprA* mutations but had mutations in *cecR* (*ybiH*), whose product upregulates drug pumps YbhG and YbhFSR, and in *gadE*, which upregulates the multidrug pump MdtEF. A *cecR*:*kanR* deletion conferred partial resistance to CCCP. Other multidrug efflux genes that had mutations included *ybhR* and *acrAB*. The *acrB* isolate was sensitive to the AcrAB substrates chloramphenicol and tetracycline. Other mutant genes in CCCP-evolved strains include *rng* (RNase G) and *cyaA* (adenylate cyclase). Overall, experimental evolution revealed a CCCP-dependent fitness advantage for mutations increasing CCCP efflux via EmrA and for mutations that may deactivate proton-driven pumps for drugs not present (*cecR*, *gadE*, *acrAB*, and *ybhR*). These results are consistent with our previous report of drug sensitivity associated with evolved benzoate tolerance.

IMPORTANCE The genetic responses of bacteria to depletion of proton motive force (PMF), and their effects on drug resistance, are poorly understood. PMF drives export of many antibiotics, but the energy cost may decrease fitness when antibiotics are absent. Our evolution experiment reveals genetic mechanisms of adaptation to the PMF uncoupler CCCP, including selection for increased CCCP efflux but also against the expression of PMF-driven pumps for drugs not present. The results have implications for our understanding of the gut microbiome, which experiences high levels of organic acids that decrease PMF.

KEYWORDS CCCP, *acrAB*, antibiotic resistance, *cecR*, *emrA*, evolution, *gadE*, *mprA* (*emrR*), proton motive force, *ybhR*

The proton motive force (PMF) is diminished or abolished by uncouplers of oxidative phosphorylation such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (1). Uncouplers such as CCCP are hydrophobic molecules with an acidic proton that reside in the cell's inner membrane. The molecule shuttles protons across the membrane via

Citation Griffith JM, Basting PJ, Bischof KM, Wrona EP, Kunka KS, Tancredi AC, Moore JP, Hyman MRL, Slonczewski JL. 2019.

Experimental evolution of *Escherichia coli* K-12 in the presence of proton motive force (PMF) uncoupler carbonyl cyanide *m*-chlorophenylhydrazone selects for mutations affecting PMF-driven drug efflux pumps. *Appl Environ Microbiol* 85:e02792-18. <https://doi.org/10.1128/AEM.02792-18>.

Editor Rebecca E. Parales, University of California, Davis

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

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

J.M.G. and P.J.B. contributed equally to this article.

Received 21 November 2018

Accepted 6 December 2018

Accepted manuscript posted online 21 December 2018

Published 20 February 2019

TABLE 1 Generation number at increases in CCCP concentration during evolution of *E. coli*

Generation no.	New CCCP concn (μM) for:	
	pH 6.5 condition	pH 8.0 condition
0	20	60
20	20	50
66	30	60
299	40	70
345	50	80
523	60	90
536	70	100
556	80	110
616	120	130
782	150	150

protonation/deprotonation (1–3). The proton flux equilibrates both the transmembrane pH difference (ΔpH) and the transmembrane electrical potential ($\Delta\psi$) and thus depletes PMF (1, 2). Respiration runs a futile cycle, as protons continue to be pumped but PMF is not maintained (4–6). The uncoupler effect is most pronounced during growth at low external pH, where the electron transport system is upregulated and a higher PMF is maintained (7).

The PMF powers many low-level multidrug resistance (MDR) efflux pumps, which provide bacteria with a first-line defense against antibiotics and other growth-inhibiting molecules (8, 9). The effect of uncouplers on MDR efflux is poorly understood. Experimental evolution in benzoic acid, a membrane-permeant aromatic acid and partial uncoupler, incurs the loss of antibiotic resistance and decreased expression of multi-drug efflux pumps (10). For example, benzoate stress selects for mutations that delete or downregulate the Gad acid fitness island, including the *mdtEF* drug efflux system (10, 11). At a high concentration, benzoic acid partly uncouples PMF (12, 13) and thus could increase the fitness cost of efflux pumps driven by proton flux. Other evolution experiments on pH stress reveal surprising fitness tradeoffs, such as the loss of amino acid decarboxylases that are highly induced by acid (10, 14–16).

It was of interest, therefore, to test the fitness effect of long-term exposure to a strong uncoupler, CCCP, that more completely abolishes PMF. One system of interest for CCCP tolerance is EmrAB-TolC. EmrA, EmrB, and TolC form a multidrug efflux pump that exports CCCP and various ionophores and antibiotics (17–19). The *emrAB* operon is upregulated by MprA (EmrR) (20). MprA binds CCCP and becomes inactivated, allowing higher expression and activity of EmrA and EmrB. It was unknown whether long-term CCCP exposure would select for increased activity of this multidrug efflux pump or its regulators or for loss of this CCCP-responsive system, and perhaps other proton-driven MDR pumps, as was found in the benzoate evolution experiment (10).

Thus, we performed experimental evolution to test the long-term effects of exposure to a full uncoupler, CCCP. We included the factor of external pH in CCCP tolerance by conducting serial dilution of *Escherichia coli* at pH 6.5 and 8.0 with increasing concentrations of CCCP. After 1,000 bacterial generations, we sequenced the CCCP-evolved isolates and analyzed their mutations.

RESULTS

CCCP-evolved populations show increased relative fitness in the presence of CCCP. To investigate the selection effects of CCCP on *E. coli*, populations of strain W3110 were subcultured daily with CCCP in medium buffered at pH 6.5 or at pH 8.0 (Table 1). The initial CCCP concentrations, 20 μM for low pH and 60 μM for high pH, were determined by culturing W3110 in a range of CCCP concentrations at pH 6.5 and at pH 8.0 (Fig. 1A and B). The ancestral strain W3110 failed to grow consistently above 40 μM CCCP (pH 6.5) or above 60 μM CCCP (pH 8.0). Culture densities at 16 h showed that 20 μM CCCP at pH 6.5, and 50 μM CCCP at pH 8.0, resulted in a significant decrease of growth without full loss of viability. Although we had started the populations at pH

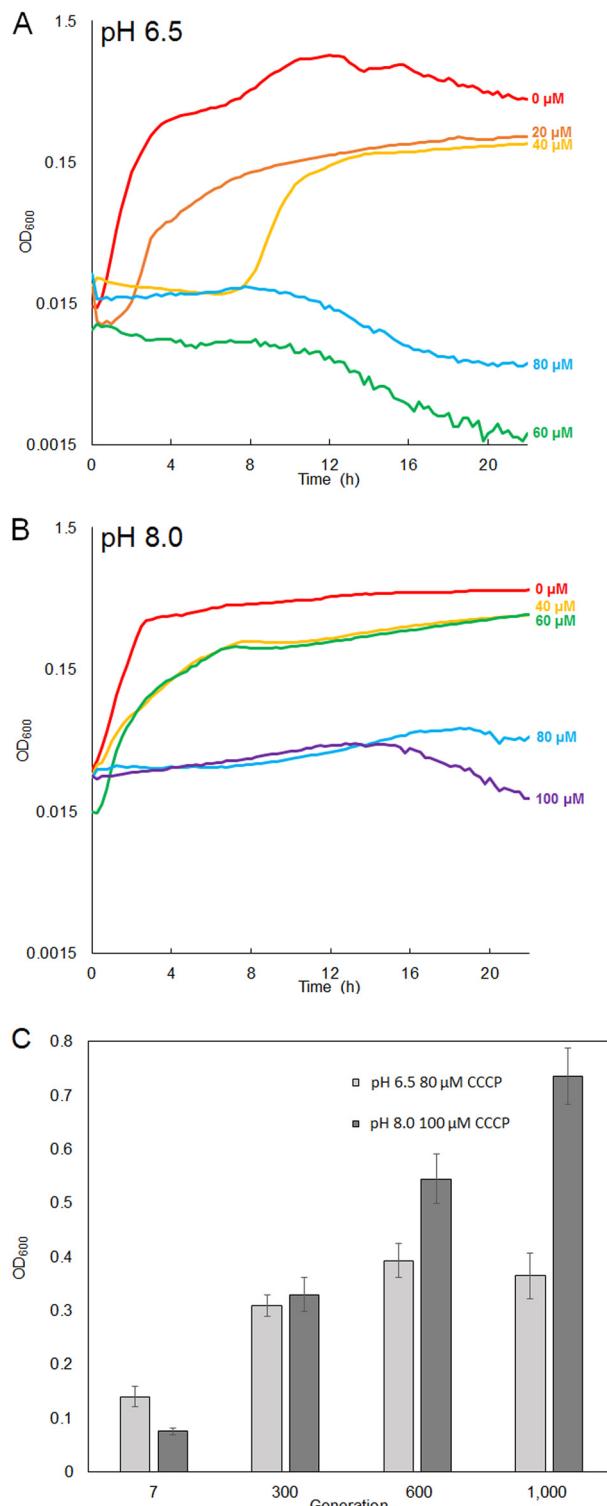


FIG 1 (A and B) Culture densities attained by populations of strain W3110 serially diluted and exposed to increasing CCCP concentrations. Microplate 200-μl populations were diluted in LBK medium containing 100 mM Na-PIPES, pH 6.5 (A), and 100 mM TAPS, pH 8.0 (B). (C) Mean endpoints at $t = 16$ h for evolving populations cultured with 80 μM CCCP at pH 6.5 (light bars) and with 100 μM CCCP at pH 8.0 (dark bars). At pH 6.5 and 80 μM CCCP, 8 samples were excluded from the generation 10 OD₆₀₀ data because no growth occurred. Tukey's test gave a P value of <0.05 for growth of generations 300, 600, and 1000 compared to growth of generation 7 at both pH 6.5 with 80 μM CCCP and pH 8.0 with 100 μM CCCP.

TABLE 2 Strains generated by experimental evolution or by P1 phage transduction

Strain	Population isolate	Description or genotype	Generation
W3110		<i>Escherichia coli</i> K-12	
JLSC0001	C-A1-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0005	C-E1-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0009	C-A3-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0010	C-B3-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0013	C-E3-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0016	C-H3-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0017	C-A5-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0023	C-G5-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0024	C-H5-1	W3110 evolved at pH 6.5 with CCCP	1000
JLSC0028	C-D7-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0031	C-G7-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0033	C-A9-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0038	C-F9-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0042	C-B11-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0044	C-D11-1	W3110 evolved at pH 8.0 with CCCP	1000
JLSC0058	C-B11-3	W3110 evolved at pH 8.0 with CCCP	2000
JLSC0059	C-B11-4	W3110 evolved at pH 8.0 with CCCP	2000
JLS1718		W3110 Δ mpmA::kanR	
JLS1740		W3110 Δ ybiH::kanR	
JLS1809		W3110 Δ adhE::kanR	
JLS1706		W3110 Δ emrA::kanR	
JLS1701		JLSC0001 (C-A1-1) Δ emrA::kanR	
JLS1702		JLSC0009 (C-A3-1) Δ emrA::kanR	
JLS1703		JLSC0023 (C-G5-1) Δ emrA::kanR	
JLS1704		JLSC0042 (C-B11-1) Δ emrA::kanR	
JLS1705		JLSC0044 (C-D11-1) Δ emrA::kanR	
JLS1733		JLSC0042 (C-B11-1) rng^+	

8.0 with 60 μ M CCCP, we decreased it to 50 μ M after 20 generations due to this poor growth. From this point forward, the CCCP concentration was increased in a stepwise fashion, reaching 150 μ M CCCP by generation 1000 (Table 1).

Figure 1C compares the 16-h endpoint culture densities attained by the evolving populations. Aliquots were obtained from frozen plates, starting from the first plate stored and followed by populations frozen at succeeding generations up to 1,000. The pH 8.0 populations showed a steeper increase in fitness than those exposed to CCCP at pH 6.5, where fitness leveled off after 600 generations.

After 1,000 generations, isolates were obtained from selected microplate populations by sequential restreaks. Isolated strains are named by the position on the plate and isolate number; for example, strain C-A1-1 was the first CCCP-evolved strain from the well in row A and column 1. Strain names are listed in Table 2. Figure 2 shows growth curves obtained for isolates from populations following evolution at pH 6.5 (Fig. 2A and B) or at pH 8.0 (Fig. 2C and D). For each isolate, eight replicate curves were obtained. Figure 2A and C show the curve exhibiting median density at 16 h for each strain and condition; Fig. 2B and D show all eight replicate curves. Isolates that had evolved at pH 6.5 (C-A1-1, C-A3-1, and C-G5-1) as well as isolates that had evolved at pH 8.0 (C-B11-1, C-D11-1, C-F9-1, and C-G7-1) showed an increase in tolerance to 150 μ M CCCP.

Genomes of CCCP-evolved strains show independent recurring mutations in common genes. The genomes of CCCP-evolved strains were resequenced and analyzed using the computational pipeline breseq (see Materials and Methods) to characterize mutations acquired over the course of the evolution (Table 3 shows selected mutations; see Table S1 in the supplemental material for all mutations). Isolate C-B3-1 behaved as a mutator, showing an approximately 20-fold-higher mutation frequency than the other strains; this strain contained a mutation to *mutS* (see Table S2 in the supplemental material). Isolates C-E1-1 and C-A1-1 were genetically highly similar, indicating that the strains were nearly clones. This high degree of similarity may have occurred via inadvertent transfer during the subculturing process. For these reasons, isolates C-B3-1 and C-E1-1 were excluded from further study.

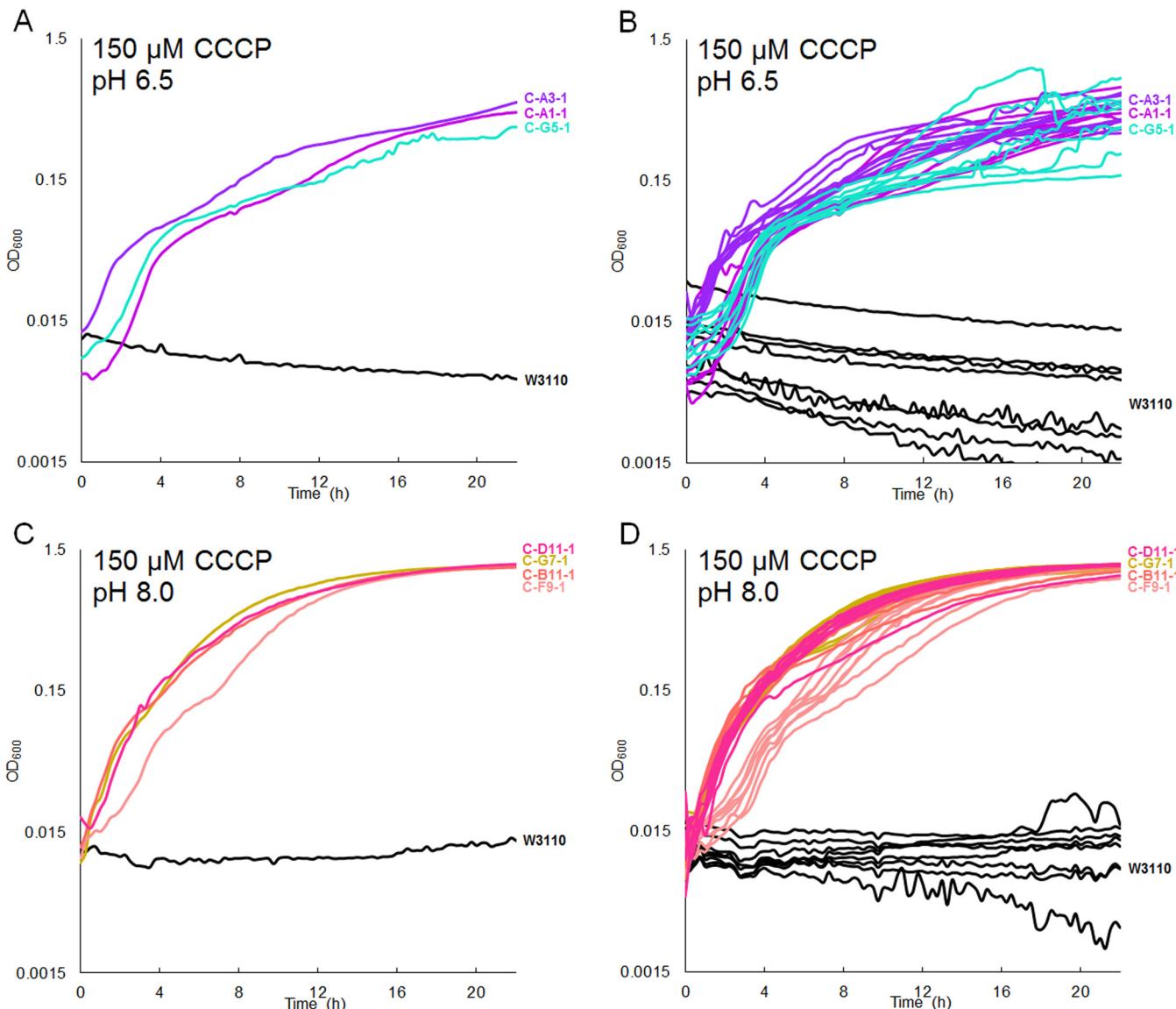


FIG 2 Isolates from CCCP-evolved populations show increased tolerance for 150 μM CCCP. (A and B) Low-pH-evolved strains in medium buffered with 100 mM Na-PIPES, pH 6.5; (C and D) high-pH-evolved strains in medium buffered with 100 mM TAPS, pH 8.0. Panels A and C show representative curves with median density at 16 h; panels B and D show all 8 replicates. For the growth of all low-pH-evolved strains compared to W3110 (A and B), Tukey's test gave a *P* value of <0.05. Tukey's test also gave a significant *P* value of <0.05 for growth of all high-pH-evolved strains compared to W3110 (C and D).

For seven CCCP-evolved populations at pH 6.5 and six at pH 8.0, all mutations predicted by breseq are shown in Table S1. Selected mutations are shown in Table 3, including all genes that showed mutations in more than one population and all mutations in the *emrAB* operon or its repressor gene *mprA*. Table 3 is organized by condition, with mutations found in populations cultured at pH 6.5 shaded blue and mutations found at high pH shaded red. Mutations that occurred in both the low-pH and high-pH projects are shaded purple.

All populations that evolved with CCCP at pH 6.5, and five out of six that evolved at pH 8.0, showed mutations in *mprA* (*emrR*), which encodes the repressor of *emrAB* (Table 3) (17, 21). Of the 12 strains with mutations in *mprA*, there were a total of 10 unique mutations to the coding region of MprA and 6 mutations to the intergenic region between *mprA* and *emrA*. Two mutations caused early stop codons in MprA, and two other mutations caused deletions of 50 bp or greater (Table 3). These early stop codons and large deletions are likely to knock out the function of MprA. MprA represses the

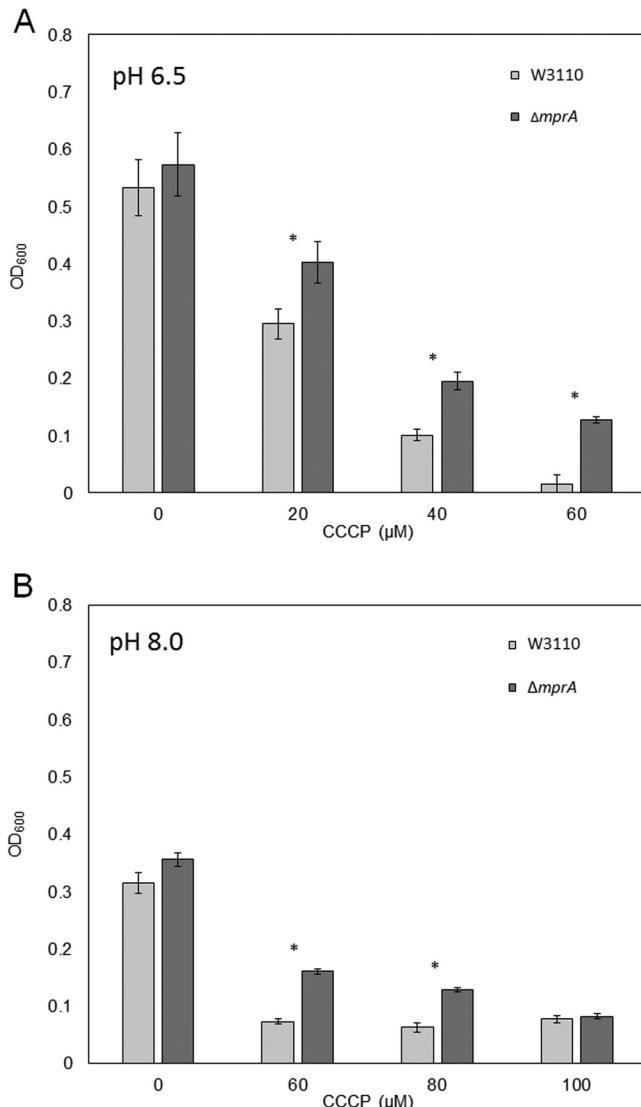


FIG 3 $\Delta mprA$ increases resistance of W3110 to CCCP at both low and high pH. W3110 $\Delta mprA::kanR$ (dark gray) and W3110 (light gray) were grown with increasing concentrations of CCCP in LBK with 100 mM Na-PIPES, pH 6.5 (A), and LBK with 100 mM TAPS, pH 8.0 (B). *, significant Tukey's test P value of <0.05 .

(Fig. 3). In addition, loss of MprA caused no growth difference in the absence of CCCP. Thus, the growth improvements due to $\Delta mprA::kanR$ are associated with CCCP, probably by efflux via the EmrAB complex. The increased CCCP tolerance of the $\Delta mprA::kanR$ strain is consistent with the hypothesis that loss of MprA repressor function in CCCP-evolved isolates increases fitness in the presence of CCCP.

Deletion of emrA in CCCP-evolved strains decreases CCCP fitness. Three of the CCCP-evolved strains (C-E3-1, C-A5-1, and C-F9-1) showed missense mutations in *emrA*. We therefore tested whether *emrA* activity was required for these and other CCCP-evolved strains. Deletion of *emrA* (by transduction of $\Delta emrA::kanR$) in strains C-A1-1, C-A3-1, C-G5-1, and C-D11-1 substantially decreased growth compared to that of the strains with *emrA* intact, both at pH 6.5 and at pH 8.0 with CCCP (Fig. 4). Thus, *emrA* appears to be required for the CCCP fitness advantage of these evolved strains.

The one CCCP-evolved strain that lacked any *emrA* or *mprA* mutation (strain C-B11-1) showed a fitness advantage that was independent of *emrA*, during culture at pH 8.0 (Fig. 4B). Another strain, C-D11-1, also showed partial CCCP tolerance at pH 8.0 with *emrA* deleted. However, at pH 6.5, where the uncoupler effect of CCCP is greatest, all strains, including C-B11-1 required *emrA* for growth.

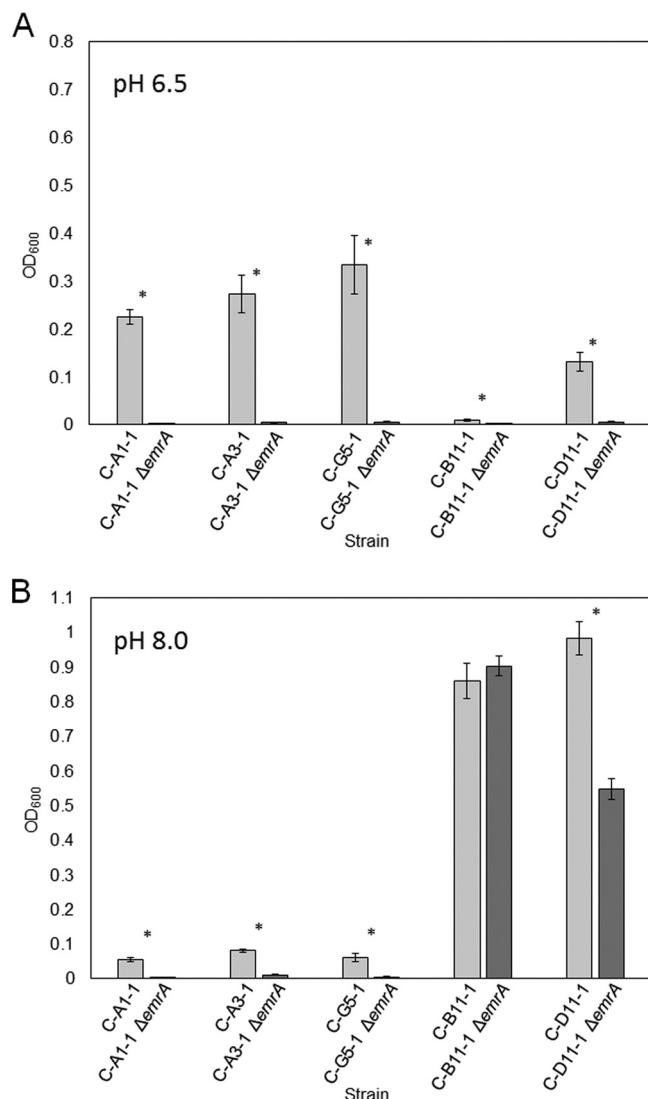


FIG 4 $\Delta emrA$ decreases growth of CCCP-evolved strains in the presence of 150 μM CCCP. The growth of C-A1-1, C-A3-1, C-G5-1, C-B11-1, and C-D11-1 (light gray) with 150 μM CCCP in LBK (pH 6.5) with 100 mM Na-PIPES (A) and LBK (pH 8.0) with 100 mM TAPS (B) is compared to the growth of C-A1-1 $\Delta emrA::kanR$, C-A3-1 $\Delta emrA::kanR$, C-G5-1 $\Delta emrA::kanR$, C-B11-1 $\Delta emrA::kanR$, and C-D11-1 $\Delta emrA::kanR$ (dark gray). *, significant Tukey's test P value of <0.05.

Mutations affecting *cecR* in strain C-B11-1 increase CCCP tolerance. The CCCP-evolved isolate C-B11-1 contains no mutations in *mprA* or *emrAB*, so we sought to identify other major components of CCCP tolerance in this strain. C-B11-1 notably sustained an *insH*-mediated deletion of nearly 20 kb which includes *phoE*, *proA* and *proB*, *perR*, *pepD*, *gpt*, *frsA*, and *crl* (Table S1). In addition to this large deletion, C-B11-1 also showed a mutation to *cecR* (*ybiH*) and a mutation in the intergenic region between *cecR* and *rhlE*. Mutations to this region were also present in another high-pH- and CCCP-evolved strain, C-D11-1. *CecR* regulates genes that affect sensitivity to cefoperazone and chloramphenicol (23). We found that W3110 $\Delta cecR::kanR$ showed increased CCCP tolerance at pH 6.5 (Fig. 5). In the presence of 30 μM CCCP, the *cecR* deletion strain reached a cell density comparable to that of isolate C-B11-1, whereas the ancestral strain W3110 grew significantly less. Thus, a *cecR* defect could contribute a major part of the CCCP fitness advantage for C-B11-1 and for C-D11-1 (Fig. 4B).

Multidrug components *gadE*, *acrA*, and *acrB* had mutations in CCCP-evolved strains. Strain C-B11-1 showed an additional mutation of interest for drug efflux, in

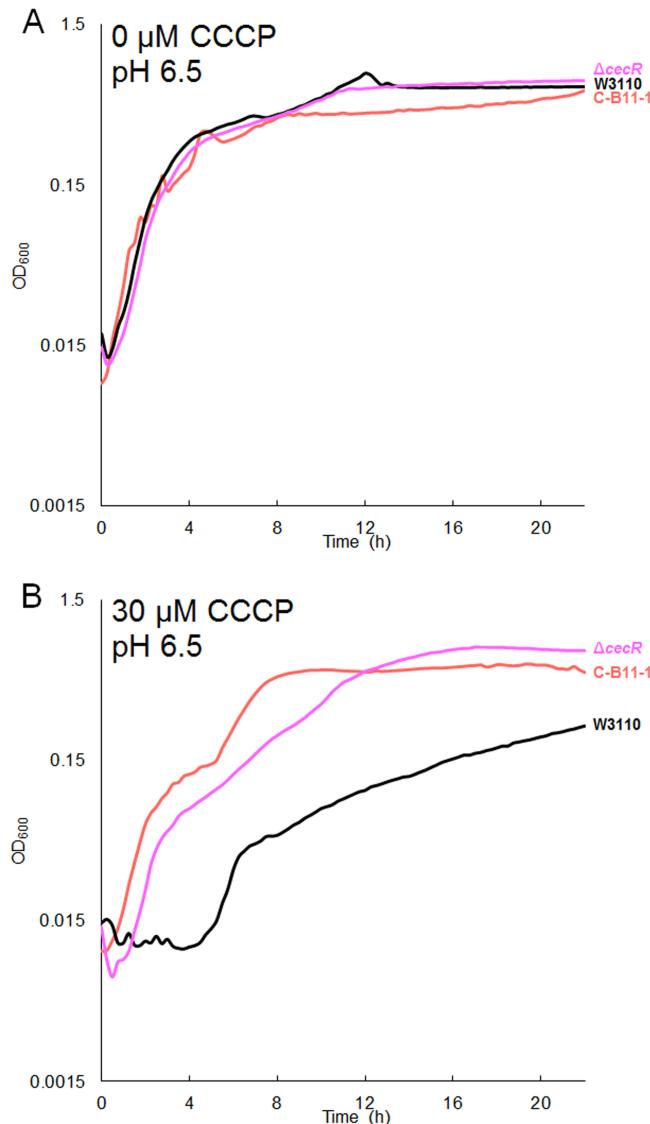


FIG 5 W3110 Δ cecR and C-B11-1 show enhanced growth in CCCP. Growth of C-B11-1, W3110 Δ cecR::kanR, and W3110 in 100 mM Na-PIPES, pH 6.5, with 0 μ M CCCP (A) and with 30 μ M CCCP (B) is shown. At 30 μ M CCCP, both W3110 Δ cecR::kanR and C-B11-1 grew significantly more than W3110. ANOVA with Tukey's test, $F = 0.135$ and $P = 0.875$ (A) and $F = 93.63$ and $P = 3.45e^{-11}$ (B) ($n = 8$ for each strain).

gadE (24, 25). Also, an intergenic mutation near *gadE* was found in strain C-H3-1 (Table 3). GadE activates expression of the Gad acid fitness island, which includes drug efflux genes *mdtFE* (26). The MdtFE multidrug efflux pump is not known to transport CCCP. Negative mutation of *gadE* would decrease expression of *mdtFE*, an effect seen in evolution under benzoate stress (10).

Strains C-A1-1 and C-A5-1 had mutations in *acrB* and *acrA*, respectively (27, 28); these are components of the major multidrug efflux pump AcrAB-TolC that confers resistance to chloramphenicol and tetracycline. C-A1-1 showed resistance to chloramphenicol (Fig. 6A) and tetracycline (Fig. 6B), indicating knockout of *acrB* function. We tested whether Δ acrB::kanR confers resistance to CCCP in the W3110 background, but we did not see an effect.

Several additional genes that showed mutations in CCCP-evolved strains were tested for effects on CCCP tolerance. Four strains that evolved with CCCP at pH 8.0, including strain C-B11-1, showed unique mutations in *rng*, encoding RNase G (29). A C-B11-1 *rng*⁺ construct made by recombineering showed no difference in CCCP

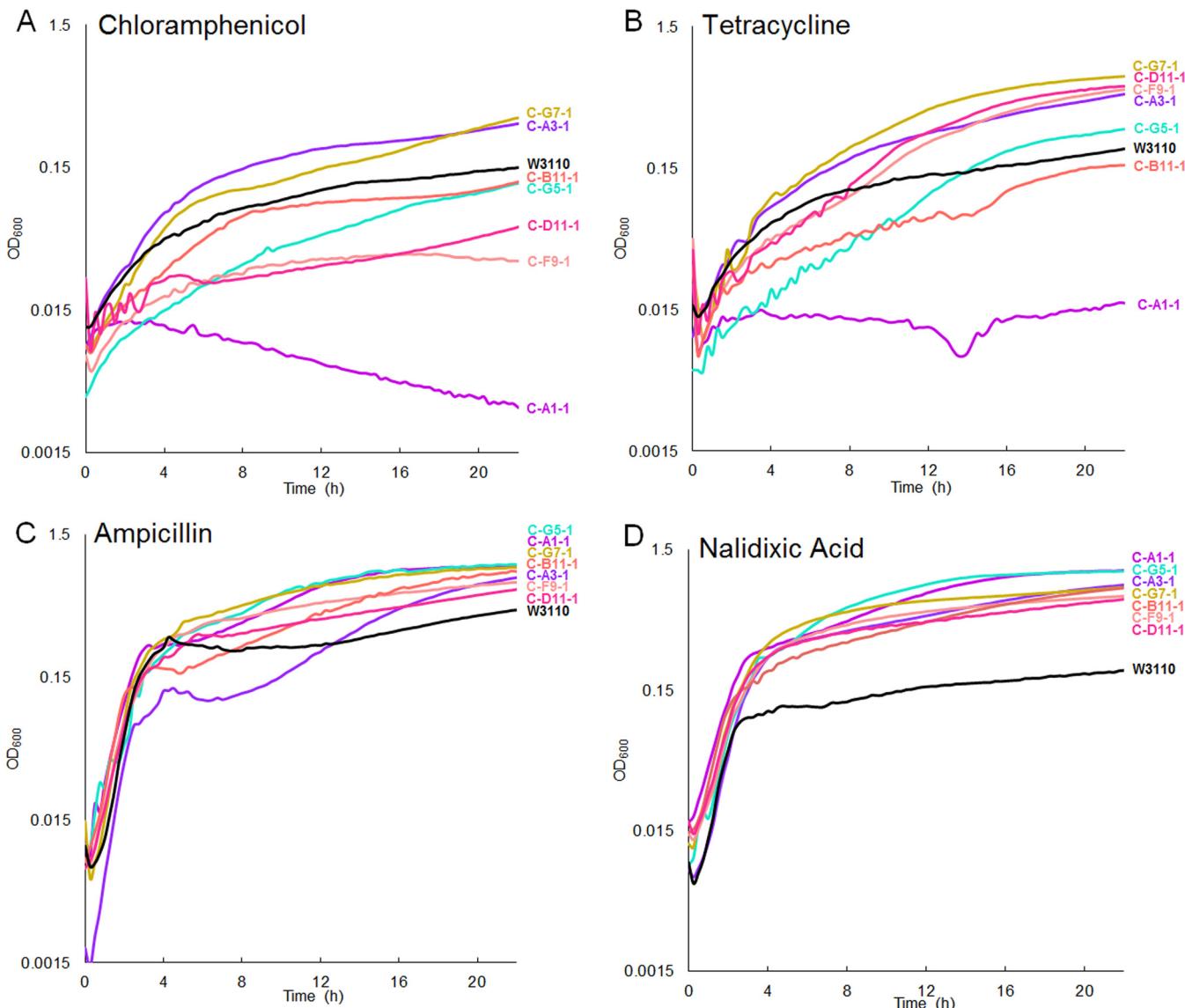


FIG 6 Growth of CCCP-evolved strains compared to the ancestor in LBK with 100 mM MOPS (pH 7.0) and 4 µg/ml chloramphenicol (A), 1 µg/ml tetracycline (B), 1 µg/ml ampicillin (C), or 6 µg/ml nalidixic acid (D). Curves shown are representative of the 8 replicates for each strain. Endpoint OD₆₀₀ data at 16 h as a measure of culture growth are shown. For chloramphenicol, growth of strains C-A1-1, C-D11-1, and C-F9-1 compared to W3110 gave a significant Tukey's test *P* value of <0.05. For tetracycline, all strains except C-B11-1 and C-G5-1 had growth differing significantly from that of W3110 (*P* < 0.05). For ampicillin and nalidixic acid, growth of all evolved isolates compared to that of W3110 gave a Tukey's test *P* value of <0.05.

tolerance compared to the parental strain C-B11-1, cultured in 150 µM CCCP at pH 8.0. CCCP-evolved strains also showed multiple mutations in genes *cyaA* (adenylate cyclase) and *nhaB* (sodium-proton antiporter). For these genes, deletions were tested in the ancestral strain background: W3110 Δ*cyaA* (50 µM CCCP, pH 6.5) and W3110 Δ*nhaB* (0 to 50 µM CCCP, pH 7.0). None of these constructs showed significant difference in growth compared to strain W3110, but small fitness increments would require competition assays to be revealed.

Mutations in 2,000-generation isolates from population C-B11. Because C-B11-1 uniquely contained no *mprA* or *emrAB* mutations and contained a mutation to *gadE*, we decided to sequence two later isolates from the C-B11 population (C-B11-3 and C-B11-4) after a total of 2,000 generations (see Table S3 in the supplemental material). Cultures of the later isolates showed growth curves comparable to those for C-B11-1. These later isolates retained most of the mutations found in C-B11-1, including the nearly 20-kb deletion of 28 genes that was unique to population C-B11. At between

1,000 and 2,000 generations, the C-B11 strains acquired additional mutations affecting genes *ybhR*, *lptD*, *tsx*, *gltA*, *cfa*, *mgrB*, *pykA*, *cpsB*, *ydhN*, and *yjjU*. Gene *ybhR* encodes a subunit of the cefoperazone efflux complex regulated by CecR (23). This result is thus consistent with the enhanced fitness shown by the *cecR* mutation found in the C-B11 lineage. The *mgrB* gene is acid inducible, via the PhoPQ response (30), and shows mutations selected after high-pH evolution (16). The gene *lptD* forms part of the lipopolysaccharide (LPS) transport slide, a hydrophobic intermembrane hole for the transport of LPS to the surface of the bacterium, (31), which could be relevant for CCCP-membrane interactions.

CCCP-evolved strains show altered resistance to antibiotics. Exposure to the partial uncoupler benzoate selects for strains sensitive to chloramphenicol and tetracycline (10). We therefore tested the growth of the CCCP-evolved strains in the presence of various antibiotics. Strain C-A1-1 (which contains an *acrB* mutation) was very sensitive to chloramphenicol and tetracycline (Fig. 6A and B), similar to the result seen for benzoate-evolved strains (10). In addition to the *acrB* mutation, strain C-A1-1 also had mutations in *sohA* and in *rpoB* that resemble the pattern of mutations selected in the benzoate-evolved strains (10). The strain did not, however, show loss of resistance to ampicillin (Fig. 6C), another substrate of AcrAB-TolC (32). Other CCCP-evolved isolates showed marginal degrees of gain or loss of resistance to chloramphenicol, tetracycline, and ampicillin (Fig. 6).

Nalidixic acid is a target of the EmrAB-TolC multidrug efflux pump (18), so mutations increasing expression of the function of this pump might increase resistance to the substrate antibiotics. In the presence of nalidixic acid, all CCCP-evolved strains grew to higher levels than the ancestor (Fig. 6D). Strain C-B11-1 does not contain mutations known to affect EmrAB, but it contains a mutation affecting CecR, which may increase resistance to nalidixic acid (23).

Evolved strains show small pH growth effects independent of CCCP. Serial culture at low or high pH is known to shift the growth range (14, 16). Thus, we tested the effect of pH alone during CCCP evolution at low or high pH. The CCCP-evolved strains showed marginal changes in pH dependence, as represented by culture density at 16 h. Strains evolved at pH 6.5 showed growth indistinguishable from that of the ancestral W3110 at pH 6.5 (Fig. 7A). At pH 8.0, we saw a small significant increase in density during stationary phase (Fig. 7B). Strains evolved at pH 8.0 consistently showed a small increase in stationary-phase growth at either pH 6.5 (Fig. 7C) or pH 8.0 (Fig. 7D).

In order to test whether these pH-dependent improvements in growth reflect some other factor associated with the stress of growing in a microplate, we conducted a growth curve with aeration in baffled flasks. We selected two evolved strains from the low-pH and high-pH conditions and measured their growth compared to that of the ancestor at pH 6.5 [100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES)] and pH 8.0 {100 mM [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS)}, respectively, over the course of 8 h using a growth assay conducted under highly aerobic conditions. We saw no significant growth differences between ancestral and evolved populations (data not shown).

To determine whether fitness differences occur under more extreme acidic and basic conditions, growth of CCCP-evolved strains was tested at pH 5.0 (100 mM morpholineethanesulfonic acid [MES]) (Fig. 8A) and pH 9.0 [100 mM N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO)] (Fig. 8B). Tests at pH 5.0 showed small significant differences. At pH 9.0, the pH 8.0-evolved strains (C-B11-1, C-D11-1, C-F9-1, and C-G7-1) grew to a higher density than the ancestor; these results show some shifting of growth range in favor of the pH during serial culture. The pH 6.5-evolved strains (C-A1-1, C-A3-1, and C-G5-1) showed smaller, mixed effects (Fig. 8B). At pH 7.0, most of the CCCP-evolved strains showed marginal or no differences from the ancestor (Fig. 9).

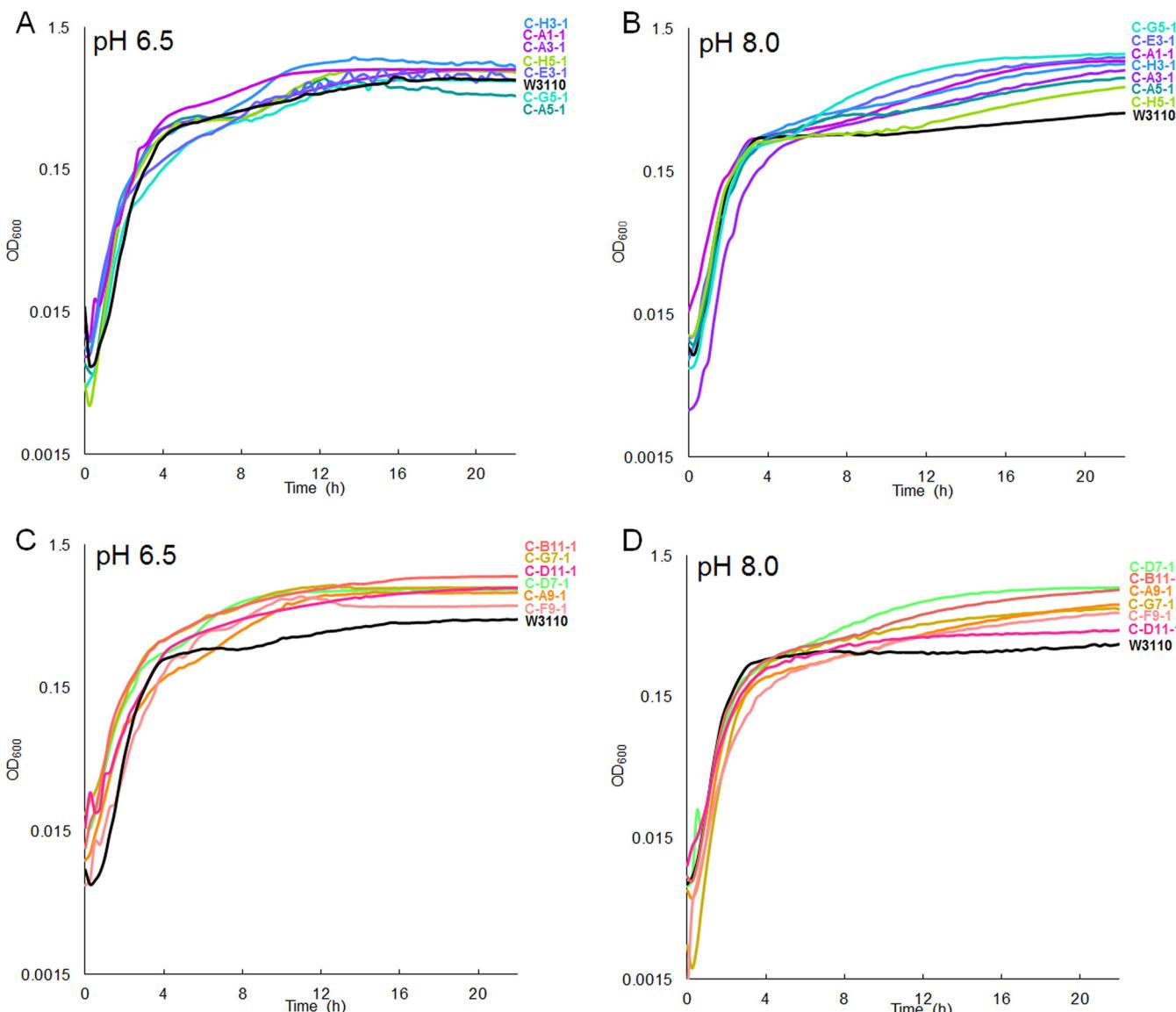


FIG 7 OD₆₀₀ values for CCCP-evolved strains cultured over 22 h at pH 6.5 or at pH 8.0. Low-pH-evolved strains were cultured with 100 mM K-PIPES, pH 6.5 (A), and with 100 mM TAPS, pH 8.0 (B). High-pH-CCCP-evolved strains were cultured with 100 mM K-PIPES pH 6.5 (C), and with 100 mM TAPS, pH 8.0 (D). Each curve represents the median 16-h culture density of eight replicates. At pH 6.5, growth of C-A1-1 and C-H3-1 is significantly different from growth of W3110 (Tukey's test, $P < 0.05$) (A). At pH 8.0, the W3110 16-h culture density differs significantly from those of all strains but C-H5-1 ($P < 0.05$; ANOVA with Tukey post hoc test, $F = 34.698$, $P < 0.001$; $n = 8$ for each strain) (B). At both pH 6.5 (C) and pH 8.0 (D), growth of all high-pH-evolved strains is significantly different from growth of the W3110 ancestor (Tukey's test, $P < 0.05$).

Evolved strains show mutations to *adhE*. Mutations occurred in *adhE* (acetaldehyde coenzyme A [acetaldehyde-CoA] dehydrogenase) in the strains that evolved in CCCP at pH 8.0. The CCCP stock had been dissolved in ethanol, leading to 1.5% ethanol at the highest concentration used in growth media (150 μ M CCCP for both pH 8.0 and pH 6.5). We tested the ability of the high-pH-evolved strains to grow in 1.5% ethanol and found that evolved strains grew to a significantly higher optical density (OD) than the ancestor (Fig. 10A). We also tested the CCCP tolerance of our strains using a stock dissolved in dimethyl sulfoxide (DMSO) instead of ethanol. The CCCP fitness advantage remained in the absence of ethanol (Fig. 10B).

DISCUSSION

In our experimental evolution of *E. coli* with CCCP, all strains but one (C-B11-1) showed mutations to *mprA*, the repressor of *emrAB*. The *emrAB* genes encode a

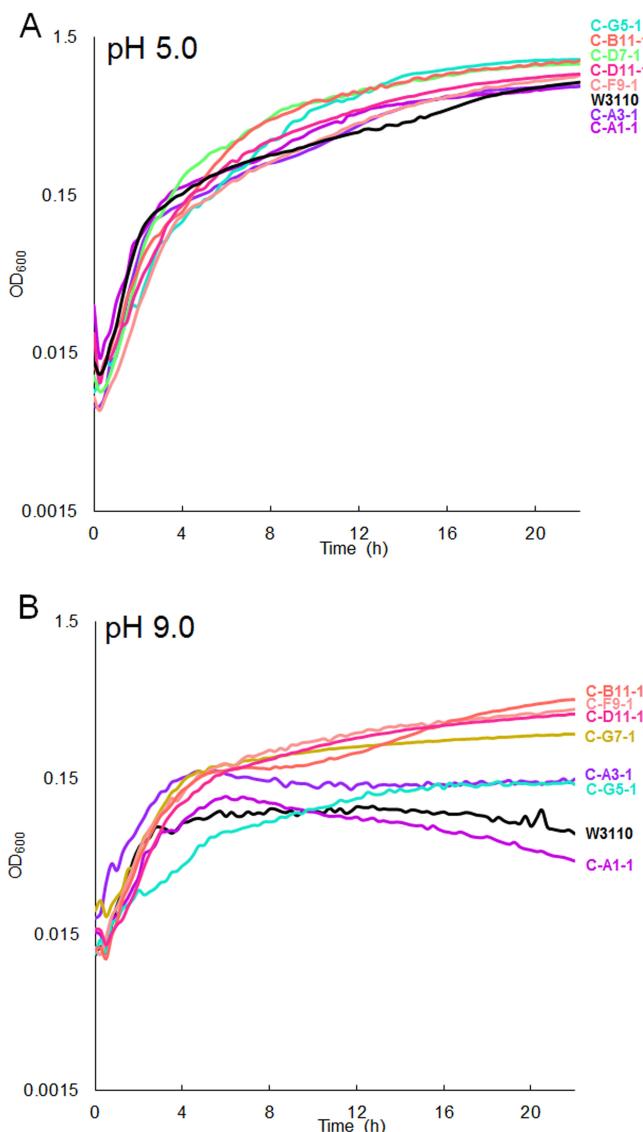


FIG 8 OD₆₀₀ values for CCCP-evolved strains cultured over 22 h at with 100 mM MES, pH 5.0 (A), and with 100 mM AMPSO, pH 9.0 (B). With 100 mM MES (pH 5.0), W3110 differs significantly from all evolved strains except A3-1 ($P < 0.05$; ANOVA with Tukey post hoc test, $F = 23.481$, $P < 0.001$; $n = 8$ for each strain) (A). With 100 mM AMPSO (pH 9.0), all low-pH-CCCP-evolved strains differ from all high-pH-evolved strains ($P < 0.05$; ANOVA with Tukey post hoc test, $F = 34.9$, $P = 2e^{-16}$; $n = 8$ for each strain) (B).

multidrug efflux transport system that has been previously linked to CCCP resistance (17). Deleting the repressor may increase expression or activity of CCCP efflux via EmrAB-TolC (17, 19). The upregulation of EmrAB in our strains is consistent with their increased resistance to nalidixic acid (Fig. 6D), another substrate of the efflux pump (17, 33). The *emrAB* genes also showed point mutations in many of the strains. These *emrA* and *emrB* point mutations probably arose secondary to the *mprA* mutations that derepress *emrAB* expression. The appearance of secondary mutations contingent on fitness changes from an earlier mutation is a common feature of experimental evolution (34).

Besides the apparent *mprA* knockouts, CCCP selected for mutations affecting several drug efflux pumps that are not known to transport CCCP. Strain C-B11-1 (lacking mutations in *mprA*) attained a comparable fitness increase via mutations in the regulator *gadE* (activates *mtdEF*) or *cecR* (represses *ybhG* [resistance to chloramphenicol and cefoperazone]). Other strains showed mutations in MDR components *acrAB* and *ybhR*.

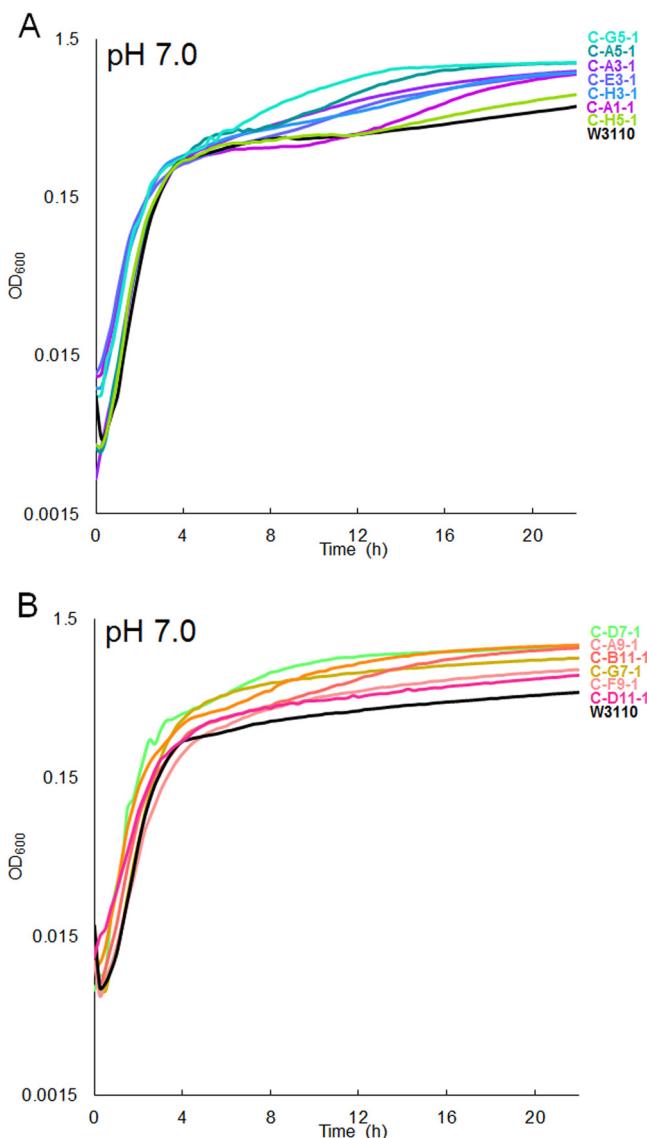


FIG 9 OD₆₀₀ values for CCCP-evolved strains cultured with 100 mM MOPS, pH 7.0, over 22 h. (A) Strains evolved at low pH; W3110 differs significantly from all strains but H5-1 ($P < 0.05$; ANOVA with Tukey post hoc test, $F = 15.925$, $P < 0.001$; $n = 8$ for each strain). (B) Strains evolved at high pH; W3110 differs significantly from A9-1 and B11-1 ($P < 0.05$; ANOVA with Tukey post hoc test, $F = 10.721$, $P < 0.001$; $n = 8$ for each strain).

The decreased expression or activity of non-CCCP drug pumps is comparable to our previous results for evolution in the presence of benzoate. Table 4 compiles all the MDR-related mutations we have found to be selected during evolution with CCCP or with benzoate (10). In contrast, microplate experimental evolution under other conditions does not show loss of multiple drug pumps (15, 16). The one exception might be mutations decreasing *mdtEF* expression in the Gad acid fitness island during evolution in near-extreme acid, but acid response components of Gad could also affect fitness in this case.

Overall, our results add to a pattern of selection against drug efflux pumps and their regulators under evolution with an uncoupler or a partial uncoupler (such as benzoate). However, unlike CCCP, benzoate acts at relatively high aqueous concentrations that equilibrate across the membrane. These concentrations cannot be effluxed by multi-drug efflux pumps; therefore, adaptation to the presence of benzoate and other permeant acids may be more dependent upon mutations that decrease transmem-

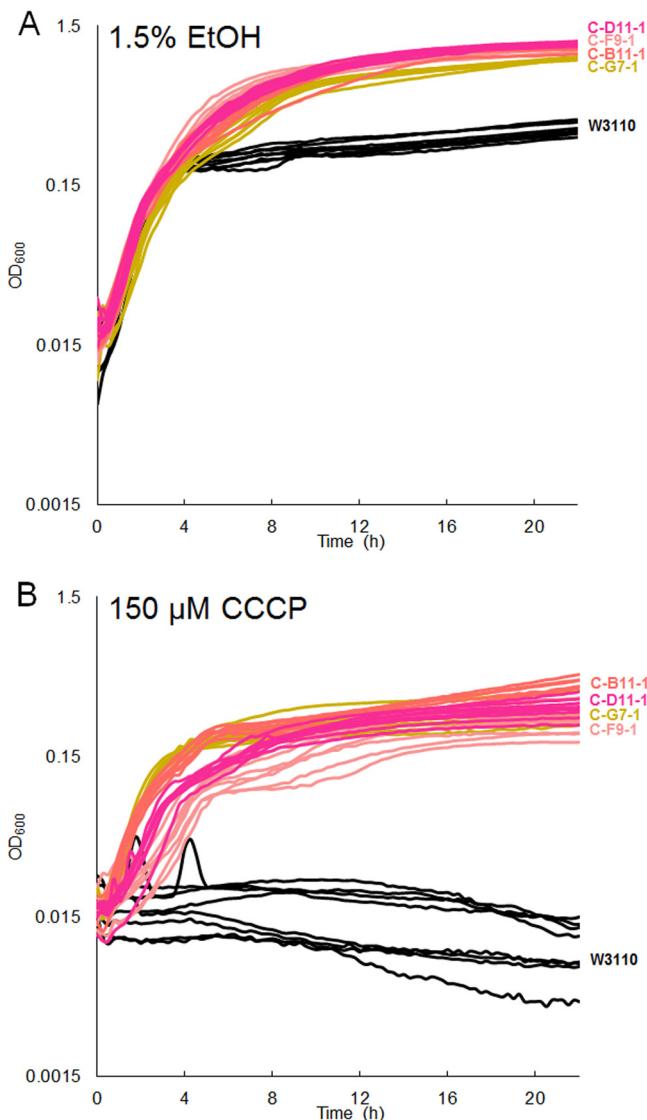


FIG 10 Growth of high-pH-CCCP-evolved strains in LBK with 100 mM TAPS, pH 8.0, and 1.5% ethanol (EtOH) (A) or 150 μ M CCCP dissolved in DMSO (B). Growth of all evolved strains in both 1.5% ethanol and 150 μ M CCCP differed significantly from growth of W3110 (Tukey's test, $P < 0.05$).

brane proton flux through unneeded pumps. Organic permeant acids are found in high concentrations in the gut. These organic acids decrease PMF for enteric bacteria in the human microbiome. As such, our findings have implications for the potential application of microbiome evolution in the human intestinal tract. We are following up this work with a more-extended comparison of the relative fitness costs of drug pump genes, using flow cytometry competition assays (35).

MATERIALS AND METHODS

Experimental evolution with CCCP at pH 6.5 and 8.0. For experimental evolution, *E. coli* K-12 W3110 populations were cultured at 37°C in a 96-well microplate in modified LBK medium (10 g/liter tryptone, 5 g/liter yeast extract, and 7.45 g/liter KCl) (14). The medium was modified with buffers to compare the effects of CCCP at low pH versus high pH. The pH 6.5 medium contained 100 mM Na-PIPES (0.55 M PIPES buffer and 1.4 M NaOH), and the pH 8.0 medium contained 100 mM TAPS. The initial and subsequent microplates contained 32 evolving populations of 200 μ l each. These included 16 populations with 20 μ M CCCP buffered at pH 6.5 and 16 populations with 50 μ M CCCP buffered at pH 8.0. The well cultures grew to stationary phase and after a total of 22 h were diluted 1:100 daily, undergoing approximately 6.6 generations per day. A multichannel pipettor with filter tips was used for daily transfer of 2 μ l culture into 200 μ l fresh medium. Over the course of their serial dilutions, the concentration of

TABLE 4 Multidrug pumps and regulators showing mutations selected by long-term exposure to benzoate or to CCCP^a

Evolution conditions	Mutation	Coding	Gene(s) ^b	Function related to MDR
CCCP, pH 6.5	Δ1 bp G→A	Coding (nt 247/3150) Q116 (CAG→TAG)	acrB ← acrA ←	MDR efflux (Tet, Chl, Amp, Nal, Rif) MDR efflux (Tet, Chl, Amp, Nal, Rif)
CCCP, pH 8.0	T→C A→T T→C A→T C→A	I321V (ATT→GTT) Y51N (TAC→AAC) Y50C (TAC→TGC) Intergenic (−36/−193) Intergenic (−61/−168)	ybhR ← cecR ← cecR ← cecR ← / → rhIE cecR ← / → rhIE	MDR efflux (Cef) MDR regulator of YbhFSR, YbhG (Cef, Chl) MDR regulator of YbhFSR, YbhG (Cef, Chl) MDR regulator of YbhFSR, YbhG (Cef, Chl) MDR regulator of YbhFSR, YbhG (Cef, Chl)
Benzoate, pH 6.5	IS5 (+)	Coding (nt 79–82/267)	ariR (ymgB) →	Acid stress; upregulates Gad regulon (<i>mdtEF</i>)
CCCP, pH 6.5	(C)5→6	Coding (nt 936/993)	sapD ←	Putrescine efflux
Benzoate, pH 6.5	Δ6,115 bp C→A IS5 (+) IS2 (-)	Coding inclusive deletion L191M (CTG→ATG) Coding (nt 430–433/1539) Coding (635–639/1173)	[ydeA]–[ydeH] mdtA → emrY ← emrA →	MarRAB multidrug efflux regulon MDR efflux (fluoroquinolones, macrolides) MDR efflux (Tet) MDR efflux (fluoroquinolones)
CCCP, pH 8.0	T→C	Intergenic (−91/−59)	cpxP ← / → cpxR	MDR regulator (with CpxA)
Benzoate, pH 6.5	G→A G→T A→G Δ14,146 bp Δ7,921 bp Δ78 bp G→T IS5 (+) Δ10,738 bp	G92S (GGC→AGC) R106L (CGT→CTT) N107S (AAC→AGC) InsH IS5 mediated InsH IS5 mediated Coding (nt 42–119/825) L199F (TTG→TTT) Coding (nt 626–629/825) InsH mediated	cpxA → cpxA → cpxA → gadX–[yhiS] gadX–gadE gadX → gadX → gadX → [gadW]–slp	Sensory kinase with CpxR Sensory kinase with CpxR Sensory kinase with CpxR gadE, gadXW, mdtFE (fluoroquinolones, penams, macrolides) gadE, gadXW, mdtFE (fluoroquinolones, penams, macrolides) Upregulates mdtFE Upregulates mdtFE Upregulates mdtFE Upregulates mdtFE gadX, mdtFE, gadE
CCCP, pH 8.0 CCCP, pH 6.5 Benzoate, pH 6.5 CCCP, pH 6.5 CCCP, pH 8.0	IS IS5 A→G C→T T→G	Coding (nt 234/528) Intergenic (−119/+680) S34P (TCC→CCC) E94K (GAA→AAA) T3P (ACC→CCC)	gadE ← gadE ← / ← hdeD rob ← arcA ← arcA ←	Upregulates mdtEF Upregulates mdtEF Upregulates marA regulon Anaerobic; upregulates mdtEF Anaerobic; upregulates mdtEF

^aIncludes CCCP data from the present paper, as well as benzoate data from reference 10. Abbreviations: nt, nucleotides; Amp, ampicillin; Cef, cefoperazone; Chl, chloramphenicol; Nal, nalidixic acid; Rif, rifampin; Tet, tetracycline.

^bArrows next to genes indicate the direction of transcription within the genome; brackets represent only partial deletion of the gene coding sequence.

CCCP was increased, with the pH 6.5 condition increasing from 20 μM to 150 μM and the pH 8.0 condition increasing from 50 μM to 150 μM CCCP (Table 1).

After the populations were cultured for approximately 1,000 generations, clonal isolates were obtained from microplate well populations. Of the populations evolved at pH 6.5, seven isolates from different wells were selected for whole-genome sequencing, and of those at pH 8.0, six isolates were selected for whole-genome sequencing. Clonal isolates were generated by streaking samples three times on LBK plates. Each isolate was given a population designation: "C" for CCCP evolution, the alphanumeric position in the well plate, and a digit for the isolate number from the given well population.

Whole-genome sequencing and sequence analysis. The genomes of fourteen isolates from generation 1000 and the ancestor were sequenced (see Table S1 in the supplemental material). DNA extraction was performed using the Epicentre Masterpure purification kit. The genomes were validated and quantitated using the Illumina TruSeq Nano DNA library preparation kit by the Research and Technology Support Facility at Michigan State University. Sequencing was then performed utilizing the Illumina MiSeq platform in 2- by 300-bp format, resulting in 20 to 25 million read pairs. Read alignment and mutation predictions were performed using the computational pipeline breseq (v.0.28.1, v.0.30.0, and v.0.30.2) with default parameters (36, 37). The reads were mapped to the *E. coli* K-12 W3110 reference (accession no. NC_00779.1). Mutations found in both the ancestor and evolved strains were excluded from analysis.

Knockouts in genes of interest. To determine the fitness impact of specific genes on growth in CCCP, certain genes were deleted from both the ancestor and evolved strains. Donor *E. coli* strains containing *kanR* insertion knockouts were obtained from the Keio collection (38). Deletion strains with the W3110 background were constructed by P1 phage transduction (10). Knockout constructs were confirmed by PCR amplification of the *kanR* insert and were checked against the recipient and donor strains using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for *lacZ*⁺ (W3110 background). Colony PCR was performed using Lucigen ClonetD colony PCR master mix to determine whether the *kanR* cassette had been inserted into the correct gene and that the recipient strain was as expected. Primers included gene of interest forward direction, gene of interest reverse direction, internal kanamycin (KAN) gene forward, internal KAN gene reverse, and primer KT for within the KAN marker.

Recombineering. In order to generate strain C-B11-1 *rng*⁺, a section of gene *rng* containing the nonsense mutation in CCCP-evolved isolate C-B11-1 was replaced with the wild-type sequence using recombineering (39, 40). A counterselectable *cat-sacB* cassette was amplified from colonies of *E. coli* strain XLT241 using primers with 5' homology to regions surrounding the *rng* mutation site in C-B11-1 (5'-CAGCGCCAAAAACCTAACGCTGGTTTCACCCGGCTTGTGACCGAAGATCACTTCGAGAATA-3' and 5'-ATAATGAAGATCACCGCCGCGAGTGCCTGCACTCGCTGGAGCAATCAAAGGGAAAATGTCCATAT-3'). Isolate C-B11-1 transformed with the heat-inducible pSIM6::ampR plasmid was cultured to mid-log phase at 32°C and then transferred to 42°C to induce recombineering proteins. Cells were then made electrocompetent and electroporated with the *cat-sacB* PCR product containing edges homologous to regions flanking the *rng* mutation site in B11-1. The cells were grown out at 32°C for 3 to 5 h and plated on LB with 10 µg/ml chloramphenicol. Colonies were then screened for chloramphenicol resistance and sucrose sensitivity. To replace the *cat-sacB* cassette with the wild-type *rng* sequence, successful colonies were made electrocompetent and electroporated with a DNA oligonucleotide containing the wild-type sequence of the region replaced by *cat-sacB* as well as homology to the flanking regions (5'-CCATTAA CGCTGGTTTCACCCGGCTTGTCAACGCCGCTGCAGCGAGTGCAGCAGTCGGCGGGTG-3'). The cells were then plated on LB lacking NaCl and containing 6% sucrose. Colonies were further screened for resistance to sucrose and chloramphenicol sensitivity. The *rng* sequence of the new construct was then confirmed by PCR sequencing (5'-GCATGGTGGACACGAACAAT-3' and 5'-CGCTGGAACGCAAAG TAGAA-3').

Growth assays. Batch culture growth was assayed under semiaerobic conditions based on previous procedures (10, 15, 16). A 96-well microplate was filled with the desired media and inoculated at a concentration of 1:200 from an overnight culture in each test. Eight replicates from two independent overnight cultures (4 each) were performed for each strain within a single plate. Microplates were placed in the SpectraMax 384 spectrophotometer and cultured for 22 h at 37°C, with the OD at 600 nm (OD₆₀₀) measured every 15 min. Each entire set of growth curves (including 8 replicates for each strain under comparison) was run in triplicate.

Growth curve determinations for antibiotic tests were conducted by inoculation from overnight cultures in LBK-100 mM MOPS (morpholinepropanesulfonic acid), pH 7.0. These antibiotic growth curve determinations were conducted in LBK-100 mM MOPS (pH 7.0) supplemented with 1 µg/ml ampicillin, 1 µg/ml tetracycline, 4 µg/ml chloramphenicol, or 6 µg/ml nalidixic acid.

For growth curves under aeration, 125-ml baffled flasks containing 20 ml of LBK (pH 8.0 [100 mM TAPS] or pH 6.5 [100 mM Na-PIPES]) were inoculated 1:200 from overnight cultures. The cultures (three per strain) were incubated in a water bath set to 37°C with rotation at 200 rpm. Aliquots (200 µl) were removed from the baffled flasks and read in a 96-well plate (wavelength, 600 nm) every thirty minutes for 8 h.

Statistics. Means are reported with standard error of the mean (SEM). Statistics were computed using R packages. Samples were compared using analysis of variance (ANOVA) tests with Tukey *post hoc* tests.

Accession number(s). Sequence data have been uploaded in the NCBI Sequence Read Archive (SRA) under accession number [SRP157768](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02792-18>.

SUPPLEMENTAL FILE 1, XLSX file, 1.6 MB.

ACKNOWLEDGMENTS

This work was supported by award MCB-1613278 from the National Science Foundation and by summer science funds from Kenyon College.

We thank Ellen Broeren for excellent technical support.

REFERENCES

- Mitchell P. 2011. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biochim Biophys Acta Bioenerg* 1807:1507–1538. <https://doi.org/10.1016/j.bbabiobio.2011.09.018>.
- Kasianowicz J, Benz R, McLaughlin S. 1984. The kinetic mechanism by which CCCP (carbonyl cyanide m-chlorophenylhydrazone) transports protons across membranes. *J Membr Biol* 82:179–190. <https://doi.org/10.1007/BF01868942>.
- McLaughlin SG, Dilger JP. 1980. Transport of protons across membranes by weak acids. *Physiol Rev* 60:825–863. <https://doi.org/10.1152/physrev.1980.60.3.825>.
- Gould JM. 1979. Respiration-linked proton transport, changes in external pH, and membrane energization in cells of *Escherichia coli*. *J Bacteriol* 138:176–184.
- Lobritz MA, Belenky P, Porter CBM, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial cellular respiration. *Proc Natl Acad Sci U S A* 112:8173–8180. <https://doi.org/10.1073/pnas.1509743112>.
- Diez-Gonzalez F, Russell JB. 1997. Effects of carbonylcyanide-m-chlorophenylhydrazone (CCCP) and acetate on *Escherichia coli* O157:H7 and K-12: uncoupling versus anion accumulation. *FEMS Microbiol Lett* 151:71–76. <https://doi.org/10.1111/j.1574-6968.1997.tb10396.x>.
- MacLeod RA, Wisse GA, Stejskal FL. 1988. Sensitivity of some marine bacteria, a moderate halophile, and *Escherichia coli* to uncouplers at alkaline pH. *J Bacteriol* 170:4330–4337. <https://doi.org/10.1128/jb.170.4330-4337.1988>.
- Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, Luisi BF. 2018. Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* 16:523–539. <https://doi.org/10.1038/s41579-018-0048-6>.
- Schuldiner S. 2018. The *Escherichia coli* effluxome. *Res Microbiol* 169: 357–362. <https://doi.org/10.1016/j.resmic.2018.02.006>.
- Creamer KE, Ditmars FS, Basting PJ, Kunka KS, Hamdallah IN, Bush SP, Scott Z, He A, Penix SR, Gonzales AS, Eder EK, Camperchioli DW, Berndt A, Clark MW, Rouhier KA, Slonczewski JL. 2017. Benzoate- and salicylate-

- tolerant strains of *Escherichia coli* K-12 lose antibiotic resistance during laboratory evolution. *Appl Environ Microbiol* 83:e02736-16. <https://doi.org/10.1128/AEM.02736-16>.
- Deng Z, Shan Y, Pan Q, Gao X, Yan A. 2013. Anaerobic expression of the *gadE-mdtEF* multidrug efflux operon is primarily regulated by the two-component system ArcBA through antagonizing the H-NS mediated repression. *Front Microbiol* 4:194. <https://doi.org/10.3389/fmicb.2013.00194>.
 - Norman C, Howell KA, Millar AH, Whelan JM, Day DA. 2004. Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. *Plant Physiol* 134:492–501. <https://doi.org/10.1104/pp.103.031039>.
 - Slonczewski JL, Macnab RM, Alger JR, Castle AM. 1982. Effects of pH and repellent tactic stimuli on protein methylation levels in *Escherichia coli*. *J Bacteriol* 152:384–399.
 - Harden MM, He A, Creamer K, Clark MW, Hamdallah I, Martinez KA, Kresslein RL, Bush SP, Slonczewski JL. 2015. Acid-adapted strains of *Escherichia coli* K-12 obtained by experimental evolution. *Appl Environ Microbiol* 81:1932–1941. <https://doi.org/10.1128/AEM.03494-14>.
 - He A, Penix SR, Basting PJ, Griffith JM, Creamer KE, Camperchioli D, Clark MW, Gonzales AS, Sebastian Chávez EJ, George NS, Bhagwat AA, Slonczewski JL. 2017. Acid evolution of *Escherichia coli* K-12 eliminates amino acid decarboxylases and reregulates catabolism. *Appl Environ Microbiol* 83:e00442-17. <https://doi.org/10.1128/AEM.00442-17>.
 - Hamdallah I, Torok N, Bischof KM, Majdalani N, Chadalavada S, Mdluli N, Creamer KE, Clark M, Holdener C, Basting PJ, Gottesman S, Slonczewski JL. 2018. Experimental evolution of *Escherichia coli* K-12 at high pH and RpoS induction. *Appl Environ Microbiol* 84:e00520-18. <https://doi.org/10.1128/AEM.00520-18>.
 - Lomovskaya O, Lewis K, Matin A. 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. *J Bacteriol* 177: 2328–2334. <https://doi.org/10.1128/jb.177.9.2328-2334.1995>.
 - Lomovskaya O, Lewis K. 1992. emr, an *Escherichia coli* locus for multidrug resistance. *Proc Natl Acad Sci U S A* 89:8938–8942. <https://doi.org/10.1073/pnas.89.19.8938>.
 - Nikaido H. 1996. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 178:5853–5859. <https://doi.org/10.1128/jb.178.20.5853-5859.1996>.
 - Xiong A, Gottman A, Park C, Baetens M, Pandza S, Matin A. 2000. The EmrR protein represses the *Escherichia coli* emrRAB multidrug resistance operon by directly binding to its promoter region. *Antimicrob Agents Chemother* 44:2905–2907. <https://doi.org/10.1128/AAC.44.10.2905-2907.2000>.
 - Brooun A, Tomashek JJ, Lewis K. 1999. Purification and ligand binding of EmrR, a regulator of a multidrug transporter. *J Bacteriol* 181:5131–5133.
 - Gage DJ, Neidhardt FC. 1993. Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-dinitrophenol. *J Bacteriol* 175:7105–7108. <https://doi.org/10.1128/jb.175.21.7105-7108.1993>.
 - Yamanaka Y, Shimada T, Yamamoto K, Ishihama A. 2016. Transcription factor CecR (YbiH) regulates a set of genes affecting the sensitivity of *Escherichia coli* against ceftazidime and chloramphenicol. *Microbiology* 162:1253–1264. <https://doi.org/10.1099/mic.0.000292>.
 - Hommais F, Krin E, Coppée JY, Lacroix C, Yeramian E, Danchin A, Bertin P. 2004. GadE (YbiE): a novel activator involved in the response to acid environment in *Escherichia coli*. *Microbiology* 150:61–72. <https://doi.org/10.1099/mic.0.26659-0>.
 - Seo SW, Kim D, O'Brien EJ, Szubin R, Palsson BO. 2015. Decoding genome-wide GadEWX-transcriptional regulatory networks reveals multifaceted cellular responses to acid stress in *Escherichia coli*. *Nat Commun* 6:7970. <https://doi.org/10.1038/ncomms8970>.
 - Zhang Y, Xiao M, Horiyama T, Zhang Y, Li X, Nishino K, Yan A. 2011. The multidrug efflux pump MdtEF protects against nitrosative damage during the anaerobic respiration in *Escherichia coli*. *J Biol Chem* 286: 26576–26584. <https://doi.org/10.1074/jbc.M111.243261>.
 - Okusu H, Ma D, Nikaido H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 178:306–308. <https://doi.org/10.1128/jb.178.1.306-308.1996>.
 - Baucheron S, Tyler S, Boyd D, Mulvey MR, Chaslus-Dancla E, Cloeckaert A. 2004. AcrAB-TolC directs efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium DT104. *Antimicrob Agents Chemother* 48:3729–3735. <https://doi.org/10.1128/AAC.48.10.3729-3735.2004>.
 - Deana A, Belasco JG. 2004. The function of RNase G in *Escherichia coli* is constrained by its amino and carboxyl termini. *Mol Microbiol* 51: 1205–1217. <https://doi.org/10.1046/j.1365-2958.2003.03905.x>.
 - Roggiani M, Yadavalli SS, Goulian M. 2017. Natural variation of a sensor kinase controlling a conserved stress response pathway in *Escherichia coli*. *PLoS Genet* 13:1007101. <https://doi.org/10.1371/journal.pgen.1007101>.
 - Li X, Gu Y, Dong H, Wang W, Dong C. 2015. Trapped lipopolysaccharide and LptD intermediates reveal lipopolysaccharide translocation steps across the *Escherichia coli* outer membrane. *Sci Rep* 5:11883. <https://doi.org/10.1038/srep11883>.
 - Lim SP, Nikaido H. 2010. Kinetic parameters of efflux of penicillins by the multidrug efflux transporter AcrAB-TolC of *Escherichia coli*. *Antimicrob Agents Chemother* 54:1800–1806. <https://doi.org/10.1128/AAC.01714-09>.
 - Sulavik MC, Housewear C, Cramer C, Jiwani N, Murgolo N, Greene J, DiDomenico B, Shaw KJ, Miller GH, Hare R, Shimer G. 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob Agents Chemother* 45:1126–1136. <https://doi.org/10.1128/AAC.45.4.1126-1136.2001>.
 - Blount ZD, Lenski RE, Losos JB. 2018. Contingency and determinism in evolution: replaying life's tape. *Science* 362:eaam5979. <https://doi.org/10.1126/science.aam5979>.
 - Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. 2011. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* 7:e1002158. <https://doi.org/10.1371/journal.ppat.1002158>.
 - Deatherage DE, Barrick JE. 2014. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using *breseq*. *Methods Mol Biol* 1151:165–188. https://doi.org/10.1007/978-1-4939-0554-6_12.
 - Deatherage DE, Traverse CC, Wolf LN, Barrick JE. 2015. Detecting rare structural variation in evolving microbial populations from new sequence junctions using *breseq*. *Front Genet* 5:468. <https://doi.org/10.3389/fgene.2014.00468>.
 - Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:2006.0008. <https://doi.org/10.1038/msb4100050>.
 - Sharan SK, Thomason LC, Kuznetsov SG, Court DL. 2009. Recombineering: a homologous recombination-based method of genetic engineering. *Nat Protoc* 4:206–223. <https://doi.org/10.1038/nprot.2008.227>.
 - Thomason LC, Sawitzke JA, Li X, Costantino N, Court DL. 2014. Recombineering: genetic engineering in bacteria using homologous recombination. *Curr Protoc Mol Biol* 106:1.16.1–1.16.39. <https://doi.org/10.1002/0471142727.mb0116s106>.