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Effects of *emrAB* and *mprA* mutants on relative fitness in carbonyl cyanide m-chlorophenylhydrazone evolved *Escherichia coli*

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Abstract

Escherichia coli was evolved for 1,000 generations in the presence of the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Half of the 48 populations were evolved at in a low pH condition (6.5) and the other half were evolved in a high pH condition (8.0). After 1,000 generations all populations could survive in 150uM CCCP, an increase from a starting concentration of 20uM for the low pH and 50uM for the high pH.

Selected populations from this evolution were sequenced using Illumina MiSeq next generation sequencing. The sequences were referenced against the ancestral genome. The mutations were then evaluated using the computational pipeline breseq.The most prevalent mutations by far were found in the *mprA*, *emrA* and *emrB* multidrug efflux complex. The *emrA* and *emrB* genes form a multidrug efflux pump and *mprA* codes for a repressor of the *emrAB* genes.All evolved strains except for one, B11-1, had one or more mutations in the *emrAB* operon. Although we knew the locations and prevalence of these mutants we couldn’t be sure what the mutations in the *emrAB* and *mprA* efflux complex were responsible for in terms of growth difference.Growth curve analyses were run to determine the growth effects of the removal of the *emrA* gene.In concentrations of CCCP that the ancestor could survive in (40uM CCCP for low pH and 60uM CCCP for high pH) it was found that the high pH strains were equally fit with or without an intact *emrA* gene (Figure 1A). In low pH strains, those with *emrA* present performed better than those with emrA removed, but even the *emrA* knockouts did better than the ancestor indicating mutations outside of *emrA* that are of importance to low pH survival. When low pH strains were grown in high pH all *emrA* knockouts grew as poorly as the ancestor except for A3-1 (Figure 1B).When high pH strains were grown in low pH all strains, with or without *emrA*, died except for D11-1. These findings suggest that mutations outside of the *emrAB* operon are conferring fitness to strains.

When *mprA* was removed from the ancestral strain, the growth differences are minimal in the high pH condition, but in low pH D13C with no *mprA* gene could survive in levels of CCCP beyond that which the ancestral strain with *mprA* in tact could (Figure 2). This leads us to believe that the *emrAB* operon is much more important to strains facing the stress of low pH than those at high pH.

Introduction

Escherichia coli are a unique specimen for study because of their position in the human body and the many stresses they face while moving through the gastrointestinal tract. The pH stress in particular allows for advantageous mutations to become prevelant in the populations that survive pH stress as low as is found in our bodies. *E. coli’s* pH stress mitigation finds its roots in the ability of the bacteria to expel protons through the cell membrane to maintain a survivable internal pH [2]. The difference between the internal pH of the bacteria and the external pH of the environment allows for an energetic potential to be formed which can be harnessed by the *E. coli* to synthesize energy. This potential is known as proton motive force or PMF, and it is key to survival of *E. coli* in varying pHs [3].

Our choosing of CCCP as the stressor molecule in this evolution relies on the fact that uncouplers by nature break down the PMF and create an energy stress for the *E. coli* in the face of pH stress. CCCP accomplishes this by posessing a hydrophobic region that can pass through the cell membrane whether it is protonated or not [4]. This allows the internal pH of the cell to be unmaintainable as the CCCP shuttles protons into the cell at an unregulated rate. This pressure over the evolution allowed for interesting mutations to sustain certain populations in the face of the uncoupler.

Methods

Evolution: 24 populations of *E. coli* K-12 W3110 were grown in medium buffered at pH 6.5, and 24 populations were grown at pH 8.0 for 1000 generations. These 48 populations were cultured to stationary phase, then were diluted 1:100 daily into a new mircowell plate containting concentrations of CCCP that increased over the course of the evolution (20uM -150uM).

Transduction: Bacteriophage was allowed to infect a Keio strain with a kanamycin deletion insertion at emrAB. The lysate this formed was then added to the chosen strains which then gained the kanR deletion at emrAB gene.The same methods were used for mprA knockout.

Growth Curves: Sterile 96-well plates were inoculated with 200uL of LBK 100mM PIPES or TAPS (pH 6.5 and 8.0, respectively), CCCP , and 1uL of the strain being tested. Od values were taken every 15 minutes for 22 hours. Growth was read kinetically in a SpectraMax Spectrophotometer.

Mutations Relevant to the multidrug efflux system composed of the genes *emrAB* and *mprA*

		A1-1	A3-1	G5-1	G7-1	F9-1	B11-1	D11-1			
position	mutation	C01	C09	C23	C31	C38	C42	C44	annotation	gene	description
2,809,388	G→A		■						intergenic (+53/‑38)	ygaH → / → mprA	inner membrane protein/DNA‑binding transcriptional regulator
2,809,535	(CATGCA)2→3				■	■			coding (110/531 nt)	mprA →	DNA‑binding transcriptional regulator
2,809,616	T→G							■	L64R (CTG→CGG)	mprA →	DNA‑binding transcriptional regulator
2,809,751	A→T	■							D109V (GAT→GTT)	mprA →	DNA‑binding transcriptional regulator
2,809,752	T→G			■					D109E (GAT→GAG)	mprA →	DNA‑binding transcriptional regulator
2,809,761	C→A		■						C112* (TGC→TGA)	mprA →	DNA‑binding transcriptional regulator
2,810,026	C→T				■	■			intergenic (+70/‑57)	mprA → / → emrA	DNA‑binding transcriptional regulator/multidrug efflux system
2,810,153	T→A					■			L24H (CTC→CAC)	emrA →	multidrug efflux system
2,812,161	A→T	■							E297V (GAG→GTG)	emrB →	multidrug efflux system protein
2,812,341	C→T				■				P357L (CCA→CTA)	emrB →	multidrug efflux system protein
2,812,502	T→G				■				S411A (TCT→GCT)	emrB →	multidrug efflux system protein

Table 1. Mutations in the *emrAB* and *mprA* multidrug efflux pump in CCCP evolved strains of *E. coli.* Whole genome sequences were matched to the *E. coli* K-12 W3110 reference genome and mutations were uncovered using the computational pipeline *breseq.* This mutation chart was compiled by Preston Basting.

emrA mutants display different fitness than the ancestor in the presence of CCCP

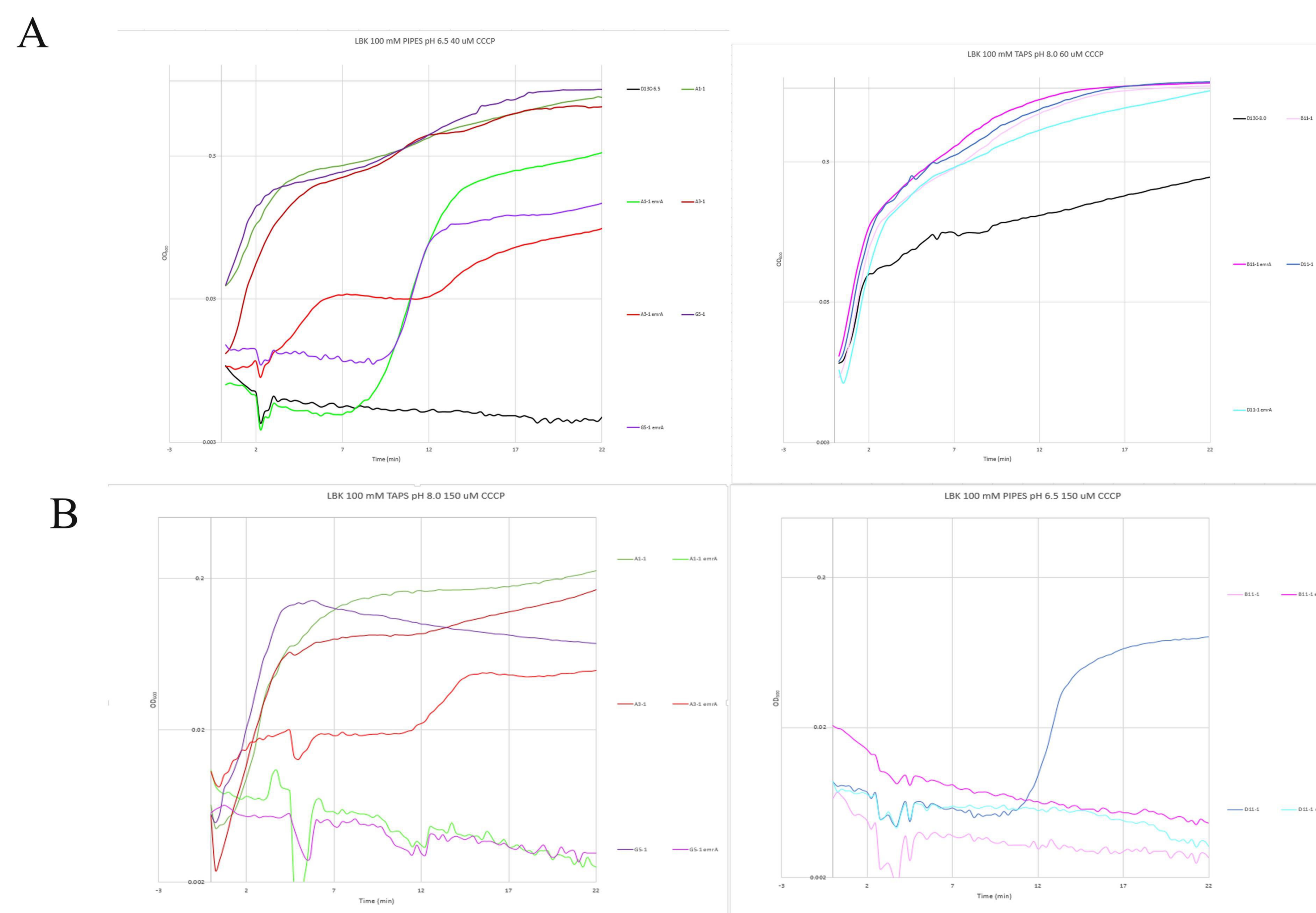

Figure 1. A) The effect of the removal of the emrA gene from low and high pH strains in 40uM and 60uM CCCP respectively (F=34.91, p=2e^-16). The label *emrA* next to a strain name denotes the removed gene. B) The effect of pH inversion on *emrA* mutants and knockouts (F=6.102, p=4.56e^-7). All strains were evolved in CCCP and compared to the ancestor W3110D13 (shown in black). Curves shown are representative of the 8 replicates for each strain. Endpoint data from t=16 hours analyzed. (ANOVA w/Tukey n=8 for each strain.)

Growth variance in *mprA* knockouts under CCCP stress

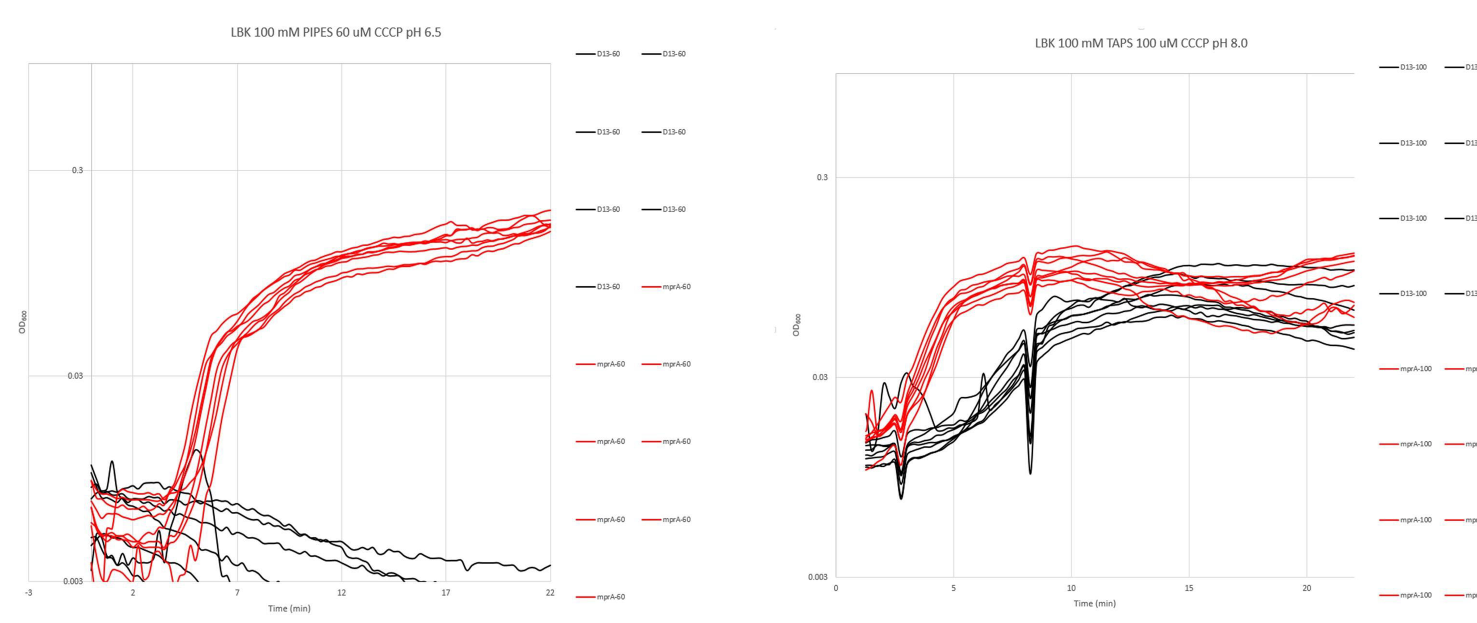

Figure 2. Differential growth between *mprA* knockouts and the ancestral W3110D13 strain with *mprA* gene in tact. (F=7.24, p=2.36e^-7). All replicatesare shown. Endpoint data taken from t=16 hours. (ANOVA w/ Tukey n=8 for each strain.

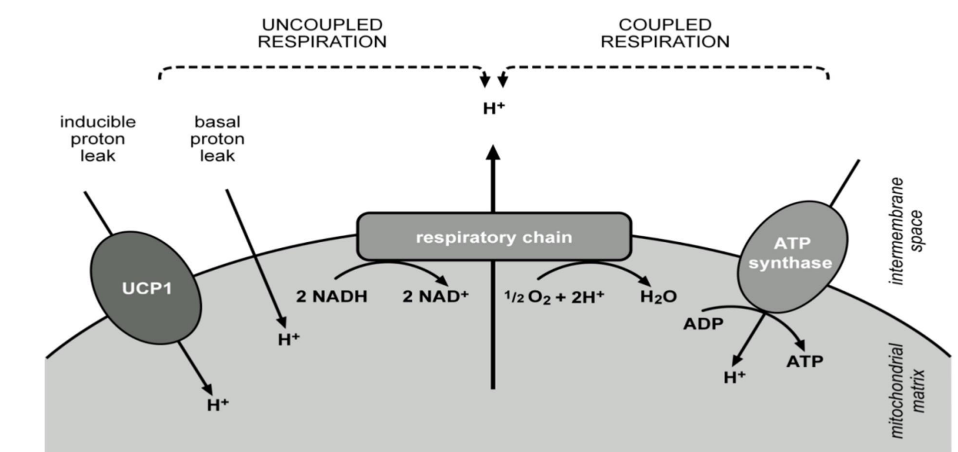

Figure 3. A schematic diagramming the system that CCCP uses to break down PMF. Source: http://keywordsuggest.org/gallery/786803.html

Conclusions

In low pH strains it is apparent that the *emrAB* operon is necessary to the survivability of those populations. Figure 2 As shown in Figure 1A and 1B, the low pH evolved strains with *emrA* removed could not survive as well as those strains with the mutated *emrA* gene in tact. This is true for both their evolved pH (6.5) and the inverse pH growth curves (8.0).An important finding in Figure 1A is that the *emrA* knockouts grow significantly better than the ancestor, W3110D13. This suggests that there are mutations outside of the *emrAB* complex that are necessary to the fitness of the populations. The *emrAB* complex appears to be more important to specimen combatting a low pH stress than those in a high pH environment. Out of seven strains of major interest to the post-evolution CCCP project, only one did not possess any mutation to *emrAB* or *mprA* and that strain (B11-1) was grown in a high pH environment.Furthermore, as displayed in Figure 1A, the high pH strains grew as well with or without an *emrA* gene present. Figure 2 supports this claim because the *mprA* knockouts in low pH have much greater fitness than the ancestor, whereas for the high pH strains the difference is not nearly as drastic. For further research it will be important to address key mutations that are unique to high pH strains to discover the mechanism by which they are surviving the stress of CCCP without the *emrAB* complex.

In future research, these strains, through recombineering, a method of gene editing using red lambda phage and genetic homologies[1], will be engineered to possess complete, ancestral forms of the entire *emrAB* operon. This will allow for us to determine what growth differences *emrAB* and *mprA* are accountable for and move forward with other novel mutations that could be responsible for other discrepancies.

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