

Supplemental Information For:

Cytochrome *bd* promotes *Escherichia coli* biofilm antibiotic tolerance by regulating accumulation of noxious chemicals

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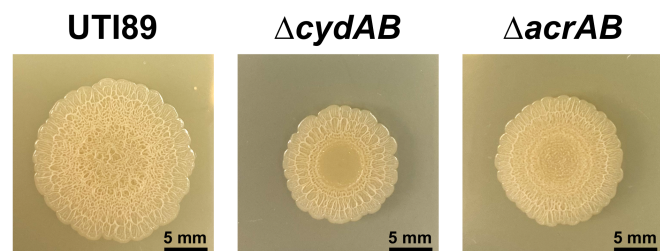
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Antibiotic Name	Antibiotic Class	Mechanism of Action	Breakpoint (mm)	UTI89 (mm)	S/R	$\Delta cydAB$ (mm)	S/R
meropenem	carbapenem	cell wall inhibitor	16	33.2	S	36.3	S
cefazolin	cephalosporin	cell wall inhibitor	15	26.0	S	28.5	S
cefepime	cephalosporin	cell wall inhibitor	18	38.6	S	41.5	S
ceftazidime	cephalosporin	cell wall inhibitor	21	32.0	S	36.2	S
ceftriaxone	cephalosporin	cell wall inhibitor	23	36.6	S	38.7	S
aztreonam	monobactam	cell wall inhibitor	21	36.7	S	41.2	S
ampicillin	penicillin	cell wall inhibitor	17	21.6	S	22.7	S
ampicillin-sulbactam	penicillin- β lactamase inhibitor	cell wall inhibitor	15	24.7	S	26.2	S
amoxicillin-clavulanic acid	penicillin- β lactamase inhibitor	cell wall inhibitor	18	21.2	S	22.7	S
piperacillin-tazobactam	penicillin- β lactamase inhibitor	cell wall inhibitor	21	30.6	S	31.5	S
sulfamethoxazole-trimethoprim	sulfonamide-dihydrofolate reductase inhibitor	folate biosynthesis inhibitor	16	30.3	S	32.5	S
nitrofurantoin	nitrofuran	reactive cellular damage	17	23.2	S	25.7	S
ciprofloxacin	fluoroquinolone	topoisomerase inhibitor	21	39.6	S	42.0	S
levofloxacin	fluoroquinolone	topoisomerase inhibitor	17	37.0	S	38.2	S
amikacin	aminoglycoside	translation inhibitor	17	24.6	S	24.7	S
gentamicin	aminoglycoside	translation inhibitor	15	25.6	S	26.2	S
tobramycin	aminoglycoside	translation inhibitor	15	24.3	S	24.7	S
tetracycline	tetracycline	translation inhibitor	15	24.3	S	26.5	S

Supplementary Table 1: Disk Diffusion Assays. Disk diffusion assays were performed to measure antibiotic sensitivity in UTI89 and $\Delta cydAB$. CLSI breakpoints were used to determine sensitivity versus resistance to each antibiotic. Reported zone of inhibition is the average of at least three biological replicates.

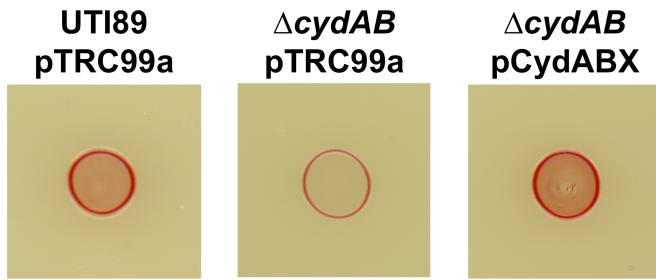
	A	B	C	D	E	F	G	H
	UniProt ID	Protein Name	Gene Name	Subcellular Location	Fold Change UTI89 vs Δ cydAB	Log2 (Fold Change)	P Value	Log10 (P Value)
2	trjQ1R227 Q1R227_ECOUT	Putative exported protein	UTI89_P011	extracellular space	0.125525147	-2.993951676	0.00337648	2.471535568
3	trjQ1R8P4 Q1R8P4_ECOUT	Beta-barrel assembly-enhancing protease	yfgC	periplasm, membrane	0.148786374	-2.748685687	2.2852E-05	4.641081337
4	trjQ1R049 Q1R049_ECOUT	Penicillin-binding protein activator	lpoB	outer membrane	0.163411049	-2.613422558	0.00157157	2.803666525
5	trjQ1R968 Q1R968_ECOUT	Bifunctional long-chain fatty acids transporter	fadL	outer membrane	0.239537481	-2.061676679	9.7553E-06	5.010759345
6	trjQ1R62 Q1R62_ECOUT	Arginine-binding periplasmic protein 1	arti	periplasm, membrane	0.266055743	-1.910199551	1.4827E-05	4.828935705
7	trjQ1R6J1 Q1R6J1_ECOUT	Putative periplasmic protein	yraP	periplasm, outer membrane	0.278227955	-1.845660714	1.3626E-05	4.865617625
8	trjQ1RCH8 Q1RCH8_ECOUT	Outer membrane protein W	ompW	outer membrane	0.295345369	-1.759525104	0.0052803	2.277341724
9	trjQ1R3A8 Q1R3A8_ECOUT	outer membrane lipoprotein B1c	b1c	outer membrane, cytosol	0.312269546	-1.679136215	0.00018313	3.373233729
10	trjQ1R8T9 Q1R8T9_ECOUT	uncharacterized lipoprotein	yfeY	outer membrane	0.328622813	-1.605495458	1.9771E-05	4.703980148
11	trjQ1R208 Q1R208_ECOUT	enterotoxin TieB	senB	extracellular space	0.362473728	-1.464051661	0.00073195	3.135517179
12	trjQ1R5B1 Q1R5B1_ECOUT	Starvation induced outer membrane protein	slp	outer membrane	0.379379633	-1.398285865	0.00126921	2.896456992
13	trjQ1RF22 Q1RF22_ECOUT	Outer membrane lipoprotein	rcsF	outer membrane	0.380701995	-1.393265964	0.00255894	2.591939804
14	trjQ1R1Q3 Q1R1Q3_ECOUT	TraT complement resistance protein	traT	outer membrane	0.424135876	-1.237401574	0.00293356	2.532605306
15	trjQ1RD74 Q1RD74_ECOUT	Flagellar hook-associated protein 3	flgL	outer membrane	0.432483284	-1.209283722	0.00580643	2.236090876
16	trjQ1R8M1 Q1R8M1_ECOUT	Outer membrane protein assembly factor BamB	yfgL	outer membrane	0.436351795	-1.196436363	0.00876317	2.057338657
17	trjQ1REY0 Q1REY0_ECOUT	Femterioerobactin receptor	fbpA	outer membrane	0.445534911	-1.166389611	0.05778582	1.238178746
18	sp Q1RGE3 LPTD_ECOUT	LPS-assembly protein	lptD	outer membrane	0.476631728	-1.069053105	0.03527219	1.452567577
19	trjQ1R5T2 Q1R5T2_ECOUT	Glucanase	yjhM	extracellular space	0.486676187	-1.03896591	0.0110896	1.955083959
20	trjQ1RBF4 Q1RBF4_ECOUT	Outer membrane lipoprotein	slxB	outer membrane	0.492971306	-1.020424419	0.00957844	2.018705394
21	trjQ1RCP9 Q1RCP9_ECOUT	Putative outer membrane receptor	prnA	outer membrane	0.498638965	-1.003932473	0.00319788	2.495137683
22	trjQ1RDS8 Q1RDS8_ECOUT	Outer membrane protein F	ompF	outer membrane	0.514037016	-0.960055844	0.00429467	2.367070229
23	trjQ1R7V0 Q1R7V0_ECOUT	Lipoprotein	nlpD	outer membrane	0.516877617	-0.952105365	0.02515595	1.599359224
24	trjQ1REB0 Q1REB0_ECOUT	Outer membrane protein X	ompX	outer membrane	0.533727755	-0.905824059	0.0125733	1.900550698
25	sp Q1R3U1 BTUB_ECOUT	Vitamin B12 transporter	btuB	outer membrane	0.579775036	-0.786434879	0.01323518	1.872701114
26	trjQ1RES9 Q1RES9_ECOUT	Endolytic peptidoglycan transglycosylase	ripA	outer membrane	0.581569816	-0.781975701	0.04173797	1.379468645
27	trjQ1RCY1 Q1RCY1_ECOUT;trjQ1RAK8 Q1RAK8_ECOUT	Outer membrane porin protein	nmpC	outer membrane	0.623544333	-0.681435958	0.02540088	1.595151319
28	trjQ1RS73 Q1RS73_ECOUT	Cellulose synthase operon protein C	bcsC	outer membrane	0.627095163	-0.673243703	0.13242154	0.878041367
29	trjQ1REF8 Q1REF8_ECOUT	Pectinesterase	ybhC	outer membrane	0.637639149	-0.649187886	0.01691269	1.771787222
30	trjQ1R9K0 Q1R9K0_ECOUT;trjQ1RC18 Q1RC18_ECOUT	Outer membrane protein 1b	ompC	outer membrane	0.638605134	-0.647003943	0.03768109	1.423876549
31	trjQ1R6U8 Q1R6U8_ECOUT	Outer membrane protein	toIC	outer membrane	0.643739109	-0.635451977	0.0269808	1.57383837
32	trjQ1RAF5 Q1RAF5_ECOUT	Putative pectin receptor	flyA	outer membrane	0.670084888	-0.577584223	0.11315881	0.946311633
33	trjQ1RAL6 Q1RAL6_ECOUT	Flagellar hook-associated protein 2	flhD	outer membrane	0.675164748	-0.566688516	0.04052614	1.392264766
34	trjQ1REI3 Q1REI3_ECOUT	Peptidoglycan-associated protein	pal	outer membrane	0.696262048	-0.522297707	0.04713933	1.32661659
35	trjQ1RD80 Q1RD80_ECOUT	Flagellar basal body protein	flgF	outer membrane	0.700667349	-0.513198427	0.04105226	1.388662971
36	trjQ1RD75 Q1RD75_ECOUT	Flagellar hook-associated protein 1	flgK	outer membrane	0.704544719	-0.505236814	0.03292098	1.482527244
37	trjQ1R8Q0 Q1R8Q0_ECOUT	Outer membrane protein assembly factor BamC	nlpB	outer membrane	0.708268662	-0.497653184	0.04341877	1.362322534
38	trjQ1R2T5 Q1R2T5_ECOUT	Hemolysin A	hlyA	extracellular space	0.723240021	-0.467453583	0.14764304	0.830787033
39	trjQ1R8N7 Q1R8N7_ECOUT	Polyphosphate kinase	ppk	outer membrane, inner membrane	0.73509184	-0.444003588	0.18628135	0.729830624
40	trjQ1RDQ7 Q1RDQ7_ECOUT	Outer membrane protein A	ompA	outer membrane	0.801838419	-0.31861655	0.3514231	0.454169693
41	trjQ1R3B9 Q1R3B9_ECOUT	Putative toxin of osmotically regulated toxin-antitoxin system	ecnB	outer membrane, inner membrane	0.864984147	-0.209254402	0.79934703	0.097264636
42	trjQ1R8B9 Q1R8B9_ECOUT	Murein lipoprotein	lpp	outer membrane, inner membrane, periplasm	0.875936704	-0.191101472	0.63511162	0.197149939
43	trjQ1RDC4 Q1RDC4_ECOUT	Curl production assembly/transport component	csqG	outer membrane, inner membrane, periplasm	0.891002758	-0.166498197	0.66324838	1.78323803
44	sp Q1RG12 BAMA_ECOUT	Outer membrane protein assembly factor	bamA	outer membrane	0.891893486	-0.165056667	0.42241878	0.37425678
45	trjQ1RBD1 Q1RBD1_ECOUT	Pertactin domain-containing protein	ydhQ	outer membrane	0.912285994	-0.132441927	0.69261387	1.59508816
46	trjQ1R5W6 Q1R5W6_ECOUT	DNA-binding protein HU-alpha	hupA	extracellular space, cytosol	0.966763547	-0.04876502	0.92445578	0.034113857
47	trjQ1R386 Q1R386_ECOUT	Protein HflK	hflK	periplasm, inner membrane, cytosol	0.977974497	-0.032131251	0.91034821	0.04079246
48	sp Q1R7R4 ENO_ECOUT	enolase	eno	extracellular space, cytosol	1.010975792	0.015748452	0.94221423	0.025850341
49	trjQ1REI6 Q1REI6_ECOUT	Membrane spanning protein	tolA	outer membrane, inner membrane	1.107158163	0.146861333	0.65309655	1.85022611
50	sp Q1R6U6 YGI8_ECOUT	UPF0441 protein	ygiB	outer membrane	1.148782818	0.200106077	0.40380723	0.393825907
51	trjQ1R2K0 Q1R2K0_ECOUT	Type 1 fimbriae major subunit FimA	fimA	outer membrane	1.217698428	0.284156883	0.50750697	0.294557987
52	trjQ1R8L2 Q1R8L2_ECOUT	Alpha-2-macroglobulin	yfhM	extracellular space	1.245572315	0.316808784	0.3205	0.494171967
53	trjQ1RD79 Q1RD79_ECOUT	Flagellar basal-body rod protein	flgG	outer membrane	1.304408614	0.383395873	0.32475714	0.488441298
54	sp Q1R3Q0 LAM8_ECOUT	Maltoporin	lamB	outer membrane	1.366544176	0.450532098	0.12537694	0.901782342
55	trjQ1RD81 Q1RD81_ECOUT	Flagellar hook protein	flgE	outer membrane	1.510549001	0.595072985	0.13068864	0.883762158
56	trjQ1RF95 Q1RF95_ECOUT	DNA-binding protein HU-beta, NS1 (HU-1)	hupB	extracellular space, cytosol	1.587862307	0.667085813	0.10083737	0.996378469
57	trjQ1R2V4 Q1R2V4_ECOUT	Putative F17-like fimbrial subunit	UTI89_C4907	outer membrane	1.73218426	0.792592404	0.09736117	1.011614237
58	trjQ1RAL7 Q1RAL7_ECOUT	Flagellin	flc	outer membrane	1.970314662	0.978426049	0.03304911	1.480840229
59	trjQ1RDB7 Q1RDB7_ECOUT	Curlin major subunit	csqA	outer membrane	6.964677933	2.80005664	0.06761867	1.169933348
60	trjQ1RDB8 Q1RDB8_ECOUT	Minor curlin subunit	csqB	outer membrane	9.63756597	2.68666883	0.02463106	1.608516969

Supplementary Table 2: Mass spectrometry data. Table lists all detected outer membrane or secreted proteins detected by mass spectrometry, fold change in UTI89 relative to Δ cydAB, and *p* value. Data are representative of three biological replicates.

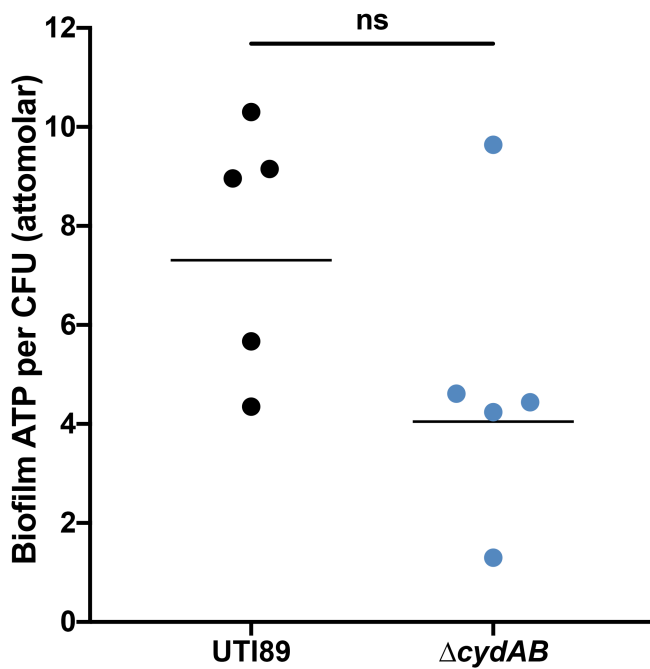


Supplementary Figure 1: AcrAB inactivation has minor effects on biofilm development.

Representative images of UTI89, Δ cydAB, and Δ acrAB biofilms grown on YESCA agar for 11 days. Data are representative of at least five biological replicates.



Supplementary Figure 2: Complementation restores TTC reduction in $\Delta cydAB$. Representative images of TTC reduction assays. Red color indicates respiratory activity. Strains were transformed with pTRC99a (empty vector) or pCydABX (*cydABX* operon under native transcriptional control). Data are representative of four biological replicates per strain.



Supplementary Figure 3: Loss of cytochrome *bd* does not impair ATP generation. Graph depicting ATP concentration in colony biofilms normalized to CFUs in the same biofilm. Line depicts geometric mean. Each dot represents a biological replicate. Data were analyzed by a Mann-Whitney test.

Primer	Sequence (5' → 3')	Purpose
acrAB_KO_Fwd	TCAATGATGATCGACAGTATGGCTGTG CTCGATATCTTCATTCTTGCGGCGTGTA GGCTGGAGCTGCTTC	<i>acrAB</i> deletion
acrAB_KO_Rev	ATGCCCCGCCGTTGGCGTAGTAACAGTC AAAACCTGAACCTCTGCAGATCACCATAT GAATATCCTCCTTAG	<i>acrAB</i> deletion
acrAB_KOtest_Fwd	TGGTTCAATACTCCTTAATGTTTCGTAG	<i>acrAB</i> deletion
acrAB_KOtest_Rev	GGCGGTCGTTCTGATGC	<i>acrAB</i> deletion
acrB_qPCR_Fwd	GTGTAGTGGTGCGTGCTCT	qPCR
acrB_qPCR_Rev	TATCGTCAGTTCTCCATTACCATTGT	qPCR
ompW_qPCR_Fwd	TATGGCGACCGACAACATTGG	qPCR
ompW_qPCR_Rev	CGTAAGGACGGAATTTGCTGC	qPCR
ompF_qPCR_Fwd	CGTTAGAGCGGCGTGTC	qPCR
ompF_qPCR_Rev	CACTGGGTTACACCGATATGCTG	qPCR
ompX_qPCR_Fwd	ACTGGCGGTTACGCACA	qPCR
ompX_qPCR_Rev	CGGACCAGCAGTGATGCC	qPCR

Supplementary Table 3: Primers used in this study.