Oxygen Shapes the Evolutionary Trajectories of Antimicrobial Resistance

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Antibiotics are a critical element of modern health care that is threatened by the microbes' natural propensity to evolve resistance. Experimental evolution of antimicrobial resistance (AMR) is a powerful *in vitro* approach that enables precise control over culture conditions and direct observation of mutational trajectories towards resistance. However, experimental evolution studies have historically overlooked the ecological context of AMR evolution in the natural world; many of the environments in which microbes are naturally selected by antibiotics are oxygen-limited. Notably, the microbially diverse human gut is largely anaerobic and can serve as both a site of selection and reservoir for antibiotic-resistant pathogens. To systematically address this gap in understanding of how oxygen impacts the *de novo* evolution of AMR, we experimentally evolved clinical isolate strains of *E. coli* towards resistance in either aerobic or anaerobic conditions. Results suggest that the microbial environment can constrain and modulate evolutionary trajectories to resistance due to its effects on the mutational landscapes of key antibiotic resistance genes. By unraveling the eco-evolutionary principles that underly antimicrobial resistance, we stand to discover novel strategies and targets to ultimately control the lethal emergence of AMR.

Microbial pathogens are frequently subject to antibiotic selective pressure in oxygen-limited environments.

How does oxygen affect the de novo evolution of antimicrobial resistance in clinical isolate strains of Escherichia coli?

Results:

Strains represent a range of phenotypic and genotypic diversity

Strain Name	Classification	Ciprofloxacin		Gentamicin		Tetracycline		Cefoxitin	
		O2	NO	O2	NO	O2	NO	O2	NO
K12	Laboratory								
BDM	Commensal								
E09	Commensal								
E11	Pathogenic								
EDL	Pathogenic								
I08	Commensal								
I14	Commensal								
I39	Pathogenic								
I48	Pathogenic								
N38	Pathogenic								
NIS	Commensal								
Z36	Commensal								

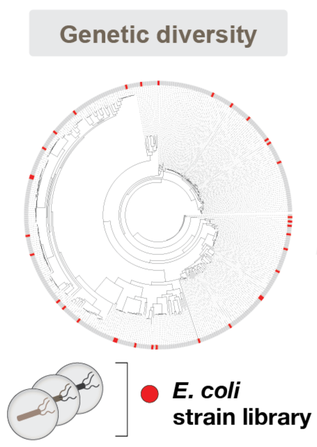

Figure 2: (A, Left) Resistance Profiles of Selected 12 Strain Panel. Minimum inhibitory concentrations demonstrate diversity of phenotypic diversity across panel. **(B, Right) Phylogenetic Tree of Expansive *E. coli* Strain Collection.** Red marks indicate the broader collection from which strain panel was chosen, demonstrating genetic diversity within panel.

Strain-level variation observed in acquisition of resistance

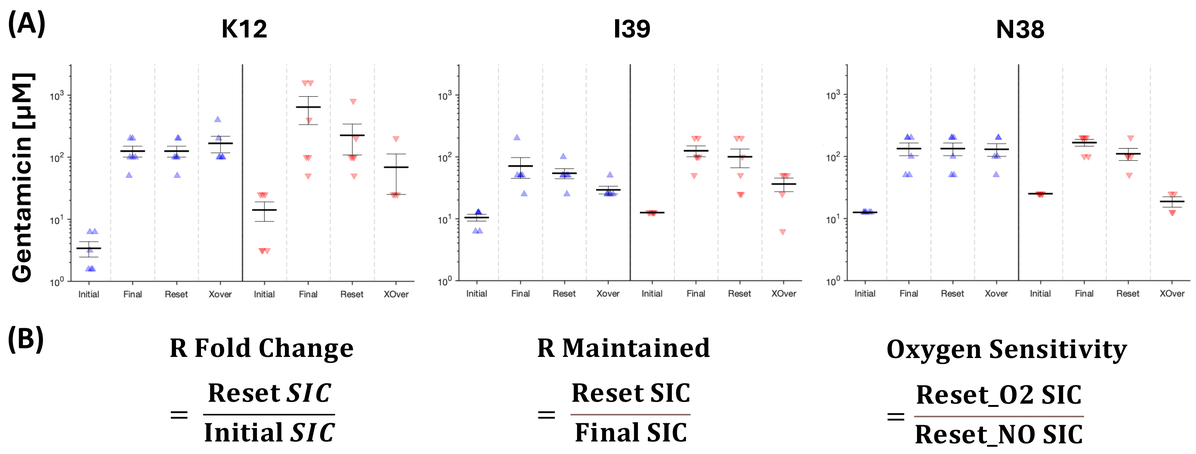

Figure 3: (A, Top) Discrete SIC Measurements for Representative Strains Evolved to Ciprofloxacin Resistance. SIC measurements shown for O2-Evolved (blue) and NO-Evolved (red) for Initial (day 1), Final (day 10), Reset, and Crossover challenge conditions. Y-scale log10-axis. **(B, Bottom) Metrics for the Calculation of Resistance Fold-Change, Resistance Maintained, and Oxygen Sensitivity of Acquired Resistance.** Equations compare discrete SIC points within each evolved lineage.

Oxygen accelerates resistance evolution for certain antibiotics

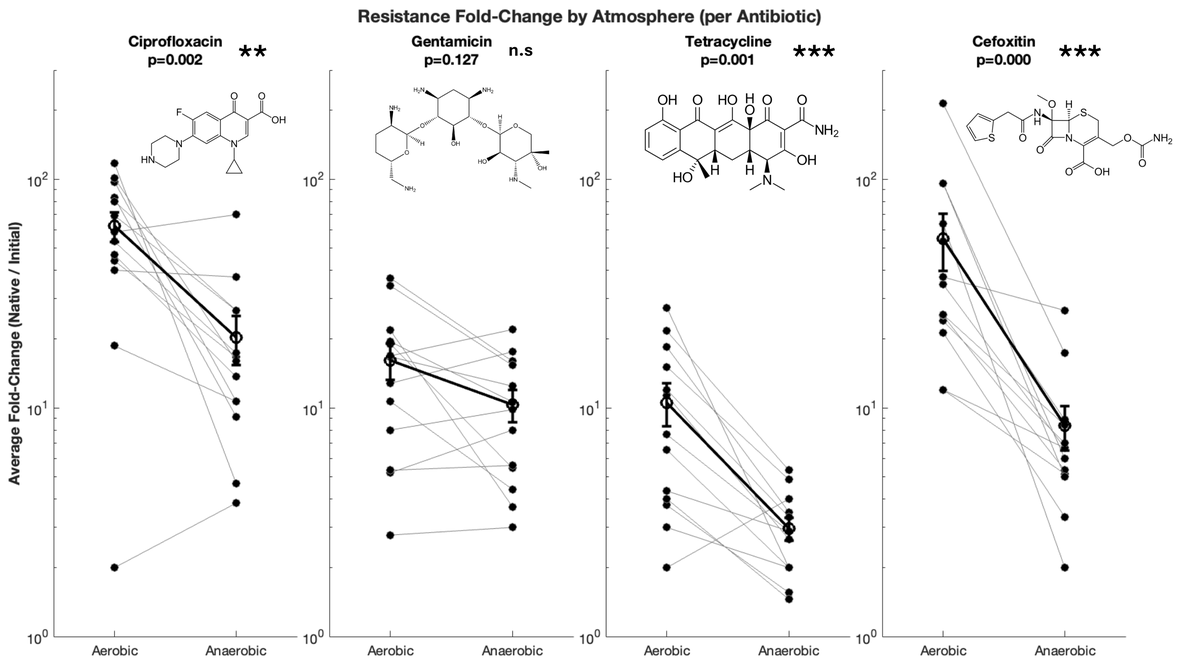

Figure 4: Oxygen availability alters antibiotic resistance outcomes in evolved populations. Paired dot plot of average fold-change (Reset/Initial SIC) per strain (12 strains, 6 replicate lineages per ABX x strain x atm.), with thick black line and error bar (total mean ± SEM). Y-scale log10 axis. Wilcoxon signed-rank test for statistical testing (**: p-val < 0.01; ***: p-val < 0.001)

Average fold-change in phenotypic resistance is **significantly higher in aerobic** conditions for ciprofloxacin, tetracycline, and cefoxitin, but not gentamicin.

Experimental Evolution Approach:

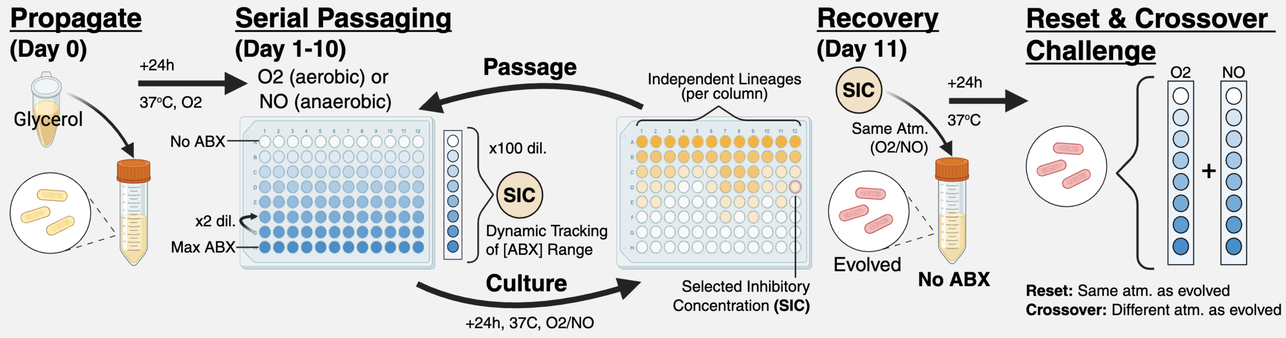

Figure 1: Schematic of Dynamic Serial Passaging Protocol. Selected Inhibitory Concentrations (SIC) cultures – highest [ABX] with positive microbial growth – were serially passaged for 10 days across dynamically tuned antibiotic gradients, which tracked the increasing resistance of independent lineages. Evolved cultures were recovered in no ABX conditions, and then susceptibility tested in both reset and crossover conditions.

Resistance is often specific to the atmosphere it was selected in

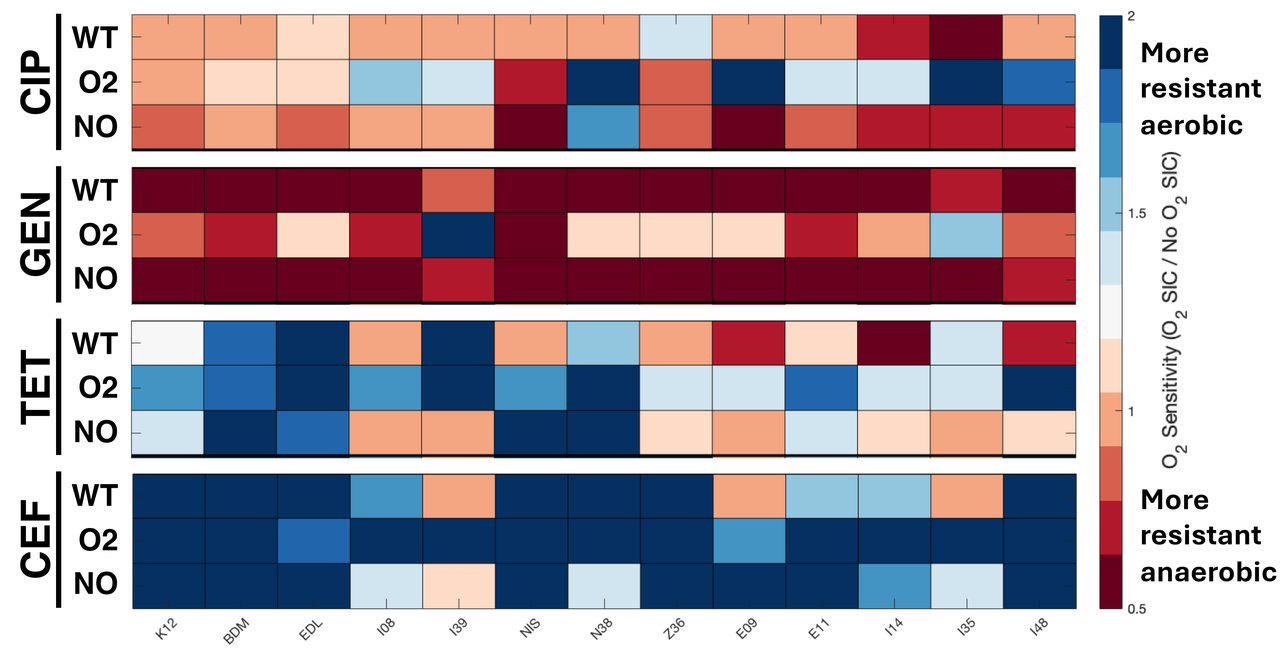

Figure 5: Heatmap of Oxygen Sensitivity in Reset Evolved Populations. Heatmap displays the oxygen sensitivity of resistance for each strain-antibiotic combination (average of 6 replicate lineages), calculated as the SIC ratio of Reset SIC in aerobic and anaerobic conditions (Reset_O2/Reset_NO SIC) following evolution in either the presence (O2) or absence (NO) of oxygen. Blue indicates higher resistance when tested aerobically, while red indicates higher resistance when tested anaerobically.

Atmosphere directly affects the mutational landscapes of resistance

GENTAMICIN	fusA	sbmA	atpD	cpxA	atpA	cydA	cydB	hemB	hscA	fdnG	atpB	rplF	yaiW	ubiB	hemL	ubiD	TOTAL SUM
SUM:	62	44	21	18	17	17	7	6	5	5	5	4	4	4	4	4	940
O2 SUM:	39	24	6	12	1	16	6	3	1	0	1	0	2	2	1	4	328
NO O2 SUM:	23	20	15	6	16	1	1	3	4	5	4	4	2	2	3	0	612
O2/NO Ratio:	1.7	1.2	0.4	2.0	0.1	16	6	1.0	0.3	NO	0.3	NO	1.0	1.0	0.3	O2	0.54

TETRACYCLINE	marR	lon	decR	robA	acrR	clpX	acrB	rpoB	cof	marC	rpoC	mlaA	TOTAL SUM
SUM:	34	31	15	13	12	11	6	6	5	5	4	4	1531
O2 SUM:	22	23	13	6	7	7	1	1	5	3	2	1	661
NO O2 SUM:	12	8	2	7	5	4	5	5	0	2	2	3	870
O2/NO Ratio:	1.8	2.9	6.5	0.9	1.4	1.8	0.2	0.2	O2	1.5	1.0	0.3	0.76

CIPROFLOXACIN	marR	gyrA	acrR	gyrB	soxR	aroK	mnmE	marC	rpoC	robA	guaA	rpoB	dinG	trnQ	deaD	TOTAL SUM
SUM:	56	54	44	35	19	11	9	8	8	7	6	6	6	4	4	629
O2 SUM:	30	32	25	25	10	6	6	5	3	0	3	2	4	4	2	358
NO O2 SUM:	26	22	19	10	9	5	3	3	5	7	3	4	2	0	2	271
O2/NO Ratio:	1.2	1.5	1.3	2.5	1.1	1.2	2.0	1.7	0.6	NO	1.0	0.5	2.0	O2	1.0	1.32

CEFOXITIN	ompC	marR	acrB	envZ	lon	ftsI	acrR	micF	galU	rrrD	essD	ydaT	clpX	frdD	marC	slyA	rseA	waaO	TOTAL SUM
SUM:	51	44	27	21	17	10	9	9	8	6	6	6	5	5	5	5	5	5	911
O2 SUM:	36	32	22	16	12	9	9	5	3	5	5	5	4	5	5	4	4	5	735
NO O2 SUM:	15	12	5	5	5	1	0	4	5	1	1	1	1	0	0	1	1	0	176
O2/NO Ratio:	2.4	2.6667	4.4	3.2	2.4	9	O2	1.25	0.6	5	5	5	4	O2	O2	4	4	O2	4.18

Figure 6: Top Mutation Hit Count Table Identifies Atmosphere-Specific Resistance-Associated Genes. Mutation hits were summed across all lineages evolved in either aerobic (O2) or anaerobic (NO) conditions. A gene was scored as a binary 1 for a given lineage if it contained one or more mutations within or directly upstream of the open reading frame. Only top-ranking genes are shown (≥4 GEN, TET, CIP; ≥5 CEF). Red-green color scale of "Sum" reflects total number of independent lineages in which each gene was mutated. Subtotals for each atmospheric condition are also shown, along with the O2/NO enrichment ratio. Blue indicates aerobic enrichment and red indicates anaerobic enrichment. On the right, total hits across all genes for each antibiotic are summarized, including atmosphere-specific subtotals and ratio therein.

For gentamicin, **cytochrome bd** was predominantly mutated under aerobic conditions, and **ATP synthase** was uniquely mutated under anaerobic conditions.

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