Anaerobic conditions unmask antimicrobial resistance in *Vibrio cholerae*

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Abstract

The antibiotic formulary is threatened by high rates of antimicrobial resistance (AMR) among enteropathogens. Enteric bacteria often undergo anaerobic respiration within the gastrointestinal tract, yet little is known about how anaerobic conditions influence AMR. The facultative enteropathogen *Vibrio cholerae* was chosen as a model to address this knowledge gap. *V. cholerae* isolates were tested for resistance under aerobic and anaerobic conditions against clinically relevant antibiotics. Using conventional breakpoints established for aerobic conditions, the odds of classifying isolates as resistant increased over 10 times for ciprofloxacin and 100 times for azithromycin under anaerobic conditions compared to aerobic conditions; for doxycycline, nearly all isolates remained sensitive. Genome-wide association studies found associations between genetic elements and AMR phenotypes that varied by oxygen exposure and antibiotic concentrations; AMR phenotypes were more heritable – and more AMR genes were discovered – under anaerobic conditions. These findings challenge the paradigm of testing facultative enteropathogens for AMR under aerobic conditions alone.

INTRODUCTION

Clinically relevant laboratory methods are essential to gauge the extent to which the antibiotic formulary is threatened by antimicrobial resistance (AMR). Knowledge gaps remain on the degree to which *in vitro* AMR assays reflect *in vivo* AMR physiology. Facultative anaerobic pathogens experience hypoxia and anoxia within the gastrointestinal tract, yet AMR assays rely on aerobic conditions [1]. How oxygen exposure effects AMR is poorly understood. To investigate this question, we chose the facultative anaerobe *Vibrio cholerae* as a model system. In *V. cholerae,* mechanisms of AMR and anaerobic growth are well characterized *in vivo* and *in vitro* [2-8], and cholera is one of the few non-invasive diarrheal diseases for which antibiotics are indicated, albeit conditionally [9-11].

Rehydration is the definitive intervention for acute diarrheal disease [9]; antibiotics are supportive and indicated for only a few diarrheal diseases, including cholera. The World Health Organization (WHO) recommends ciprofloxacin, azithromycin or doxycycline for cholera patients with severe dehydration [9-11]; antibiotics shorten the frequency and duration of diarrhea. In practice, guideline adherence in cholera endemic regions may be low out of clinical concern that a patient ‘might’ have cholera and may develop severe dehydration, contributing to rates of inappropriate antibiotic usage that can rise above 90% [12, 13]. Strong regional associations between antibiotic use and rise of AMR have been observed across enteric taxa [14, 15]. Given that AMR genetic elements frequently co-localize on mobile elements [16], inappropriate single-agent therapy also poses a risk of multidrug-resistance (MDR) selection.

Associations between AMR phenotypes and genotypes are known for the three antibiotics recommended to treat cholera; the cognate AMR mechanisms share commonality across Gram negative taxa. Ciprofloxacin (a fluoroquinolone) resistance mechanisms include mutations in genes encoding type II topoisomerases: heterotetrameric DNA gyrase ($GyrA_2GyrB_2$) and DNA topoisomerase IV ($ParC_2ParE_2$). Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* can yield additive resistance phenotypes [17]. Efflux pump upregulation, downregulation of outer membrane porins that permit quinolone entry and Qnr expression that protects the target gyrase also convey fluoroquinolone resistance [17]. Resistance can increase over 30-fold compared to wild-type when strains harbor *qnr* family genes.

In *V. cholerae*, diverse AMR genes, including *qnr,* often reside on an integrative and conjugative element (ICE; 'SXT' in *V. cholerae*) [16, 18, 19]. Azithromycin (a macrolide) resistance mechanisms are similarly diverse and include mutations in the 23S ribosomal RNA (rRNA) target genes and ribosomal protein genes. Macrolide resistance is also conveyed by carriage of rRNA methyltransferase genes (*erm*) and associated induction mechanisms, *cis*-acting peptides, efflux systems (e.g. *mef, msr)*, macrolide esterases (e.g. *ere*), and macrolide phosphotransferases (e.g. *mphA); mphA* often resides in the *V. cholerae* SXT element [20]. Doxycycline (a tetracycline) resistance is conferred by mutations in the 16S rRNA component of the 30S ribosomal subunit [21]. Additional mechanisms include tetracycline-specific ribosomal protection proteins (RPPs), tetracycline specific efflux pumps (e.g. *tet(59)*) that can reside on SXT element intrinsic efflux pumps, AraC-family transcriptional activators (e.g. MarA), and cytoplasmic ATP-dependent serine proteases [21].

Associations between AMR phenotypes and genotypes have been studied by random mutagenesis, phenotypic screening, and network analyses, and applied in *V. cholerae* [22]. These approaches uncovered how the effect of an antibiotic is shaped by a large number of often more subtle physiologic perturbations, including altered DNA synthesis/repair, central metabolism/growth, and SOS response [23, 24]. A limitation of AMR assays conducted under aerobic conditions alone may not reflect these physiologic perturbations experienced in the host. Aerobic oxidative phosphorylation generates reactive oxygen species (ROS) that are lethal unless a sufficient defense is mounted by factors like superoxide dismutase, catalase, and glutathione systems [25, 26]. Under anaerobic conditions, growth rate typically slows and proton motive force is reduced [27, 28], which can have both synergistic and antagonistic effects on antibiotics [24, 29]. In *Escherichia coli,* ROS are generated after fluroquinolone treatment under aerobic conditions[30] and fluoroquinolone resistance increases under anaerobic conditions [31, 32]. The extent to which tetracyclines and macrolides induce ROS and how anaerobiosis influences resistance is less known [23, 33].

The objective of this study was to compare AMR phenotypes, with underlying genotypes, under aerobic and anaerobic conditions. We found that resistance increased significantly under anaerobic conditions for select antibiotics, and distinct genetic elements associated with AMR with and without oxygen exposure.

METHODS

Clinical sample collection. The two sample collections analyzed were part of previously published IRB approved studies [12, 34]. In the primary collection, stool

samples were obtained during the spring cholera outbreak period of 2006 at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Samples were collected prior to hospital administration of antibiotics; patient histories were negative for known antibiotic exposure. The library consisted of 67 *V. cholerae* isolates (Supplementary Table 1); paired stool supernatant for mass spectrometry was available for 50 isolates. In the secondary collection, samples were obtained in 2018 as part of a cholera surveillance study conducted across Bangladesh [12]. Samples were collected at hospital admission independent of reported antibiotic exposure; 120 isolates were analyzed to assess generalizability of the AMR profiles identified in the primary collection.

Antimicrobial resistance testing. Growth kinetics and the minimal inhibitory concentration (MIC) determinations for ciprofloxacin, azithromycin, and doxycycline were performed on isolates from the primary collection in LB broth with twelve two-fold serial dilutions with concentrations approximated for CLSI MIC breakpoints[1] for *V. cholerae* (ciprofloxacin = 2 µg/ml; azithromycin = 8 µg/ml; doxycycline = 8 µg/ml)[1]. Isolates were prepared and grown aerobically at 37˚C in 15-ml tubes containing 5-ml LB broth at 220 rpm Bacteria were back-diluted to a final OD_{600} of 0.01 (200 µl/well) in LB with or without the respective antibiotic dilution-series in black Corning CoStar clear-bottom 96-well plates. Plates were placed in a BioTek Synergy H1 reader pre-warmed to 37˚C with the lid on. Anaerobic conditions were generated using a continuous chamber flow (5% CO_2, 95% N_2) and a BioTek CO_2/O_2 gas controller; anaerobic growth plates were given a 10-minute equilibration period. OD_{600} was measured every 2 minutes for 8 hours at 37˚C with orbital shaking at 220 rpm. A standard logistic equation

was fit to growth curve data using the R package *growthcurver* version 0.3.0[35]. Outcome measures were intrinsic growth velocity (growth rate that would occur if there were no restrictions on total population size), carrying capacity (K; maximum possible population size), and area under the curve (AUC). The MIC was determined using a logistic fit for growth over the twelve, two-fold serial dilutions of the test antibiotic. Binary phenotypic resistance categories were set in concordance with Clinical and Laboratory Standards Institute (CLSI); intermediate and resistant isolates were scored as resistant. CLSI standards are based on assays conducted aerobically and break points are set by clinical correlates of disease.

Use of catalase to determine if ROS contribute to antibiotic sensitivity. To test if the reduction of ROS was associated with increased resistance to antibiotics under aerobic conditions, MICs were determined for two select strains (E7946, EN160) with/without catalase (10 U/ml) added to the media. Growth curves were performed with viable counts as endpoints to determine the minimum dose for lethality by H_2O_2 or protection by catalase.

Molecular procedures. Whole-genome sequencing was performed with conventional methods (Supplementary Material). Known resistance genes were identified using the comprehensive antibiotic resistance database (CARD) [36] and ResFinder [37]. A series of genome-wide association studies (GWAS) were conducted on the primary collection to detect genomic variants associated with antibiotic resistance phenotypes under both aerobic and anaerobic conditions; a total of 30 GWAS were generated by 3 antibiotics x 5 antibiotic concentrations x 2 oxygen conditions. The sample size for analysis was 67 genomes. GWAS bioinformatic methods are provided (Supplementary Material).

Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS). The approach was based on a former study [38]. Preparation of stool supernatants for analysis using high-performance liquid chromatography (Thermo UltiMate 3000 series) and an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific) are provided (Supplementary Material).

Statistical analysis. Bivariate analyses of categorical data were analyzed using Fisher's Exact Test, and continuous data were analyzed using the Mann-Whitney U Test (alpha = 0.05). McNemar's test was used to analyze paired data (alpha = 0.05).

RESULTS

Comparison of antimicrobial resistance under aerobic and anaerobic conditions. Pairwise analyses for baseline growth parameters found that carrying capacity, AUC, and growth velocity of the population were significantly lower under anaerobic compared to aerobic conditions (Supplementary Table 2). In this physiologic context, AMR differed depending on anaerobic versus aerobic conditions (Figure 1); distributions of single and multi-agent AMR phenotypes are shown (Figure 2A). The MIC modes for ciprofloxacin were 7 µg/ml (min=0.0016 µg/ml; max=32 µg/ml) and 2 µg/ml (min=0.004 µg/ml; max=8 µg/ml) under anaerobic and aerobic conditions, respectively (Supplementary Table 3); the rates of resistance under anaerobic (93%; N=62/67) and aerobic (54%; N=36/67) conditions were significantly different (Figure 1a; Supplementary Table 4). For azithromycin, the MIC modes were 32 µg/ml (min= 8 µg/ml; max=128 µg/ml) and 4 µg/ml (min=1 µg/ml; max=32 µg/ml) under anaerobic and aerobic conditions respectively (Supplementary Table 3). The rates of resistance under

anaerobic (n=67/67; 100%) and aerobic (n=15/67; 22%) conditions were significantly different (Figure 1b; Supplementary Table 4). For doxycycline, the MIC modes were 1 µg/ml under both aerobic and anaerobic conditions, respectively (Supplementary Table 3); only two isolates were found to be resistant under anaerobic conditions and one under aerobic conditions. The odds of classifying isolates as resistant increased over 10 times for ciprofloxacin (OR= 10.5; 95% CI= 3.61-37.7) and over 200 times for azithromycin (OR = 213; 95% CI= 31.9->5000) under anaerobic compared to aerobic conditions.

To evaluate the generalizability of these findings from the primary sample collection, pairwise analyses were conducted on 120 isolates from the secondary sample collection (Supplementary Table 5). For ciprofloxacin, the rates of resistance were significantly different under anaerobic (39%; n= 47/120) and aerobic conditions (1.6%; n= 2/120; McNemar's test $p<0.001$). For azithromycin, the rates of resistance were significantly different under anaerobic (100%; N= 120/120) and aerobic conditions (47%; N= 56/120; McNemar's test $p<0.001$); only two isolates were resistant under anaerobic conditions and zero under aerobic conditions. The odds of classifying isolates as resistant increased over 37 times for ciprofloxacin (OR= 37.5; 95% CI= 9.34-328) and over 136 times under azithromycin (OR = 136; 95% CI= 22.3 - >5000) for anaerobic compared to aerobic conditions.

Addition of catalase to test if reactive oxygen species effect antibiotic resistance/sensitivity under aerobic conditions. For ciprofloxacin, the MICs for the sensitive reference strain E7946 (Cip^S, Azi^S, Dox^S) and the resistant clinical isolate EN160 (Cip^R, Azi^R, Dox^S) remained unchanged when catalase was added to the media

174 under aerobic conditions. The addition of catalase was not associated with differences
175 in AUCs for both E7946 and EN160 in media containing ciprofloxacin, azithromycin, or
176 doxycycline at 2-fold below the MIC. The AUCs in LB media with and without catalase
177 alone for E7946 and EN160 were not statistically different (Supplementary Table 6).

178 **Molecular AMR correlates under aerobic and anaerobic conditions**. The distribution
179 of known AMR genetic elements are shown (Fig 2B). AMR associated point mutations
180 (likely transmitted vertically, not on an established mobilizable element), and genes on
181 known horizontally transferred mobilizable elements, are provided (Supplementary
182 Material). The integrative conjugative element (ICE) SXT/R391 was found in 90%
183 (60/67) of isolates. The ICE elements contained a pentapeptide repeat protein
184 conferring fluoroquinolone resistance (qnr_{Vc}), a macrolide-inactivating
185 phosphotransferase (*mph*(A)), and a major facilitator superfamily (MFS) efflux pump
186 conferring tetracycline resistance (*tet*(59)) [39-42]. The genes qnr_{Vc}, *mph*(A), and *tet*(59)
187 were found in 78% (52/67), 33% (22/67), and 78% (52/67) of isolates, respectively.
188 Ciprofloxacin resistance under both anaerobic and aerobic conditions was significantly
189 associated with qnr_{VC}, *gyrA* and *parC* (Supplementary Table 7). Identification of the
190 known azithromycin AMR gene *mph(A)* was significantly associated with resistance
191 under aerobic conditions alone (P<0.001). The gene *tet(59)* was not associated with
192 doxycycline resistance under aerobic or aerobic conditions (both p=0.566).

193 GWAS was conducted to more broadly explore the genetic basis of AMR. This
194 approach used the phenotype of AUC to represent 'growth' with or without exposure to
195 the three antibiotics at five concentrations under either aerobic or anaerobic conditions.
196 Phenotypes (AUCs) at similar antibiotic concentrations were positively correlated within

aerobic and anaerobic conditions for all three antibiotics (Figure 3). Phenotypes were also positively correlated between aerobic and anaerobic conditions for ciprofloxacin (Figure 3a). However, AUCs were weakly, or even negatively, correlated between aerobic and anaerobic conditions for azithromycin and doxycycline (Figure 3b,c). These results reinforce the proposal that anaerobic and aerobic growth are distinct under antibiotic pressure.

The heritability of the AMR phenotypes (AUCs) was estimated prior to the GWAS. Heritability (h^2) was defined as the proportion of phenotypic variation explained by genetic variation, measured as unique contiguous tracts of the genome (unitigs) that tag both single nucleotide variants, indels, and gene content changes (Methods). We found relatively high heritability (h^2 in the range 0.60-0.99) of growth across concentrations of ciprofloxacin under both aerobic and anaerobic conditions, yielding statistically significant GWAS hits (Table 1; Supplementary Data Files). In contrast, heritability tended to be much lower under aerobic compared to anaerobic conditions for both azithromycin and doxycycline, yielding significant GWAS hits only under anaerobic conditions (Table 1; Supplementary Data Files).

AMR genes identified by GWAS were diverse (Figure 4; Supplementary Data Files). These candidates included known AMR genes, such as *qnrVC* and *dfrA,* which were associated with ciprofloxacin resistance under both aerobic and anaerobic conditions. We identified seven genes associated with ciprofloxacin resistance under anaerobic conditions alone (including the stress response gene *barA* and a *radC* homolog involved in DNA repair), and ten genes under aerobic conditions alone (including *rtxB*). Under anaerobic conditions, most genes were identified at ciprofloxacin concentrations at or

above 0.25 µg/ml; however, four genes, including *barA*, were identified under one of the lowest tested ciprofloxacin concentrations (0.13 µg/ml). GWAS hits for azithromycin and doxycycline resistance were found only under anaerobic conditions. For azithromycin, two genetic elements were identified: *mphA* and a region between *ompT* and *dinG* (*ompT-dinG)*. For doxycycline, 23 genes were shared across concentrations; however, the gene discovery rate was highest at the lower concentrations (n=53 at 0.13 µg/ml; n =26 µg/ml). GWAS hits included the major facilitator superfamily antibiotic efflux pump *tet(59)* (Figure 4, Supplementary Data Files). The majority of genetic elements identified have unknown function, yet the expected detection of known AMR genes suggests that the genes of unknown function may indeed play a role in AMR.

Antibiotics detected in stool by LC-MS/MS. A total of 196 antibiotics were detected in the 51 stool supernatants tested (Figure 5). At least one antibiotic was detected in 98% (n=50/51), at least two antibiotics were detected in 94% (n=48/51), and three or more antibiotics were detected in 90% (n=46/51) of stool supernatants (Figure 5). Antibiotics detected were ciprofloxacin (n=48/51; 94%), tetracycline / doxycycline (n=46/51; 90%), nalidixic acid (n=41/51; 80%), metronidazole (n=37/51; 73%), bactrim (sulfamethoxazole/trimethoprim; n=22/51; 43%), and amoxicillin (n=2/51; 4%); azithromycin was not detected. Detection of quinolone/fluoroquinolone and tetracycline/doxycycline in stool by LC-MS/MS was not associated with AMR genotypes or phenotypes (Supplementary Table 8). Associations for azithromycin could not be tested because azithromycin was not detected in any stool supernatant.

DISCUSSION

In this study, the rate of resistance among clinical isolates differed significantly when tested under aerobic versus anaerobic conditions. The extent to which isolates were resistant under anaerobic conditions differed by antibiotic class. The genetic basis of resistance was uniquely discoverable under anaerobic conditions, and also differed by antibiotic class. The approach of using AUC growth curve data as a dependent continuous variable for GWAS to identify AMR genetic elements under aerobic and anaerobic conditions may provide a new framework to investigate AMR.

The odds of classifying isolates as resistant under anaerobic conditions compared to aerobic conditions increased over 10 times for ciprofloxacin and over 200 times for azithromycin. These results are likely generalizable across *V. cholerae* taxa in that analysis of a second collection separated by more than 10 years found that the odds of classifying isolates as resistant under anaerobic conditions compared to aerobic conditions increased over 30 times for ciprofloxacin and over 100 times for azithromycin. Physiologic mechanisms for increased resistance to antibiotics under anaerobic conditions may include reduced growth velocity limiting the impact of agents that directly or indirectly disrupt cell envelope integrity. Additional mechanisms may include ROS that induce both intracellular and cell wall stress and can act synergistically to potentiate antibiotic lethality. The assays that utilized catalase to quench hydrogen peroxide were conducted to evaluate this possibility. The MIC for all three antibiotics did not increase with the addition of catalase suggesting that the reduction of ROS alone cannot account for increased MICs observed under anaerobic conditions.

Multiple knowledge gaps on the genetic basis of AMR remain. This study prioritized the factor of oxygen exposure as a determinant of AMR phenotype because facultative enteropathogens experience hypoxia and anoxia in the animal host. The first phase of the analysis focused on known AMR genotypes with known AMR phenotypes. For ciprofloxacin, mutations in *parC* and carriage of qnr_{Vc} significantly associated with phenotypic resistance under aerobic and anaerobic conditions; mutations in *gyrA* significantly associated with resistant phenotypes under anaerobic conditions alone. For azithromycin, *mphA* was identified and significantly associated with AMR phenotypes under aerobic conditions alone. While *tet*(59) was identified, very few isolates were identified as resistant to doxycycline under aerobic (n=1) or anaerobic conditions (n=2). These data demonstrate that the relationship between AMR genotype and phenotype may differ between aerobic and anaerobic conditions.

The second phase of analysis sought to identify unknown genetic elements associated with AMR. Initial attempts to use categorical data (resistant vs sensitive) in the GWAS resulted in modest gene discovery rates (data not shown). To address this problem, the continuous variable of AUC strengthened the GWAS. Breakpoint and sub-breakpoint concentrations were chosen based on a rationale that different genetic elements might contribute differently to AMR phenotypes at different antibiotic concentrations. GWAS expectedly identified qnr_{Vc} for ciprofloxacin exposure under aerobic and anaerobic conditions, *mphA* for azithromycin exposure under anaerobic conditions alone and *tet*(59) for doxycycline exposure under anaerobic conditions alone. The lack of detection of *mphA* under aerobic conditions by GWAS conflicted with the conventional analysis. For ciprofloxacin, seven genes were discovered in anaerobic conditions alone; these

included a homolog for DNA repair (*radC*), 2-component histidine kinase involved in stress response (*barA*), and an ATP-dependent zinc protease. The genes *dfrA31* and APH(6)-Id were identified for both aerobic and anaerobic conditions and encode a trimethoprim-resistant dihydrofolate reductase and a streptomycin phosphotransferase enzyme, respectively. These genes are located on SXT with qnr_{Vc} and may therefore be associated due to genetic linkage rather than due to causal roles in ciprofloxacin resistance. For azithromycin, two genetic elements under anaerobic conditions alone were identified: *mphA* (mentioned above) and an intergenic region between *ompT* (porin; known to be associated with AMR)[43, 44] and *dinG* (ATP-dependent DNA helicase). For doxycycline, a diverse set of 57 genetic elements under anaerobic conditions alone were identified that included *tet(59)* (mentioned above), *vexK* (efflux RND transporter permease associated with AMR) [45-47], and *zorA* (anti-phage defense system ZorAB subunit A; a putative proton channel that may respond to membrane perturbation by depolarization)[48].

Genes associated with an AMR phenotype were found on SXT (e.g. *tet(59), aph(6)-Id, radC, dfrA31*) and discovered on the Vibrio Pathogenicity Island II (VSPII; N16961 VC0506-VC0512 / E7946 loci RS02705-RS02745); these loci are genetically diverse in Bangladesh[49]. The GWAS hits in VSPII encode both biofilm/ autoaggregation associated factors as well as an aerotaxis protein (AerB; VC0512)[50]; findings consistent with roles in AMR and aerobic/ anaerobic conditions. The GWAS were limited by sample size and were at risk of increased false positive discovery rates at AMR 'hot-spots' like SXT. Despite these limitations, GWAS enabled the discovery of an

intriguing list of genetic targets that were associated with AMR and require future molecular manipulation to test for causal relationships.

LC-MS/MS analysis on the stools, from which the isolates were obtained, was conducted to test the hypothesis that the rates of AMR genotypes and phenotypes were higher when the stool samples contained the cognate antibiotic. Nearly all patients shed at least one antibiotic making it difficult to identify AMR correlates to exposure.

Conclusions

Facultative enteropathogens are exposed to antibiotics under aerobic and anaerobic conditions in both the human gut and in the environment. We used the facultative enteropathogen *V. cholerae* as a model to test for differences in AMR phenotypes under aerobic and anaerobic conditions. The odds of classifying isolates as resistant under anaerobic compared to aerobic conditions increased over 10 times for two of the three antibiotics tested. While several known resistance genes were associated with AMR under both conditions, many genes were only associated with AMR under one condition. Heritability tended to be higher and more genes associated with resistance under anaerobic conditions, suggesting that key genetic determinants of resistance may be missed when experiments are only performed aerobically. Taken together, these findings challenge clinical and experimental paradigms of testing facultative enteropathogens for AMR under aerobic conditions alone.

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Data availability

Data analyzed in the manuscript have been made available in the online supplementary material.

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354 **Disclaimer**

355 The funders had no role in study design, data collection and analysis, decision to
356 publish, or preparation of the manuscript.

357 **Potential conflicts of interest.**

358 All authors: No reported conflicts.

FIGURES

FIGURE 1

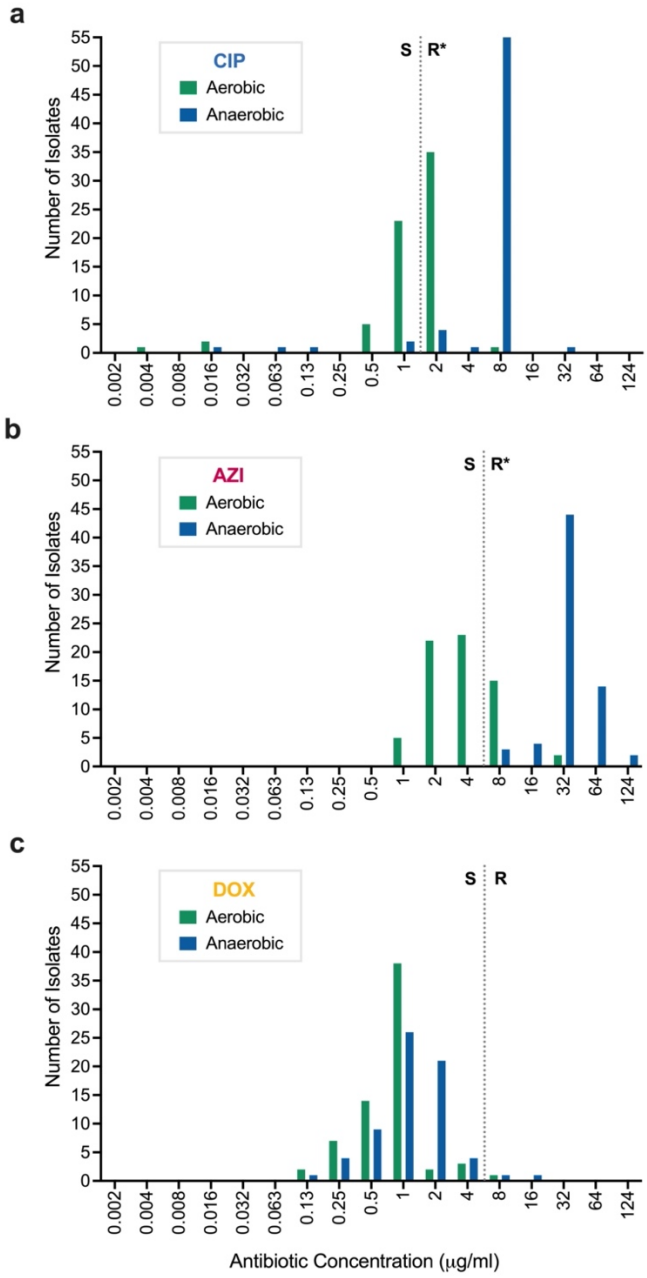
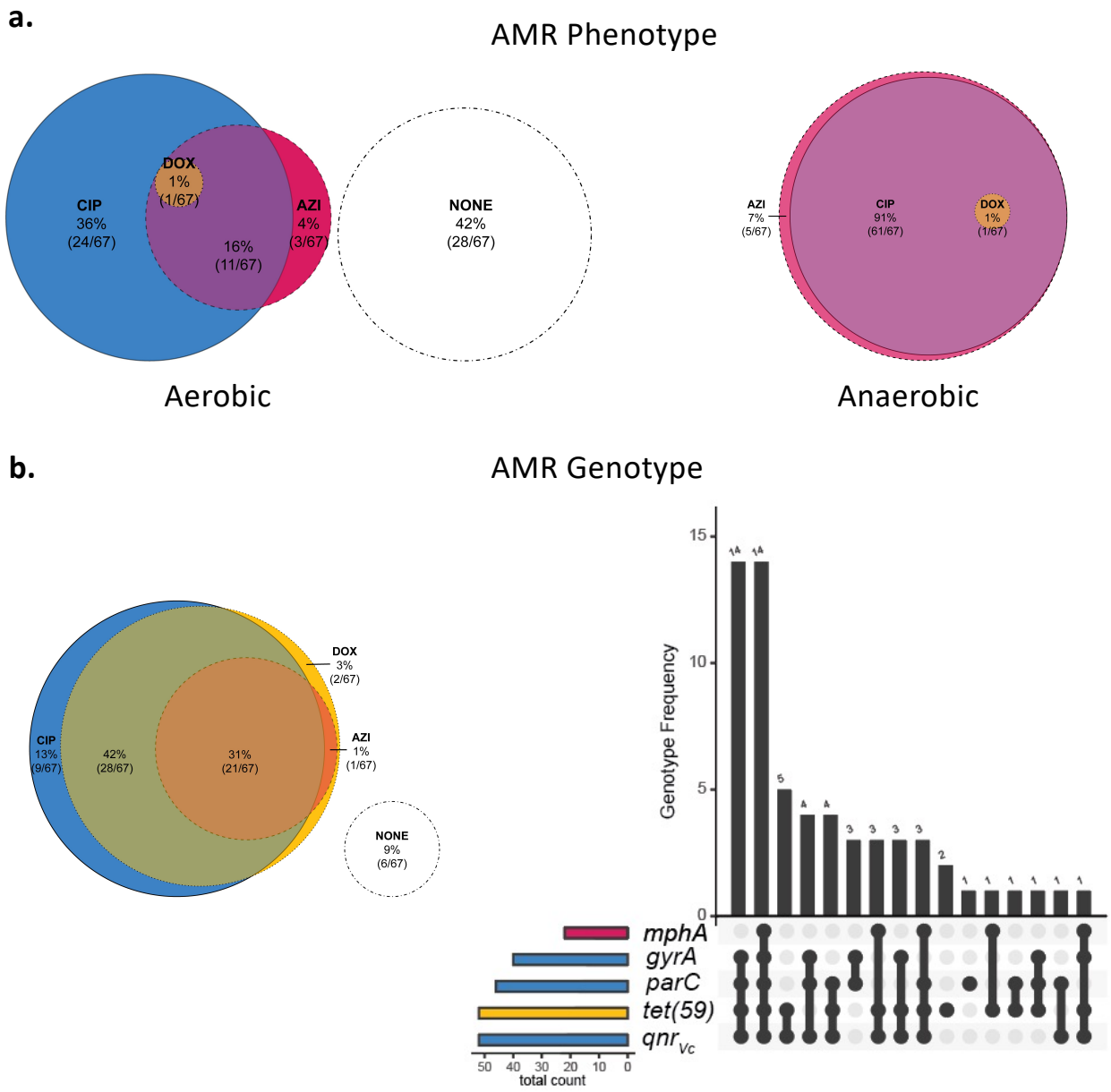


Figure 1. Distribution of antibiotic resistance among clinical isolates under aerobic and anaerobic conditions. Ciprofloxacin (CIP; **a**), Azithromycin (AZI; **b**), and Doxycycline (DOX; **c**). Resistance was determined by MIC under aerobic (green) and anaerobic conditions (blue). Data are from 67 human-shed *V. cholerae* isolates. Dotted line is the breakpoint for resistance per CLSI standards which is based on assays under aerobic conditions (CIP = 2 μg/ml; AZI = 8 μg/ml; DOX = 8 μg/ml). "*" represents a significant difference in the frequency of isolates identified as resistant to ciprofloxacin and azithromycin by McNemar's test (both $p<0.001$). S=sensitive. R = Resistant.

370 **FIGURE 2**

371



372

373 **Figure 2. AMR phenotypes and known AMR genetic elements in human-shed *V. cholerae* isolates**.
374 **a,** Proportional Venn diagram (Euler) of AMR phenotypes to azithromycin (AZI), doxycycline (DOX) and/or
375 ciprofloxacin (CIP) under aerobic (left) and anaerobic conditions (right). **b,** Distribution of specific known
376 AMR genetic elements by proportional Venn diagram (Euler; left) and bar chart (right). AMR genetic
377 elements to other antibiotics are not shown.

FIGURE 3

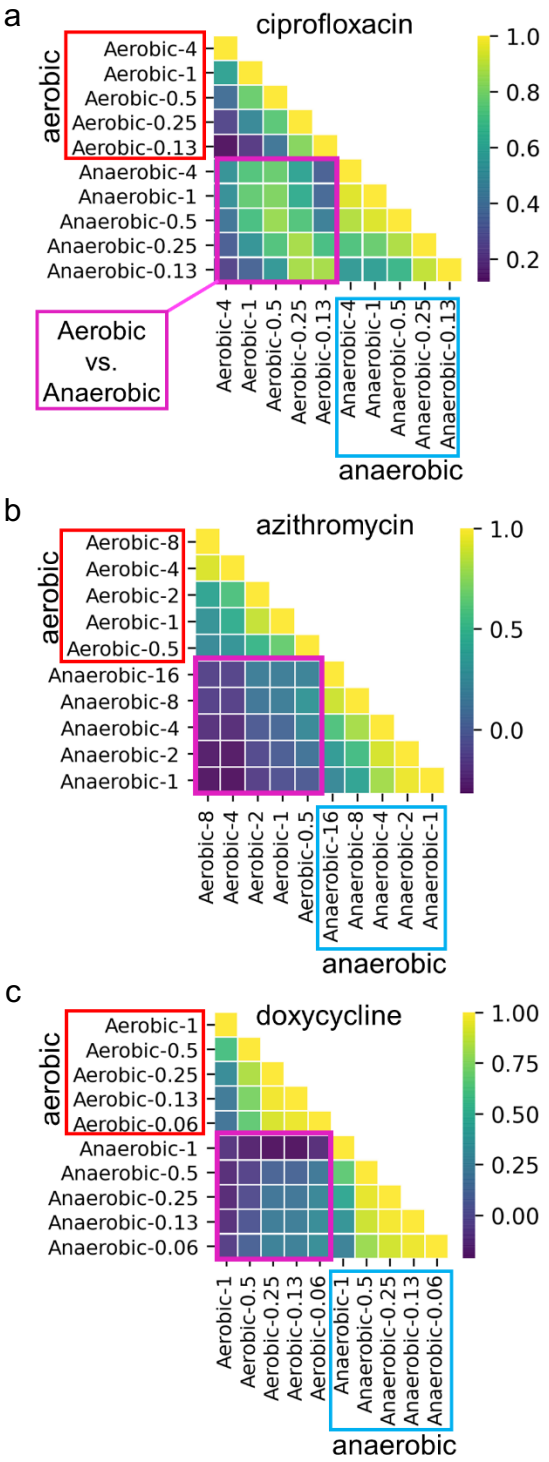
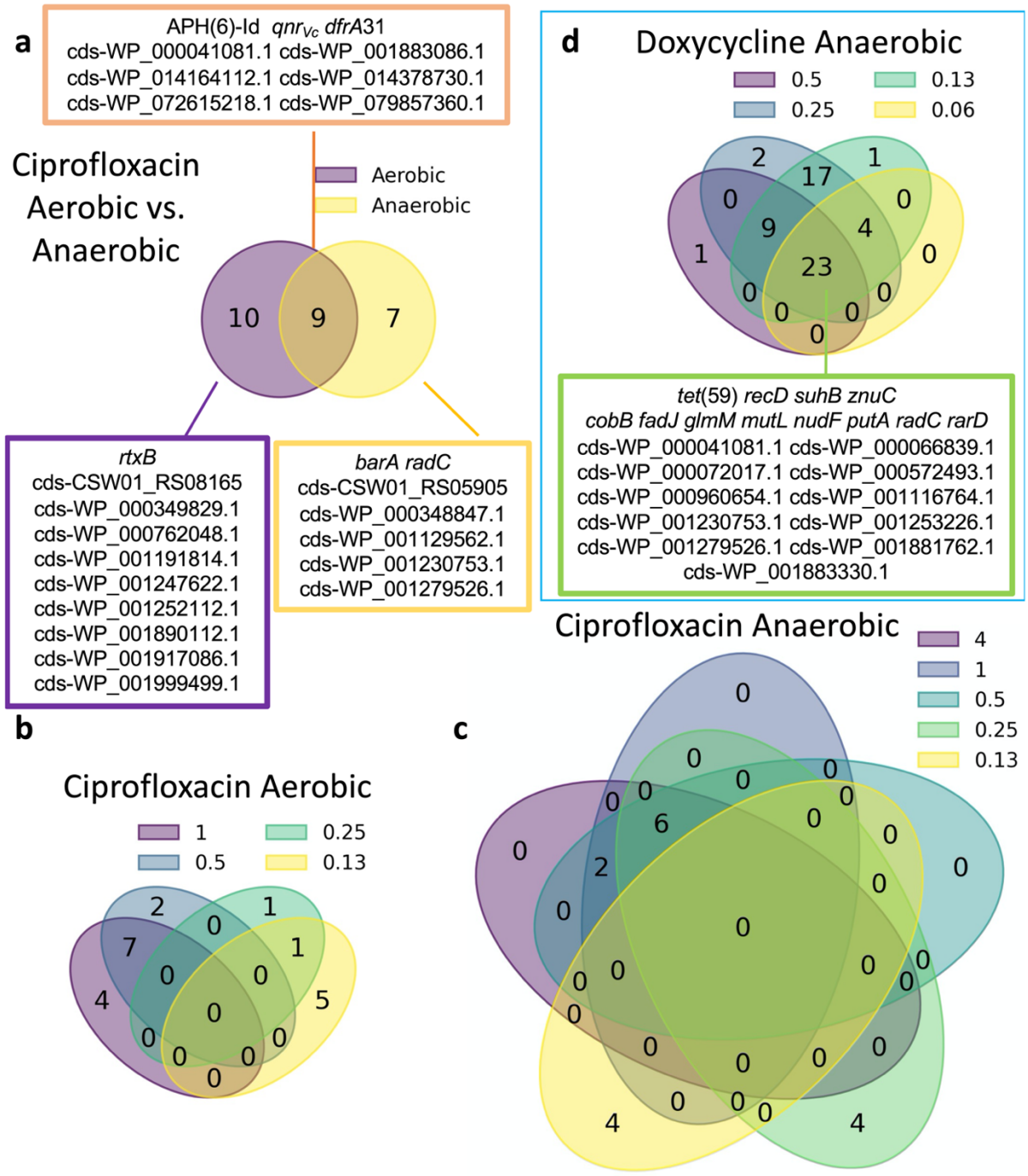


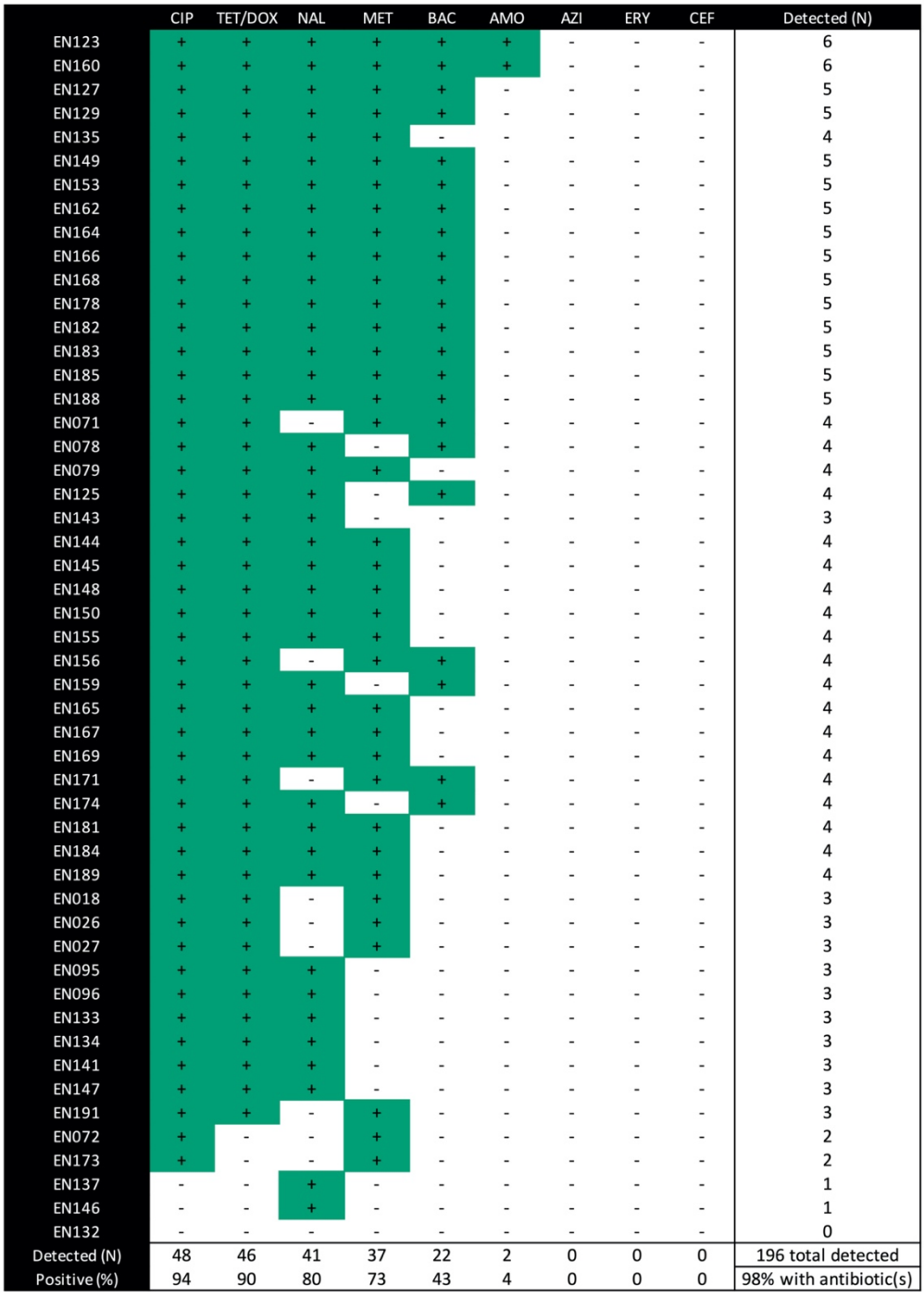
Figure 3. Correlations among *V. cholerae* growth phenotypes at different concentration of antibiotics and respiratory conditions. Correlations of *V. cholerae* growth phenotypes (AUC) to ciprofloxacin (CIP; **a**), azithromycin (AZI; **b**), and doxycycline (DOX; **c**) at different antibiotic concentrations under aerobic and anaerobic conditions (e.g., “Anaerobic-0.06”). Heatmaps show correlation coefficients (scale bar is to right).

386 **FIGURE 4**



387
388 **Figure 4. Distribution of AMR genes associated with AMR growth phenotypes at different**
389 **concentrations of antibiotics under aerobic and anaerobic conditions.** Venn diagrams show the
390 overlap between genes associated with **(a)** ciprofloxacin resistance under aerobic vs. anaerobic
391 conditions, **(b)** ciprofloxacin at different concentrations (μg/ml) under aerobic conditions, **(c)** ciprofloxacin
392 at different concentrations (μg/ml) under anaerobic conditions, and (**d)** doxycycline at different
393 concentrations (μg/ml) under anaerobic conditions. Genes shown in boxes had statistically significant
394 associations.

FIGURE 5.



	CIP	TET/DOX	NAL	MET	BAC	AMO	AZI	ERY	CEF	Detected (N)
EN123	+	+	+	+	+	+	-	-	-	6
EN160	+	+	+	+	+	+	-	-	-	6
EN127	+	+	+	+	+	-	-	-	-	5
EN129	+	+	+	+	+	-	-	-	-	5
EN135	+	+	+	+	-	-	-	-	-	4
EN149	+	+	+	+	+	-	-	-	-	5
EN153	+	+	+	+	+	-	-	-	-	5
EN162	+	+	+	+	+	-	-	-	-	5
EN164	+	+	+	+	+	-	-	-	-	5
EN166	+	+	+	+	+	-	-	-	-	5
EN168	+	+	+	+	+	-	-	-	-	5
EN178	+	+	+	+	+	-	-	-	-	5
EN182	+	+	+	+	+	-	-	-	-	5
EN183	+	+	+	+	+	-	-	-	-	5
EN185	+	+	+	+	+	-	-	-	-	5
EN188	+	+	+	+	+	-	-	-	-	5
EN071	+	+	-	+	+	-	-	-	-	4
EN078	+	+	+	-	+	-	-	-	-	4
EN079	+	+	+	+	-	-	-	-	-	4
EN125	+	+	+	-	+	-	-	-	-	4
EN143	+	+	+	-	-	-	-	-	-	3
EN144	+	+	+	+	-	-	-	-	-	4
EN145	+	+	+	+	-	-	-	-	-	4
EN148	+	+	+	+	-	-	-	-	-	4
EN150	+	+	+	+	-	-	-	-	-	4
EN155	+	+	+	+	-	-	-	-	-	4
EN156	+	+	-	+	+	-	-	-	-	4
EN159	+	+	+	-	+	-	-	-	-	4
EN165	+	+	+	+	-	-	-	-	-	4
EN167	+	+	+	+	-	-	-	-	-	4
EN169	+	+	+	+	-	-	-	-	-	4
EN171	+	+	-	+	+	-	-	-	-	4
EN174	+	+	+	-	+	-	-	-	-	4
EN181	+	+	+	+	-	-	-	-	-	4
EN184	+	+	+	+	-	-	-	-	-	4
EN189	+	+	+	+	-	-	-	-	-	4
EN018	+	+	-	+	-	-	-	-	-	3
EN026	+	+	-	+	-	-	-	-	-	3
EN027	+	+	-	+	-	-	-	-	-	3
EN095	+	+	+	-	-	-	-	-	-	3
EN096	+	+	+	-	-	-	-	-	-	3
EN133	+	+	+	-	-	-	-	-	-	3
EN134	+	+	+	-	-	-	-	-	-	3
EN141	+	+	+	-	-	-	-	-	-	3
EN147	+	+	+	-	-	-	-	-	-	3
EN191	+	+	-	+	-	-	-	-	-	3
EN072	+	-	-	+	-	-	-	-	-	2
EN173	+	-	-	+	-	-	-	-	-	2
EN137	-	-	+	-	-	-	-	-	-	1
EN146	-	-	+	-	-	-	-	-	-	1
EN132	-	-	-	-	-	-	-	-	-	0
Detected (N)	48	46	41	37	22	2	0	0	0	196 total detected
Positive (%)	94	90	80	73	43	4	0	0	0	98% with antibiotic(s)

Figure 5. Antibiotic detection in stool supernatants by mass spectrometry (LC-MS/MS). Green with "+" = Detected. White with "-" = not detected. CIP= ciprofloxacin, TET/DOX= tetracycline and/or doxycycline, NAL = nalidixic acid, MET = metronidazole, BAC = sulfamethoxazole and/or trimethoprim, AMO = amoxicillin, ERY = erythromycin, CEF = ceftriaxone. Stool supernatants were not available for EN80, 86, 88, 92, 100, 103, 109, 116-120, 126, 124, 130, 131.

403 **TABLE**

404

405

406 **Table 1.** Identification of genetic elements by GWAS that associate with AMR.

Condition	Outcome [b]	Antibiotic Concentration (µg/ml)				
Ciprofloxacin[a]		CIP4	CIP1	CIP0.5	CIP0.25	CIP0.13
Aerobic	Heritability (h^2)	0.99	0.73	0.60	0.74	0.92
	Associated genes	0	11	9	2	6
Anaerobic	Heritability (h^2)	0.81	0.73	0.72	0.72	0.87
	Associated genes	8	8	8	10	4
Azithromycin[a]		AZI16	AZI8	AZI4	AZI2	AZI1
Aerobic	Heritability (h^2)	-	0.00	0.02	0.00	0.11
	Associated genes	-	-	-	-	-
Anaerobic	Heritability (h^2)	0.30	0.77	0.66	0.38	0.00
	Associated genes	2	1	0	0	-
Doxycycline[a]		DOX1	DOX0.5	DOX0.25	DOX0.13	DOX0.06
Aerobic	Heritability (h^2)	0.00	0.27	0.23	0.30	0.50
	Associated genes	-	0	0	0	0
Anaerobic	Heritability (h^2)	0.10	0.61	0.72	0.72	0.77
	Associated genes	0	23	55	54	17

407 [a] Ciprofloxacin = CIP; Azithromycin = AZI; Doxycycline = DOX
408 [b] Heritability is the proportion of phenotypic variation that is explained by genetic variation. Associated
409 genes are all significant GWAS hits after correction for multiple hypothesis testing ($P < 0.05$ after
410 Bonferroni correction).
411

REFERENCES

1. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Wayne, PA (USA): Clinical and Laboratory Standards Institue (CLSI), **2017** CLSI supplement M100).

2. Bueno E, Sit B, Waldor MK, Cava F. Genetic Dissection of the Fermentative and Respiratory Contributions Supporting *Vibrio cholerae* Hypoxic Growth. J Bacteriol **2020**; 202.

3. Bueno E, Pinedo V, Cava F. Adaptation of *Vibrio cholerae* to Hypoxic Environments. Front Microbiol **2020**; 11:739.

4. Xu Q, Dziejman M, Mekalanos JJ. Determination of the transcriptome of *Vibrio cholerae* during intraintestinal growth and midexponential phase in vitro. Proc Natl Acad Sci U S A **2003**; 100:1286-91.

5. Mandlik A, Livny J, Robins WP, Ritchie JM, Mekalanos JJ, Waldor MK. RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. Cell Host Microbe **2011**; 10:165-74.

6. Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature **2004**; 427:72-4.

7. Narendrakumar L, Gupta SS, Johnson JB, Ramamurthy T, Thomas S. Molecular Adaptations and Antibiotic Resistance in *Vibrio cholerae*: A Communal Challenge. Microb Drug Resist **2019**; 25:1012-22.

8. Das B, Verma J, Kumar P, Ghosh A, Ramamurthy T. Antibiotic resistance in *Vibrio cholerae*: Understanding the ecology of resistance genes and mechanisms. Vaccine **2020**; 38 Suppl 1:A83-A92.

435 9. The treatment of diarrhoea -- A manual for physicians and other senior health
436 workers. —4th rev. 4th Rev. ed. Vol. WHO/CAH/03.7. Geneva, Switzerland: World
437 Heath Organization.
438 10. Nelson EJ, Nelson DS, Salam MA, Sack DA. Antibiotics for both moderate and
439 severe cholera. N Engl J Med **2011**; 364:5-7.
440 11. Leibovici-Weissman Y, Neuberger A, Bitterman R, Sinclair D, Salam MA, Paul M.
441 Antimicrobial drugs for treating cholera. Cochrane Database Syst Rev **2014**:CD008625.
442 12. Khan AI, Mack JA, Salimuzzaman M, et al. Electronic decision-support improves
443 diarrhoeal disease guideline adherence (mHealth Diarrhoea Management, mHDM,
444 Trial): a cluster randomized controlled trial. Lancet DH **2020**; 2:e250-8.
445 13. Biswas D, Hossin R, Rahman M, et al. An ethnographic exploration of diarrheal
446 disease management in public hospitals in Bangladesh: From problems to solutions.
447 Soc Sci Med **2020**; 260:113185.
448 14. Ingle DJ, Levine MM, Kotloff KL, Holt KE, Robins-Browne RM. Dynamics of
449 antimicrobial resistance in intestinal *Escherichia coli* from children in community settings
450 in South Asia and sub-Saharan Africa. Nat Microbiol **2018**; 3:1063-73.
451 15. Towner KJ, Pearson NJ, Mhalu FS, O'Grady F. Resistance to antimicrobial agents
452 of *Vibrio cholerae* E1 Tor strains isolated during the fourth cholera epidemic in the
453 United Republic of Tanzania. Bull World Health Organ **1980**; 58:747-51.
454 16. Burrus V, Marrero J, Waldor MK. The current ICE age: biology and evolution of
455 SXT-related integrating conjugative elements. Plasmid **2006**; 55:173-83.
456 17. Hooper DC, Jacoby GA. Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of
457 Action and Resistance. Cold Spring Harb Perspect Med **2016**; 6.

458 18. Garriss G, Waldor MK, Burrus V. Mobile antibiotic resistance encoding elements
459 promote their own diversity. PLoS Genet **2009**; 5:e1000775.
460 19. Fonseca EL, Dos Santos Freitas F, Vieira VV, Vicente AC. New *qnr* gene cassettes
461 associated with superintegron repeats in *Vibrio cholerae* O1. Emerg Infect Dis **2008**;
462 14:1129-31.
463 20. Fyfe C, Grossman TH, Kerstein K, Sutcliffe J. Resistance to Macrolide Antibiotics in
464 Public Health Pathogens. Cold Spring Harb Perspect Med **2016**; 6.
465 21. Grossman TH. Tetracycline Antibiotics and Resistance. Cold Spring Harb Perspect
466 Med **2016**; 6:a025387.
467 22. Dorr T, Delgado F, Umans BD, Gerding MA, Davis BM, Waldor MK. A Transposon
468 Screen Identifies Genetic Determinants of *Vibrio cholerae* Resistance to High-
469 Molecular-Weight Antibiotics. Antimicrob Agents Chemother **2016**; 60:4757-63.
470 23. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common
471 mechanism of cellular death induced by bactericidal antibiotics. Cell **2007**; 130:797-810.
472 24. Dwyer DJ, Belenky PA, Yang JH, et al. Antibiotics induce redox-related
473 physiological alterations as part of their lethality. Proc Natl Acad Sci U S A **2014**;
474 111:E2100-9.
475 25. Staerck C, Gastebois A, Vandeputte P, et al. Microbial antioxidant defense
476 enzymes. Microb Pathog **2017**; 110:56-65.
477 26. Smirnova G, Muzyka N, Oktyabrsky O. Transmembrane glutathione cycling in
478 growing *Escherichia coli* cells. Microbiol Res **2012**; 167:166-72.

27. Bryan LE, Kwan S. Mechanisms of aminoglycoside resistance of anaerobic bacteria and facultative bacteria grown anaerobically. J Antimicrob Chemother **1981**; 8 Suppl D:1-8.

28. Bryan LE. General mechanisms of resistance to antibiotics. J Antimicrob Chemother **1988**; 22 Suppl A:1-15.

29. Bryant RE, Fox K, Oh G, Morthland VH. Beta-lactam enhancement of aminoglycoside activity under conditions of reduced pH and oxygen tension that may exist in infected tissues. J Infect Dis **1992**; 165:676-82.

30. Dwyer DJ, Kohanski MA, Hayete B, Collins JJ. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol Syst Biol **2007**; 3:91.

31. Hong Y, Li Q, Gao Q, et al. Reactive oxygen species play a dominant role in all pathways of rapid quinolone-mediated killing. J Antimicrob Chemother **2020**; 75:576-85.

32. Luan G, Hong Y, Drlica K, Zhao X. Suppression of Reactive Oxygen Species Accumulation Accounts for Paradoxical Bacterial Survival at High Quinolone Concentration. Antimicrob Agents Chemother **2018**; 62.

33. Zhao X, Drlica K. Reactive oxygen species and the bacterial response to lethal stress. Curr Opin Microbiol **2014**; 21:1-6.

34. Nelson EJ, Chowdhury A, Harris JB, et al. Complexity of rice-water stool from patients with *Vibrio cholerae* plays a role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A **2007**; 104:19091-6.

35. Sprouffske K, Wagner A. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC Bioinformatics **2016**; 17:172.

501 36. McArthur AG, Waglechner N, Nizam F, et al. The comprehensive antibiotic
502 resistance database. Antimicrob Agents Chemother **2013**; 57:3348-57.
503 37. Zankari E. Comparison of the web tools ARG-ANNOT and ResFinder for detection
504 of resistance genes in bacteria. Antimicrob Agents Chemother **2014**; 58:4986.
505 38. Alexandrova L, Haque F, Rodriguez P, et al. Identification of widespread antibiotic
506 exposure in cholera patients correlates with clinically relevant microbiota changes. J
507 Infect Dis **2019**.
508 39. Leclercq SO, Wang C, Zhu Y, et al. Diversity of the Tetracycline Mobilome within a
509 Chinese Pig Manure Sample. Appl Environ Microbiol **2016**; 82:6454-62.
510 40. Grossman TH, Starosta AL, Fyfe C, et al. Target- and resistance-based mechanistic
511 studies with TP-434, a novel fluorocycline antibiotic. Antimicrob Agents Chemother
512 **2012**; 56:2559-64.
513 41. Noguchi N, Emura A, Matsuyama H, O'Hara K, Sasatsu M, Kono M. Nucleotide
514 sequence and characterization of erythromycin resistance determinant that encodes
515 macrolide 2'-phosphotransferase I in *Escherichia coli*. Antimicrob Agents Chemother
516 **1995**; 39:2359-63.
517 42. Chesneau O, Tsvetkova K, Courvalin P. Resistance phenotypes conferred by
518 macrolide phosphotransferases. FEMS Microbiol Lett **2007**; 269:317-22.
519 43. Mathur J, Waldor MK. The *Vibrio cholerae* ToxR-regulated porin OmpU confers
520 resistance to antimicrobial peptides. Infect Immun **2004**; 72:3577-83.
521 44. Li CC, Crawford JA, DiRita VJ, Kaper JB. Molecular cloning and transcriptional
522 regulation of *ompT*, a ToxR-repressed gene in *Vibrio cholerae*. Mol Microbiol **2000**;
523 35:189-203.

524 45. Buckley AM, Webber MA, Cooles S, et al. The AcrAB-TolC efflux system of
525 Salmonella enterica serovar Typhimurium plays a role in pathogenesis. Cell Microbiol
526 **2006**; 8:847-56.
527 46. Taylor DL, Bina XR, Bina JE. *Vibrio cholerae* VexH encodes a multiple drug efflux
528 pump that contributes to the production of cholera toxin and the toxin co-regulated pilus.
529 PLoS One **2012**; 7:e38208.
530 47. Bina XR, Philippart JA, Bina JE. Effect of the efflux inhibitors 1-(1-naphthylmethyl)-
531 piperazine and phenyl-arginine-beta-naphthylamide on antimicrobial susceptibility and
532 virulence factor production in *Vibrio cholerae*. J Antimicrob Chemother **2009**; 63:103-8.
533 48. Doron S, Melamed S, Ofir G, et al. Systematic discovery of antiphage defense
534 systems in the microbial pangenome. Science **2018**; 359.
535 49. Baddam R, Sarker N, Ahmed D, et al. Genome Dynamics of *Vibrio cholerae* Isolates
536 Linked to Seasonal Outbreaks of Cholera in Dhaka, Bangladesh. mBio **2020**; 11.
537 50. Murphy SG, Johnson BA, Ledoux CM, Dörr T. *Vibrio cholerae*'s mysterious Seventh
538 Pandemic island (VSP-II) encodes novel Zur-regulated zinc starvation genes involved in
539 chemotaxis and autoaggregation. bioRxiv **2021**:2021.03.09.434465.
540

SUPPLEMENTARY MATERIAL

Table of Contents

Supplementary Data Files (excel file with four datafile tabs)

Date File S1. GWAS Hits. Ciprofloxacin resistance phenotype. Aerobic.
Date File S2. GWAS Hits. Ciprofloxacin resistance phenotype. Anaerobic.
Data File S3. GWAS Hits. Azithromycin resistance phenotype. Anaerobic.
Data File S4. GWAS Hits. Doxycycline resistance phenotype. Anaerobic.

Supplementary Methods.

Whole-genome sequencing. Genomic DNA was extracted from *V. cholerae* isolates from the primary collection using the Qiagen DNeasy Blood and Tissue Kit. Library construction was completed using the Illumina Nextera XT v.2 DNA Library Preparation Kit. Twenty-four genomes were pooled and sequenced on an Illumina MiSeq for 500 cycles per run. Using CLC Genomics Workbench v20, raw reads were filtered by length, trimmed, and mapped to the reference genome (*V. cholerae* O1 El Tor E7946) to identify single-nucleotide variants. To identify genes not present in the reference genome, contigs were assembled *de novo* using CLC Genomics Workbench v20.

Genome-wide association studies (GWAS). To extract genomic variants, which can potentially capture all sources of variation in the genome (i.e. single nucleotide variants, indels and gene presence/absence) without *a priori* assumption about the underlying gene content of each sample (e.g. accessory genes or plasmids), unitigs were generated from genomes assembled using GATB[1]. Unitigs are sequences of variable length (unlike k-mers of fixed length *k*) which represent the variations in the population of genomes under study in high-resolution. GWAS were performed using linear mixed models implemented in pyseer v.1.3.6 and adjusted for population stratification using the kinship matrix estimated from the phylogenetic tree[2].

To generate the phylogenetic tree, genome alignments consisting entirely of variable nucleotides were produced from whole genome SNP data generated by CLC Genomics Workbench v20 using VCF-kit 0.1.6[3]. The tree was then inferred by RaxML under the general time reversible (GTR) model with rate variation across sites following a GAMMA distribution[4]. We used the linear-mixed model approach to adjust for population

stratification and linkage disequilibrium in microbial GWAS[5]. Heritability (h^2), an estimate of the proportion of the phenotype variance that can be explained by total genomic variation represented in the unitigs, was also calculated using pyseer v.1.3.6. Likelihood-ratio test *p*-values for the association tests were adjusted for multiple-testing by Bonferroni correction (at a genome-wide false discovery rate of 0.05) for the number of unique unitig patterns (i.e. only giving one count to a unitig with an identical presence/absence profile across genomes). We also removed unitigs tagged with the errors 'bad-chisq', 'pre-filtering-failed', 'lrt-filtering-failed', 'firth-fail' and 'matrix-inversionerror' after the analysis. To further remove false positive GWAS hits, we removed any considerable clusters of unitigs (> 20) with identical *p*-values, as these are likely to be lineage-specific markers or markers with strong linkage disequilibrium comprised of mostly non-causal variants linked on the same clonal frame. GWAS hits were annotated by mapping the unitigs to two reference genomes of *V. cholerae*, namely, E7946 (NCBI assembly accession number: GCA_002749635.1) and O1 biotype El Tor strain N16961 (NCBI assembly accession number: GCA_003063785.1) using BWA. Statistically significant GWAS hits were further annotated with the CARD resistance gene identifier (RGI) after filtering the 'loose' hits and hits with identity <0.90.

Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS). Stool supernatant from the primary collection were obtained by centrifugation and filtration (0.2 µM surfactant-free cellulose acetate; Thermo Scientific Nalgene). Proteins were precipitated (1:7 ratio (v/v) of water::methanol). Supernatants were diluted with methanol and water (1:1 v/v) in 0.1% formic acid for liquid chromatography, and 5 µl of supernatant was injected for analysis. LC/MSMS was performed on a 2.1 x 150-mm

Hypersil Gold aQ column (particle size, 3 µm) using a high-performance liquid chromatography system (Thermo UltiMate 3000 series) with an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific). Mobile phases were 1% formic acid in water (A) and 1% formic acid in methanol (B) and held at a constant 5%B for 2min before ramping to 95%B at 15 min where it was held for an additional minute before returning to starting conditions for a total run time of 25 min.

Eluent was ionized using electrospray ionization (ESI) in positive mode at a spray voltage of 5 kV, a nitrogen sheath gas flow rate of 8 L min^{-1}, and capillary temperature of 300°C. Two scan events were programmed to perform an initial scan from *m/z* 100 to 1000, which was followed by targeted collision induced dissociation based on a retention time and mass list. Retention time windows ranged from 0.35 minutes to 6.50 min, depending on the elution range of the standards at high and low concentrations. Masses were targeted for the most abundant adduct or ion associated with each antibiotic (typically the $[M+H]^+$ ion) with a *m/z* 1 window. Data analysis for amoxicillin, sulfamethoxazole/trimethoprim, azithromycin, tetracycline, doxycycline, metronidazole, nalidixic acid, and ciprofloxacin was performed manually using extracted ion chromatograms and MSMS matching with an in-house antibiotic MSMS library using Xcalibur 2.2 SP 1.48 (Thermo Fisher Scientific).

Supplementary Results.

Antibiotic resistance phenotypes of known AMR genetic elements under aerobic and anaerobic conditions. The distribution of known AMR genetic elements (Figure 2) was grouped by point mutations (likely transmitted vertically, not on an established mobilizable element) and horizontal transmission (on an established mobilizable element).

Point mutations in known AMR genes. For ciprofloxacin, the most identified known resistance mutations were those in the topoisomerase encoding genes *gyrA* (VC1258/RS06370) (Ser83Ile) and *parC* (VC2430/RS12340) (Ser85Leu). These were present in 60% (40/67) and 70% (47/67) of all isolates, while 54% (36/57) contained both. For azithromycin, we found no mutations in known resistance genes encoding ribosomal proteins L4 (*rplD;*VC2595/RS13175) and L22 (*rplV;*VC2591/RS13155) [6, 7]. For tetracycline (proxy for doxycycline), one of the 9 total 16S rRNA genes (VCr001) was found to have a single nucleotide insertion of a G nucleotide at position 327 within the sequences of 15% (10/67) of isolates; the significance is unknown. No mutations were detected in the 30S ribosomal protein encoded by genes *rpsJ* (VC2597/RS13185) or *rpsC* (VC2590/RS13150), whose mutations have been associated with tetracycline class resistance in other Gram negative organisms [8-10].

Known AMR genes on mobile genetic elements. The integrative conjugative element (ICE) SXT/R391 was found in 90% (60/67) of isolates. The ICE contained a pentapeptide repeat protein conferring fluoroquinolone resistance (qnr_{Vc}), a macrolide-inactivating phosphotransferase (*mph*(A)), and a major facilitator superfamily (MFS) efflux pump conferring tetracycline resistance (*tet*(59)) [11-14]. The genes qnr_{Vc},

123 *mph*(A), and *tet*(59) were also found in 78% (52/67), 33% (22/67), and 78% (52/67) of

124 isolates, respectively.

125

126

127 **Supplementary Table 1.** Reference strains and clinical isolates

128

		Aerobic[b]			Anaerobic[b]			Source[c]
	Strain[a]	CIP^R	AZI^R	DOC^R	CIP^R	AZI^R	DOX^R	
Reference	E7946	+	-	-	+	-	-	(1)
Clinical Isolates								
EN018	L_EN1286	-	-	-	-	+	-	(2)
EN026	L_EN1291	-	-	-	-	+	-	(2)
EN027	L_EN1292	-	-	-	-	+	-	(2)
EN071	L_EN1300	+	-	-	+	+	-	(2)
EN072	L_EN1301	-	-	-	+	+	-	(2)
EN078	L_EN1303	+	-	-	+	+	-	(2)
EN079	L_EN1304	-	+	-	+	+	-	(2)
EN080	L_EN1305	+	-	-	+	+	-	(2)
EN086	L_EN1307	+	+	-	+	+	-	(2)
EN088	L_EN1308	+	-	-	+	+	-	(2)
EN092	L_EN1310	+	-	-	+	+	-	(2)
EN095	L_EN1312	+	+	-	+	+	-	(2)
EN096	L_EN1313	+	+	-	+	+	-	(2)
EN100	L_EN1314	+	+	-	+	+	-	(2)
EN103	L_En1315	-	-	-	+	+	-	(2)
EN109	L_EN1319	-	-	-	+	+	-	(2)
EN116	L_EN1325	-	-	-	+	+	-	(2)
EN117	L_EN1326	-	-	-	-	+	-	(2)
EN118	L_EN1327	-	+	-	+	+	-	(2)
EN119	L_EN1328	+	-	-	+	+	-	(2)
EN120	L_EN1329	-	-	-	+	+	-	(2)
EN123	L_EN1330	-	-	-	+	+	-	(2)
EN124	L_EN1331	+	-	-	+	+	-	(2)
EN125	L_EN1332	+	+	-	+	+	-	(2)
EN126	L_EN1333	+	-	-	+	+	-	(2)
EN127	L_EN1334	+	-	-	+	+	-	(2)
EN129	L_EN1335	-	-	-	+	+	-	(2)
EN130	L_EN1336	+	-	-	+	+	-	(2)
EN131	L_EN1337	+	-	-	+	+	-	(2)
EN132	L_EN1338	+	-	-	+	+	-	(2)
EN133	L_EN1339	+	-	-	+	+	-	(2)

EN134	L_EN1340	-	+	-	+	+	-	(2)
EN135	L_EN1341	-	-	-	+	+	-	(2)
EN137	L_EN1343	+	+	-	+	+	-	(2)
EN141	L_EN1344	+	-	-	+	+	-	(2)
EN143	L_EN1346	-	-	-	+	+	-	(2)
EN144	L_EN1347	+	-	-	+	+	-	(2)
EN145	L_EN1348	+	+	-	+	+	+	(2)
EN146	L_EN1349	+	-	-	+	+	-	(2)
EN147	L_EN1350	-	-	-	+	+	-	(2)
EN148	L_EN1351	-	-	-	+	+	-	(2)
EN149	L_EN1352	+	-	-	+	+	-	(2)
EN150	L_EN1353	-	-	-	+	+	-	(2)
EN153	L_EN1355	-	-	-	+	+	-	(2)
EN155	L_EN1357	+	-	-	+	+	-	(2)
EN156	L_EN1358	-	-	-	+	+	-	(2)
EN159	L_EN1360	+	+	-	+	+	-	(2)
EN160	L_EN1361	+	-	-	+	+	-	(2)
EN162	L_EN1363	+	+	-	+	+	-	(2)
EN164	L_EN1365	-	-	-	+	+	-	(2)
EN165	L_EN1366	+	-	-	+	+	-	(2)
EN166	L_EN1367	+	-	-	+	+	-	(2)
EN167	L_EN1368	+	-	-	+	+	-	(2)
EN168	L_EN1369	+	+	-	+	+	-	(2)
EN169	L_EN1370	-	-	-	+	+	-	(2)
EN171	L_EN1371	+	+	-	+	+	-	(2)
EN173	L_EN1372	-	-	-	+	+	-	(2)
EN174	L_EN1374	-	-	-	+	+	-	(2)
EN178	L_EN1377	+	-	-	+	+	-	(2)
EN181	L_EN1379	-	-	-	-	+	-	(2)
EN182	L_En1380	-	-	-	+	+	-	(2)
EN183	L_EN1381	-	-	-	+	+	-	(2)
EN184	L_EN1382	+	-	-	+	+	-	(2)
EN185	L_EN1383	-	-	-	+	+	-	(2)
EN188	L_EN1385	-	-	-	+	+	-	(2)
EN189	L_EN1386	+	+	-	+	+	-	(2)
EN191	L_EN1388	-	-	-	+	+	-	(2)

[a] Prefix of "L_" was used to distinguish the strain number in the library from the clinical isolate number, which is maintained to be consistent with prior publications.

[b] CIP^R = ciprofloxacin resistance. AZI^R = azithromycin resistance. DOX^R = doxycycline resistance.

[c] Sources of strains: (1) Mekalanos, J. J. Duplication and amplification of toxin genes in Vibrio cholerae. Cell 35, 253-263, (1983) ; (2) Nelson, E. J. et al. Complexity of rice-water stool from patients with Vibrio cholerae plays a role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A 104, 19091-19096, (2007).

Supplementary Table 2. Baseline growth parameters of *V. cholerae* clinical isolates under aerobic and anaerobic conditions

Growth parameter [a]	Aerobic median	Anaerobic median	P [b]
K	1.08	0.261	**< 0.001**
AUC	5.15	1.58	**< 0.001**
Velocity	0.011	0.006	**< 0.001**

[a] AUC = area under the curve. K = carrying capacity. Media was LB alone without antibiotics. Velocity = growth rate in percent increase per minute at half the carrying capacity.

[b] Wilcoxon signed-rank test for growth. Bold = statistically significant (P<0.05).

Supplementary Table 3. Minimal inhibitory concentrations (MICs) for ciprofloxacin, azithromycin, and doxycycline among *V. cholerae* clinical isolates

	Ciprofloxacin[b]		Azithromycin[b]		Doxycycline[b]	
µg/ml[a]	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0.002	0	0	0	0	0	0
0.004	1	0	0	0	0	0
0.008	0	0	0	0	0	0
0.016	2	1	0	0	0	0
0.032	0	0	0	0	0	0
0.063	0	1	0	0	0	0
0.13	0	1	0	0	2	1
0.25	0	0	0	0	7	4
0.5	5	0	0	0	14	9
1	23	2	5	0	**38**	**26**
2	**35**	4	22	0	2	21
4	0	1	**23**	0	3	4
8	1	**56**	15	3	1	1
16	0	0	0	4	0	1
32	0	1	2	**44**	0	0
64	0	0	0	14	0	0
124	0	0	0	2	0	0

[a] Concentration of antibiotic.

[b] Distribution of MICs for clinical isolates grown under aerobic and anaerobic conditions. Bold text signifies the concentration at which the MIC mode was determined among the clinical isolates.

Supplementary Table 4. Comparison of rates of resistance detected under aerobic versus anaerobic conditions among *V. cholerae* clinical isolates in the primary collection

	N	R^{Ae}/R^{An}	R^{Ae}/S^{An}	S^{Ae}/R^{An}	S^{Ae}/S^{An}	p[b]
Ciprofloxacin[a]	67	36	0	26	5	**<0.001**
Azithromycin[a]	67	15	0	52	0	**<0.001**
Doxycycline[a]	67	0	1	1	65	1

[a] Distribution of paired resistant ('R') and sensitive ('S') phenotypes for isolates under aerobic ('Ae') and anaerobic ('An') conditions.
[b] McNemar's Exact Test. Bold = statistically significant (P<0.05)

Supplementary Table 5. Comparison of rates of resistance detected under aerobic versus anaerobic conditions among *V. cholerae* clinical isolates in the secondary collection

	N	R^{Ae}/R^{An}	R^{Ae}/S^{An}	S^{Ae}/R^{An}	S^{Ae}/S^{An}	p[b]
Ciprofloxacin[a]	120	1	1	46	72	**<0.001**
Azithromycin[a]	120	56	0	64	0	**<0.001**
Doxycycline[a]	120	0	0	2	118	0.480

[a] Distribution of paired resistant ('R') and sensitive ('S') phenotypes for isolates under aerobic ('Ae') and anaerobic ('An') conditions.
[b] McNemar's Exact Test. Bold = statistically significant (P<0.05)

Supplementary Table 6. Effect of catalase on growth parameters for *V. cholerae* E7946 and EN160 under aerobic conditions

Experiment	Strain[a]	Antibiotic[b]	Catalase[c]	AUC mean[d]	AUC IQR[d]	P[e]
1.	E7946	CIP	YES	41.84	1.76	0.136
		CIP	NO	44.2	0.865	
2.	E7946	AZI	YES	196	3.37	0.533
		AZI	NO	197.5	0.294	
3.	E7946	DOX	YES	308.5	6.32	0.959
		DOX	NO	308.9	4.11	
4.	E7946	NO	YES	317.3	4.65	0.818
		NO	NO	318	5.42	
5.	EN160	CIP	YES	138.4	22.1	0.551
		CIP	NO	126.1	25.2	
6.	EN160	AZI	YES	265.8	7.12	0.571
		AZI	NO	269.9	6.75	
7.	EN160	DOX	YES	280.1	19.3	0.895
		DOX	NO	275.8	21.6	
8	EN160	NO	YES	319.5	11.26	0.092
		NO	NO	326.6	8.78	

[a] E7946 (Cip^S, Azi^S,Dox^S) is the reference strain and EN160 (Cip^R, Azi^R, Dox^S) is a clinical isolate. Biological replicates in experiments 1-3 and 5-7 were 3, each with 4 technical replicates. Biological replicates for experiments 4 and 8 were 9, each with 4 technical replicates.

[b] CIP = ciprofloxacin. AZI = azithromycin. DOX = doxycycline. Assays were run at CIP = 0.5, AZI = 2, and DOX = 0.25 µg/ml for EN160; E7946 was run at CIP = 0.002, AZI = 1, and DOX = 0.013 µg/ml.

[c] CIP and AZI were tested with 3 biological replicates; DOX with 2 biological replications; LB controls with 9 biological replicates.

[d] AUC = area under the curve. IQR = interquartile range.

[e] Student's t-test. Bold = statistically significant (P<0.05)

Supplementary Table 7. Comparison of antibiotic resistance phenotypes and known resistance genotypes among *V. cholerae* clinical isolates

Aerobic conditions [a]								
Antibiotic tested	Gene	R/P	R/NP	S/P	S/NP	OR[b]	95% CI[b]	P[b]
Ciprofloxacin	*qnrVc*	36	0	16	15	31	(4.22 - 695)	**<0.001**
	gyrA	28	8	12	19	5.4	(1.86 - 17.9)	**0.002**
	parC	31	5	16	15	5.6	(1.65 - 18.9)	**0.003**
Azithromycin	*mphA*	15	0	7	45	83	(11.2 - 1938)	**<0.001**
Doxycycline	*tet(59)*	1	0	51	15	0.62	(0.046 - 18.9)	0.566
Anaerobic conditions [a]								
Antibiotic tested	Gene	R/P	R/NP	S/P	S/NP	OR[b]	95% Cb[c]	P[b]
Ciprofloxacin	*qnrVc*	52	10	0	5	27	(3.53 - 666)	**<0.001**
	gyrA	40	22	0	5	10	(1.39 - 248)	**0.016**
	parC	47	15	0	5	17	(2.28 - 416)	**0.003**
Azithromycin	*mphA*	22	45	0	0	0.51	(0.0127 - 20.1)	1
Doxycycline	*tet(59)*	1	0	51	15	0.62	(0.046 - 18.9)	0.566

[a] R = resistant phenotype, S = sensitive phenotype, P = gene present, NP = gene not present. Enumerations include isolates with at least the specific gene named; isolates may have more than one resistance gene (e.g. *qnrVC, gyrA* and *parC*)

[b] Fisher's Exact Test. Bold = statistically significant (P<0.05)

Supplementary Table 8. Test of association between antibiotic detection by mass spectrometry and AMR genotypes and phenotypes among *V. cholerae* clinical isolates

Antibiotic detection (D) and AMR genotype present (P) [a]					
	D/P	D/NP	ND/P	ND/NP	*P*[b]
CIP	41	7	3	0	1
CIP + NAL	35	4	9	3	0.334
DOX	16	8	20	7	0.759
DOX + TET	2	0	34	15	1
Antibiotic detection (D) and resistance phenotype (R) under aerobic conditions [a]					
	D/R	D/S	ND/R	ND/S	*P*[b]
CIP	24	24	3	0	0.238
CIP + NAL	22	17	5	7	0.511
DOX	1	23	0	27	0.471
DOX + TET	0	2	1	48	1
Antibiotic detection (D) and resistance phenotype (R) under anaerobic conditions [a]					
	D/R	D/S	ND/R	ND/S	*P*[b]
CIP	44	4	3	0	1
CIP + NAL	38	1	9	3	**0.036**
DOX	1	23	0	27	0.217
DOX + TET	0	2	1	48	1

[a] D=detected, ND=not detected, R=resistant by MIC, S=sensitive by MIC, P = Present, NP = Not present.

[b] Fisher's Exact Test. Bold = statistically significant (P<0.05)

Supplementary References

1. Drezen E, Rizk G, Chikhi R, et al. GATB: Genome Assembly & Analysis Tool Box. Bioinformatics **2014**; 30:2959-61.

2. Lees JA, Galardini M, Bentley SD, Weiser JN, Corander J. pyseer: a comprehensive tool for microbial pangenome-wide association studies. Bioinformatics **2018**; 34:4310-2.

3. Cook DE, Andersen EC. VCF-kit: assorted utilities for the variant call format. Bioinformatics **2017**; 33:1581-2.

4. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **2014**; 30:1312-3.

5. Saber MM, Shapiro BJ. Benchmarking bacterial genome-wide association study methods using simulated genomes and phenotypes. Microb Genom **2020**; 6.

6. Davydova N, Streltsov V, Wilce M, Liljas A, Garber M. L22 ribosomal protein and effect of its mutation on ribosome resistance to erythromycin. J Mol Biol **2002**; 322:635-44.

7. Hansen LH, Mauvais P, Douthwaite S. The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S ribosomal RNA. Mol Microbiol **1999**; 31:623-31.

8. Williams G, Smith I. Chromosomal mutations causing resistance to tetracycline in Bacillus subtilis. Mol Gen Genet **1979**; 177:23-9.

9. Wei Y, Bechhofer DH. Tetracycline induces stabilization of mRNA in Bacillus subtilis. J Bacteriol **2002**; 184:889-94.

10. Beabout K, Hammerstrom TG, Perez AM, et al. The ribosomal S10 protein is a general target for decreased tigecycline susceptibility. Antimicrob Agents Chemother **2015**; 59:5561-6.

11. Leclercq SO, Wang C, Zhu Y, et al. Diversity of the Tetracycline Mobilome within a Chinese Pig Manure Sample. Appl Environ Microbiol **2016**; 82:6454-62.

12. Grossman TH, Starosta AL, Fyfe C, et al. Target- and resistance-based mechanistic studies with TP-434, a novel fluorocycline antibiotic. Antimicrob Agents Chemother **2012**; 56:2559-64.

13. Noguchi N, Emura A, Matsuyama H, O'Hara K, Sasatsu M, Kono M. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2'-phosphotransferase I in Escherichia coli. Antimicrob Agents Chemother **1995**; 39:2359-63.

14. Chesneau O, Tsvetkova K, Courvalin P. Resistance phenotypes conferred by macrolide phosphotransferases. FEMS Microbiol Lett **2007**; 269:317-22.