A commensal-encoded genotoxin drives restriction of *Vibrio cholerae* colonization and host gut microbiome remodeling

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**Significance**

In a polymicrobial battlefield where different species compete for nutrients and colonization niches, antimicrobial compounds are the sword and shield of commensal microbes in competition with invading pathogens and each other. The identification of an *Escherichia coli*-produced genotoxin, colibactin, and its specific targeted killing of enteric pathogens and commensals, including *Vibrio cholerae* and *Bacteroides fragilis*, sheds light on our understanding of intermicrobial interactions in the mammalian gut. Our findings elucidate the mechanisms through which genotoxins shape microbial communities and provide a platform for probing the larger role of enteric multibacterial interactions regarding infection and disease outcomes.

Members of complex microbial communities that reside in environments such as the mammalian gut have evolved mechanisms of interspecies competition, which may be directed at resident microbial and host cells. While previous work has focused mainly on metabolic or niche competition, few specific intermicrobial targeting mechanisms have been elucidated in the mammalian gut. Here, we show that a genotoxin produced by commensal *Escherichia coli*, colibactin, which was previously shown to induce DNA damage in host intestinal cells, is also able to target via a contact-dependent mechanism a variety of enteric pathogens and commensals, including the important human diarrheal pathogen *Vibrio cholerae*. We find that colibactin-mediated killing depends on accumulation of intracellular reactive oxygen species, leading to DNA damage and loss of target cell fitness. We also show that the presence of colibactin is associated with cholera outcomes in a large human metagenomic dataset and that colibactin can shape the microbiome by species-specific targeting of a common human gut-associated microbe *Bacteroides fragilis*, suggesting that genotoxin-mediated mechanisms may have broad effects in the complex polymicrobial interactions that shape commensal microbial communities and their effects on host health and disease.

*Vibrio cholerae* is an enteropathogen that infects the human small intestine and causes the severe acute diarrheal disease cholera by using cholera toxin and toxin-coregulated pilus as the primary factors for virulence and colonization, respectively (8). Over the years, several engineered or natural commensals directly targeting virulence factors or virulence signaling have been reported that enable colonization resistance (2) thus offers the possibility of identifying insights into mechanisms of microbiome-mediated colonization resistance.

In this study, we began by identifying enteric commensals in the mouse intestine with deleterious effects upon *V. cholerae* infection. Through screening more than 100 lactose-fermenting enteric bacterial isolates from both the small and large intestines of adult mice, we found a handful of strains that could potently inhibit *V. cholerae* growth. In-depth analysis of one isolate, a B2 phylogroup *Escherichia coli* harboring a polyketide synthase (*pks*) island, demonstrated that the genotoxin colibactin encoded by the *pks* island is essential for antagonizing *V. cholerae* in vitro and during mouse colonization. Produced by commensal and pathogenic enterobacteria, colibactin is a natural genotoxin that damages double-stranded DNA via DNA cross-linking (15, 16). It is closely linked to tumorigenic effects in colorectal cancer (17), but its effects on other microbes or the gut microbiota are largely unknown. Here, we determine the DNA-damaging effects of *pks E. coli* on *V. cholerae* and explore their ecological effects on the gut microbiota.

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Results

Contact-Dependent Killing of *Vibrio* by an *E. coli* Isolate from the Mouse Intestines. To investigate how commensal bacteria in the gastrointestinal tract affect *V. cholerae* growth, we spread the intestinal contents of 10 CD-1 mice on MacConkey agar plates and selected over 140 pink colonies representing lactose-fermenting enterobacteria, such as *E. coli* and *Klebsiella*. These colonies were grown in Luria–Bertani (LB) medium overnight and mixed with *V. cholerae* C6706 (referred as *Vc* hereafter) in 96-well plates containing M9 minimal medium. The plates were incubated at 37°C for 24 h without shaking (mimicking the microaerobic condition that *Vc* may encounter during colonization of the small intestine), and the cultures were diluted and spotted on selective LB polymyxin B agar plates that only permit *Vc* growth. We identified nine isolates that significantly inhibited *Vc* growth (Fig. 1A). We then performed quantitative liquid assays and confirmed that these strains inhibited *Vc* growth, effectively reducing *Vc* populations from three to five orders of magnitude compared to *Vc* monocolon (Fig. 1B). Then, 16S rRNA sequencing indicated that all nine highly *Vc*-inhibiting strains were *E. coli*. As controls, coculture of either *E. coli* K12 strain MG1655 or MP1, a mouse commensal previously isolated from CD-1 fecal samples (18), had no effect on *Vc* growth (Fig. 1B). We selected F10, which was isolated from the small intestine, for further studies, as it was susceptible to PI transduction with higher efficiency (SI Appendix; Fig. S1), which facilitated downstream genetic manipulation. We named this *E. coli* strain *E. coli* mouse small intestine isolate 1 (EMS1). Genome sequencing of EMS1 (19) revealed it to be a member of the phylogenetic group B2. Whole-genome alignment of EMS1 with other *E. coli* strains showed homology with human uropathogenic *E. coli* CFT073 and 536 as well as with the probiotic human fecal isolate Nissle 1917 (SI Appendix, Fig. S2A). Average nucleotide identity (ANI) analysis of EMS1 with representatives of phylogenetic groups A, B1, B2, D, E, and S showed clustering of EMS1 with B2 strains with >98%ANI (SI Appendix, Fig. S2B).

**EMS1 Kills *V. cholerae* In Vitro.** We then more closely examined the effects of EMS1 on *Vc* growth. Coculture of *Vc* and EMS1 at different ratios displayed an EMS1 dose-dependent inhibition of *Vc* growth (Fig. 1C). Importantly, in the presence of EMS1, *Vc* populations grew to mid-log phase before crashing (Fig. 1D), suggesting that EMS1 effectively kills *Vc*. *Vc* is sensitive to low pH, but the killing effect from EMS1 was not due to growth medium acidification, as the pH was similar between cultures with and without EMS1 cocultivation (SI Appendix, Fig. S3). When we separated *Vc* and EMS1 using dialysis membranes with a 10-kDa molecular weight cutoff, EMS1 no longer exhibited bactericidal effects toward *Vc* (Fig. 1E), suggesting that the killing effect is likely contact dependent. An alternative explanation is that the membrane may prevent the active compounds produced by EMS1 from reaching *Vc*.

*V. cholerae* has over 200 known serotypes, but only strains with O1 and O139 antigens are known to cause epidemic disease (20). The O1 serogroup is further divided into O1 Classic and El Tor biotypes on the basis of other physiological properties. We examined whether EMS1 affected the growth of different *V. cholerae* strains. We mixed EMS1 with various *V. cholerae* strains (SI Appendix, Table S1) and found that EMS1 exhibited a killing effect toward all *V. cholerae* strains tested (Fig. 1F). Moreover, EMS1 also inhibited the growth of other Vibrio species, such as *V. fischeri*, *V. fluvialis*, and *V. harveyi*

These data suggest that EMS1 has a broad spectrum of anti-*Vibrio* activity.

**EMS1-Produced Colibactins Are Responsible for the Anti-*Vibrio* Activity.** To investigate the molecular mechanism responsible for EMS1 killing of *Vc*, we performed a genetic screen by transposon mutagenizing EMS1 and screening for mutants that lost the anti-*Vibrio* activity (Fig. 2A). From ~3,000 Tn5 mutants, we obtained 3 candidates in which anti-*Vibrio* activity was abolished (Fig. 2B). Sequencing analysis showed that these mutants all had transposons inserted in different loci of the *psb* genomic island (Fig. 2C, triangles represent transposon insertion), encoding a putative hybrid nonribosomal peptide synthetase–polypeptide synthase assembly line that is responsible for synthesis of colibactin (21). Alignment of the biosynthetic gene cluster (BGC) for colibactin shows that EMS1 harbors a colibactin BGC analogous to those of Nissle 1917, CFT073, and 536 (SI Appendix, Fig. S2A). To confirm the involvement of colibactin synthesis in EMS1’s anti-*Vibrio* activity, we constructed in-frame deletion mutants in *clbN*, encoding a synthase involved in the initiation of colibactin biosynthesis (22), and in *clbl*, encoding an amidase that is required for colibactin maturation (23). Deletions in either *clbN* or *clbl* in EMS1 completely abolished the vibriocidal effect (Fig. 2D). Complementation of *clbN* or *clbl* in trans restored the observed killing. These data suggest that the anti-*Vibrio* activity of EMS1 is contingent upon colibactin synthesis.

The colibactin BGC produces several intermediates that can be detected in crude cellular extracts of *E. coli* source strains. Liquid chromatography–mass spectrometry (LC–MS) analysis of a crude extract of EMS1 showed the presence of C14-t-Asn, an abundant diagnostic metabolite of the colibactin BGC (Fig. 2B). C14-t-Asn is both the hydrolysis product of an intermediate synthesized on ClbN as well as the ClbP-catalyzed cleavage product of precolibactin-1492 (24). Deletion of *clbN* abolishes production of C14-t-Asn, and deletion of the late-stage *clbl* gene retains production, as was previously observed using a plasmid-based colibactin BGC (25). We also observed several other characteristic colibactin BGC metabolites characterized previously (22, 26–28). It should be noted that proteins encoded in the colibactin BGC are involved in the biosynthesis of other metabolites that are potentially bioactive (29, 30). However, both *clbN* and *clbl* mutants were deficient for killing *Vibrio*, suggesting that EMS1 produces classical colibactin that directly antagonizes *Vibrio*. We could detect both left and right ends of the intermediates (SI Appendix, Fig. S4), implying that these strains likely utilize the same inhibitory mechanisms as that of EMS1.

**EMS1-Produced Colibactin Inhibits *V. cholerae* Colonization of the Small Intestine.** Next, we used a streptomycin-treated adult mouse model to test whether EMS1 impacts *Vc* colonization in vivo. One day before *Vc* infection, we preinoculated mice with either a blank control or overnight cultures of either EMS1 or ∆*clbN* mutant. We examined *Vc* colonization of the small intestine 3 d postinfection. We found that compared to the blank inoculation, *Vc* colonization was reduced nearly 100-fold in the mice preinoculated with EMS1, whereas precolonization with ∆*clbN* mutants did not affect *Vc* colonization (Fig. 3A). However, this result was not due to differential colonization of EMS1 itself, since both the wild-type EMS1 and the ∆*clbN* mutant colonized equally well in the mouse small intestine (Fig. 3B). These data suggest that EMS1 inhibits *Vc* colonization in vivo and that the inhibition is dependent on the production of colibactin.
The Presence of Colibactin in the Gut Microbiome Is Associated with Less Severe Outcomes of *V. cholerae* Exposure in Human Populations. To examine whether genes involved in colibactin production are associated with divergent cholera outcomes in human populations, we performed an analysis of a shotgun metagenomic deep-sequencing study (Dataset S1) examining the fecal metagenome of a prospective human cholera exposure cohort. Household contacts were enrolled within 6 h of presentation of the index household cholera case and tracked for infection status. Individuals who developed diarrhea were categorized as exposed and symptomatic ("SYM"), and those without diarrhea but whose culture and/or serum tested positive were considered exposed and asymptomatic ("ASYM"). The rest were classified as uninfected ("UNI"). Our analysis of the published shotgun metagenomics sequencing of fecal specimens from these cohorts showed that the relative abundance of reads mapping to the *clb* biosynthetic cluster differed based on exposure outcome; *clb* reads were significantly lower in the fecal microbiomes of SYM contacts than in both ASYM and UNI (Fig. 3C). These results thus suggest that the presence of colibactin in the gut microbiome may be associated with different outcomes to *V. cholerae* exposure in humans.

Colibactin Produced by EMS1 Induces DNA Damage in Mammalian Cells and Waxworm Larvae. A signature property of colibactin is the induction of DNA double-strand breaks (DSBs) in mammalian cells (16). To confirm that EMS1 also produces colibactin with this feature, we first monitored the phosphorylation of histone H2AX (γH2AX), a sensitive marker of DSBs (21), in HeLa cells infected with EMS1 or Δ*clbN* mutants for 4 h. We found that EMS1, but not Δ*clbN* mutants, induced γH2AX foci (green) on mitotic chromosomes (stained by pH3-red)
Fig. 2. EMS1 produces colibactin that inhibits *V. cholerae* growth. (A) Screening scheme for EMS1 genes responsible for anti-*Vibrio* activity. (B) Effects of coculturing transposon mutants of EMS1 on *V. cholerae* growth. *V. cholerae* alone or *V. cholerae* with different EMS1 mutants were inoculated into M9 medium and incubated standing at 37°C for 24 h. *V. cholerae* CFU were then determined. (C) BGC of colibactin. The EMS1 colibactin BGC was extracted and plotted using EasyFig (65). Commonly observed colibactin intermediates (or hydrolysis byproducts) (1 to 4) are drawn as products of 

(31), suggesting that EMS1 produces colibactin that can induce DNA damage in mammalian cells in vitro (*SI Appendix, Fig. S5A*).

We used larvae of the wax moth *Galleria mellonella* as a model to examine whether EMS1 induces DNA damage in vivo. We injected 10 μL (10^7 colony-forming units [CFU]) of either EMS1 wild type or ΔclbN mutant into the larvae. Only 25% of larvae were alive at 48 h after infection for wild type, whereas more than 75% of larvae survived ΔclbN injection, indicative of colibactin-induced lethality in wax moth larvae (*SI Appendix, Fig. S5B*). We quantified plasma phenoloxidase (PO) activity, a proxy for immune response, in EMS1-infected insects. We found that PO levels in larvae infected with wild-type EMS1 were significantly higher than in ΔclbN-treated insects (*SI Appendix, Fig. S5C*). Similarly, the levels of antimicrobials in the hemolymph of larvae infected with EMS1...
were higher than those found in ΔclbN-infected larvae (SI Appendix, Fig. S5D), suggesting that colibactin triggers the production of antimicrobial factors. To examine whether EMS1 infection induces DNA damage in larvae, we performed comet assays (32) to detect DNA lesions in larval hemolymph cells exposed to EMS1 or ΔclbN for 48 h. The comet tail moment increased in EMS1-infected cells compared with the phosphate-buffered saline and ΔclbN controls (SI Appendix, Fig. S5 E and F), indicating that colibactin produced by EMS1 induces DNA damage in vivo.

Colibactin Induces *V. cholerae*’s SOS Response and Reactive Oxygen Species Accumulation under Microaerobic Conditions. Next, we investigated how EMS1-produced colibactin serves as an antibiotic against *V. cholerae*. We first examined whether oxygen levels affect colibactin function. Compared to microaerobic conditions, EMS1 grown under aerobic conditions was incapable of killing *Vc* (Fig. 4A). colB is the first gene of the clb operon. We measured the expression of a clbB–lacZ transcriptional fusion and found that clbB was more highly expressed under oxygen-limiting conditions (Fig. 4B), consistent with the observation that more effective killing occurred under static microaerobic conditions, which *V. cholerae* encounters during infection.

Colibactin induces DSBs in eukaryotic cells (SI Appendix, Fig. S5 and ref. 16). We examined whether EMS1-produced colibactin might similarly induce DNA damage in *V. cholerae* cells. We constructed a chromosomal recA–gfp transcriptional fusion in *Vc* to monitor DNA damage. DNA-damaging agents such as fluoroorquinolones activate the SOS response pathway, including recA (33); as expected, sublethal doses of ciprofloxacin strongly activated recA expression in *Vc* (SI Appendix, Fig. S6). Compared to *Vc* alone, cotreatment with EMS1, but not with ΔclbN, significantly induced recA–gfp expression in *Vc* cells (Fig. 4C). These results suggest that colibactin produced by EMS1 may cause DNA damage in *Vc* cells. We also measured intracellular reactive oxygen species (ROS) accumulation using the redox-sensitive, cell-permeable dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Compared to monocolonized *Vc*, the presence of wild-type EMS1, but not of ΔclbN, resulted in significantly more accumulated ROS in *Vc* (Fig. 4D). Although it is disputed whether ROS production is a common effect of antibiotics that cause antimicrobial killing (34–36), we speculate that elevated ROS levels due to EMS1-produced colibactin are likely detrimental to *Vc* survival.

Colibactin-Dependent Growth Inhibition toward Other Bacterial Species. To test whether EMS1 inhibits the growth of bacteria other than *Vibrio* in a clb-dependent manner, we cocultured either wild-type EMS1 or ΔclbN mutants with a panel of mostly pathogenic bacteria (Fig. 5A and SI Appendix, Table S2). We found that EMS1 did not affect the growth of other *E. coli* strains tested nor did it affect *Acinetobacter, Pseudomonas, Bacillus, Enterococcus*, or *Streptococcus*. However, EMS1 inhibited growth of one *Enterobacter aerogenes* strain and three *Staphylococcus* strains tested (Fig. 5A). It also mildly affected two *Clostridium difficile* strains. In one previous study (37), it has been shown that pks* E. coli elicits antibiotic activity against a number of *Staphylococcus aureus* clinical isolates, but the killing efficiency is relatively low (~fourfold). Here, EMS1 inhibited *S. aureus* growth by one to three orders of magnitude. This could be due to the potency of colibactin production by EMS1 or differences in tested culturing conditions.

To better understand why EMS1 can kill some bacteria but not others, we conducted a genomic library screen (Fig. 5B). We first constructed a plasmid-based genomic library of *E. coli* MG1655, which is resistant to EMS1 (Fig. 5A), and introduced the library into *Vc*. The resulting *Vc* transconjugants were screened for resistance to EMS1. From ~5,000 clones, we identified 1 *Vc* isolate harboring a ~6-kilobase MG1655 fragment (Fig. 5C) that increased *Vc* EMS1 survival rate by 100-fold over the vector control (Fig. 5D). Sequencing of the *E. coli* fragment revealed it to contain the recG gene, encoding a DNA helicase in *E. coli* that plays a role in DNA DSB repair. Overexpressing this *E. coli* recG in *Vc* effectively prevented EMS1 killing (Fig. 5D) and reduced EMS1-mediated ROS accumulation (Fig. 5E). These results reinforce the idea that the mechanism of colibactin killing of *Vc* is through induction of DNA damage and that the bacteria resistant to EMS1 killing may
Colibactin Expression and Specific Microbial Targeting Is Associated with Altered Microbiota Composition in Humans and Mice.

In cholela-endemic areas, human gut microbiomes recurrently enter a dysbiotic state characterized by low microbial diversity after disruption by cholera and noncholera diarrhea, malnutrition, and other regular environmental insults (6, 38). This has been demonstrated to be a driver of disease susceptibility through the loss of key biochemical functions, such as microbial quorum sensing and bile acid metabolism (5). Colibactin-specific effects on the gut microbiome under specific conditions have been previously reported in mice. A prior study inoculated pregnant mice with colibactin-producing or colibactin-knockout E. coli and evaluated the response of the gut microbiome of the resulting pups (39). Using this mother-to-pup transmission mouse model, they observed colibactin-dependent phylum-level changes and a reduction in overall abundance 35 d after birth. While this is a measure of the effect of colibactin-producing microbes on microbial succession, or the ordered colonization of microbes after birth, the microbes found within immature animals and humans can differ dramatically from those of adults (40, 41). The long time horizons of these studies, coinciding with early life development and dietary transition after weaning, may also obscure specific colibactin-dependent changes in the gut microbial community. To probe commensal–colibactin interactions that are diet and host development independent that may be relevant in human populations, we colonized streptomycin-treated mice with either EMS1 or ΔclbN mutant. One day before E. coli was inoculated, streptomycin was removed from the drinking water, and the fecal gut microbial community was monitored by 16S ribosomal RNA gene sequencing (42) (Dataset S2). We found that both EMS1 and ΔclbN mutants colonized the colon similarly (SI Appendix, Fig. S7), suggesting that colibactin is not required for EMS1 colonization in this model. In contrast to Tronnet et al. (39), we identified rapid and strong community-level differences and species-level microbial taxa in the fecal microbiome of these animals that varied strongly as a function of the presence of EMS1 versus the isogenic ΔclbN strain. Using principal coordinates analysis of weighted UniFrac distances between fecal microbiomes, we demonstrated that fecal microbial communities diverged strongly and significantly between mice colonized with EMS1 and ΔclbN 5 d postcolonization (Fig. 6A). At the species level, our data show a dramatic depletion of Bacteroidetes fragilis and Enterococcus species in mice and the simultaneous enrichment of Aerococcus sp., Clostridium paraputrificum, Actinomycetales sp., and Coprococcus sp. (Fig. 6B and SI Appendix, Fig. S8).

Since several recent studies have demonstrated that the structure of the human gut microbiome can be a driver of V. cholerae infection outcomes, we next aimed to describe commensal gut microbiome changes associated with microbial colibactin production. We used MetaPhlAn3 analysis, which predicts the taxonomic composition of microbiomes from the human prospective cholera cohort metagenomic data to examine taxa that exhibited differential relative abundance between clb+ and clb− individuals in either SYM, ASYM, or UNI groups (SI Appendix, Fig. S8). In general, we observed more similar microbial abundance patterns within the ASYM and SYM groups than in the UNI population, which exhibited higher intragroup variation. Several species belonging to the genera Corynebacterium and Prevotella were more likely to coexist with the colibactin-producing strains in SYM microbiomes, but intragroup variation was observed, with Corynebacterium afermentans and Prevotella denticilium exhibiting lower relative abundance in the presence of clb− individuals. Treponema bermilense, Campylobacter jejuni, and Trueperella bernardiae abundance was higher in the clb− subpopulation of the UNI group, while Bifidobacterium bifidum, Tissierella bacterium KA00581, and Mycoplasma hominis were reduced. Species belonging to Enterococcus displayed decreased abundance in clb−-infected contacts except Enterococcus cecorum in ASYM individuals. Among species in the genus Bacteroides, Bacteroides caccae and Bacteroides plebeius were higher. Strikingly, levels of B. fragilis were lower in clb+ ASYM individuals, similar to in vitro and in vivo killing results in microcosms with EMS1 or clb+ mutants (Fig. 6C).

We confirmed that colibactin-producing E. coli are able to target B. fragilis by repeating our in vitro assays with B. fragilis...
and either EMS1 or ΔclbN and showed strong colibactin-driven killing of *B. fragilis* by EMS1 (Fig. 6D). To confirm that this interaction occurs in vivo, we coin introduced *B. fragilis* and either EMS1 or ΔclbN into suckling mice that had their native microflora cleared with streptomycin treatment before gavage; the presence of functional colibactin biosynthesis again corresponded to significantly reduced *B. fragilis* colonization (Fig. 6D). Interestingly, this effect is species specific, as in vitro assays demonstrated no colibactin-dependent killing of other *Bacteroides* species, such as *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* (Fig. 6F). Taken together with in vitro survival assays (Fig. 5A), these findings again suggest that colibactin antibacterial effects may be highly species or strain specific outside of *Vibrio*. In complex fecal communities, colibactin-driven differences in individual species abundance may also be indirectly mediated via targeting of members of microbial syntrophic relationships or overall changes in gut environmental conditions.

Further comparisons of murine and human microbiome datasets revealed additional similarities in pattern. We examined clb+ and clb− humans by exposure outcome class for the abundance of species belonging to the genera *Bacteroides*, *Clostridium*, *Enterococcus*, and *Coprococcus* and order *Actinomycetales*, which showed the strongest colibactin-dependent differences in vivo in colonized mice (Fig. 6G and SI Appendix, Fig. S9). In accordance with the mouse gut microbiome findings, *B. fragilis* was significantly reduced in the clb+ individuals in both ASYM and SYM groups. Species specificity in association with clb status was again observed, with clb− individuals showing higher relative abundance of *B. plebeius* and *B. caccae*.
Colibactin drives changes in the gut microbiome in mice and humans and specifically kills \textit{B. fragilis}. (A) PCoA of mouse microbial communities from fecal samples based on weighted UniFrac distance. Ellipses show 95% confidence intervals. Statistical tests by permutational multivariate ANOVA indicate significantly different microbiome structures between the \(\Delta clbN\) mutant and EMS1; \(\Delta clbN\), \(n = 4\); EMS1, \(n = 5\). \(* P < 0.05\); \(** P < 0.01\). ns, no significance. (B) Relative abundance of \textit{B. fragilis} in EMS1- and \(\Delta clbN\)-treated animals day 5 after inoculation. Mann–Whitney U test; \(* P < 0.05\); \(** P < 0.01\). (C) Extrapolated \textit{B. fragilis} relative abundances by MetaPhlAn3 analysis in presymptomatic fecal samples of humans exposed to \textit{V. cholerae} by presence of colibactin biosynthetic genes; \(** P < 0.01\) edgeR. (D) Colibactin effects on \textit{B. fragilis} growth in vitro. Approximately 10^6 CFU/mL overnight cultures of \textit{B. fragilis} tested were inoculated into M9 plus 1% BHI (Brain Heart Infusion) with 10^7 CFU/mL of EMS1, \(\Delta clbN\), or \(\Delta clbL\) and incubated at 37 °C anaerobically. After 24 h, CFU values of assayed strains were determined, and the survival rate was calculated by dividing CFU with \textit{E. coli} by CFU without \textit{E. coli}. The mean of three independent assays is shown, with error bars representing SDs; \(** P < 0.01\); \(**** P < 0.0001\). (E) Effects of coinoculation of EMS1 and \(\Delta clbN\) in suckling mice. Levels of \textit{B. fragilis} in small intestine determined by plating after 16 h of colonization. Mann–Whitney U test; \(* P < 0.05\). (F) Colibactin does not kill related \textit{Bacteroides} commensals. Commensal \textit{Bacteroides} strains were incubated with EMS1, \(\Delta clbN\), or \(\Delta clbL\) at 37 °C anaerobically. After 24 h, CFU values of assayed strains were determined, and the survival rate was calculated by dividing CFU with \textit{E. coli} by CFU with \textit{Bacteroides} grown alone. The mean of three independent assays is shown, with error bars representing SD. (G) Distribution of the species in genera exhibiting highest colibactin-dependent changes in mice from household contacts of confirmed cholera patients in Bangladesh. Species with relative abundance >1% in at least one sample across the whole dataset (Dataset S3) are shown; edgeR; \(* P < 0.05\); \(** P < 0.01\); \(*** P < 0.001\).
in the ASYM group and _Bacteroides cauli-galum_ in SYM individuals. _Actinomyxodes_, _T. bernardiae_, and _Actinomyces tunicensis_ were enriched in SYM/UNI and ASYM individuals separately. _Enterococcus biurea_ was observed at a lower abundance in the ASYM _clb_ population, while _E. ceceorum_ exhibited the opposite trend. Taken together these data demonstrate that in vitro and in vivo in humans and mice, colibactin production is associated with strong effects on gut commensal bacteria, providing direct evidence that this commensal-derived genotoxin plays a role in shaping the enteric microbiome and in modulating the infectiousness of pathogenic bacteria.

**Discussion**

The host gut microbiota has a significant impact on the susceptibility of hosts to infection by enteropathogens, including _V. cholerae_ (43, 44). Gut commensals are known to produce antagonistic metabolites against _V. cholerae_ infections. Here, we identified a _iks_ B2 family commensal _E. coli_, EMS1, from the adult mouse intestine that can antagonize _V. cholerae_ growth in vitro and gut colonization in vivo. The anti-_Vibrio_ activity of EMS1 depends on the production of the genotoxin colibactin. We demonstrated that EMS1 colibactin damages bacterial DNA and reshapes the host gut microbiota, thus exemplifying the multifaceted role of a commensal-produced genotoxin in contributing to the dynamics of the host gut microbiome.

**Anti-_Vibrio_ Commensals and Their Mechanisms of Action.** Commensal microbes protect hosts from _V. cholerae_ colonization and infection (2, 45). Studies of individual gut microbes have revealed a number of possible mechanisms contributing to colonization resistance against _V. cholerae_. _Lactobacillus_ species from cholera patient fecal samples (13) or from the stool of healthy children (46) were reported to produce diffusible compounds inhibitory against _V. cholerae_ or its in vitro biofilm formation and dispersal. Oral feeding of infant mice with _Lactobacillus_ lactis reduced _V. cholerae_ growth and colonization in vivo, depending on the production of lactic acid (9). A commensal _E. coli_ from a healthy human gut or probiotic _E. coli_ Nissle 1917 can metabolize glucose to lower pH to kill _V. cholerae_ in vitro (47) or reduce _V. cholerae_ colonization in a zebrafish model (12). _V. cholerae_ is sensitive to acidic pH (pH < 5), and lowering local environmental pH by gut microbes provides an effective mechanism to prevent _V. cholerae_ infection. The mouse commensal EMS1 potently inhibited _V. cholerae_ growth in vitro (Fig. 1) and conferred colonization resistance in vivo (Fig. 3), and it is likely to use a different antagonistic mechanism because coculture did not lower pH compared to _Vc_ monoculture (SI Appendix, Fig. S3).

Gut commensals can also secrete small molecules to interfere with the virulence signaling of _V. cholerae_. _Blautia obeurn_, a human gut commensal, provided major colonization resistance to _V. cholerae_ via production of autoinducer-2 (AI-2) (6). AI-2 is a cell density–dependent signal molecule that mediates bacterial cell–cell communication; _B. obeurn_ AI-2 led to repression of several factors critical for _V. cholerae_ colonization. You et al. (14) reported that a mouse commensal _B. vulgatus_ suppresses _V. cholerae_ infection in mice by competing for compounds that enhance _V. cholerae_ proliferation and promoting the accumulation of compounds inhibitory toward _V. cholerae_ growth, including short-chain fatty acids. Here, we found that EMS1-mediated antagonism relies on a _iks_ genomic island frequently found in _E. coli_ belonging to the B2 phylogroup and encoding the genotoxin colibactin. _iks_ _E. coli_ have been linked to human colorectal cancer, as the secondary metabolite colibactin, functioning as a genotoxin, cross-links DNA, transforms gut epithelial cells, and contributes to tumorigenesis (15, 16). The discovery of bactericidal activity of _iks_ _E. coli_ suggested that this family of bacteria may protect the host from pathogen infections as well as affect the composition of gut microbiota more generally.

**_iks_ _E. coli_ and Colibactin.** EMS1 was isolated from the small intestine of an adult mouse, a niche where _V. cholerae_ colonization takes place. EMS1 shares extensive genomic synteny with _iks_ _B2 E. coli_, including uropathogenic _E. coli_ CFT073 and the probiotic _E. coli_ Nissle 1917 (SI Appendix, Fig. S2), both of which are _iks_ _B2 E. coli_. These _iks_ _E. coli_ strains all produce colibactin and are frequently found in colon cancer patients and, to a lesser extent, in healthy individuals. EMS1 colibactin potently killed _Vibrio_ spp. and moderately inhibited other pathogens, including _E. aerogenes_ and a subgroup of _Staphylococcus_ spp. These findings expand upon a recent report that found that colibactin exhibited modest antibiotic activity to _S. aureus_ (37) and also suggest that colibactin is able to act like a bacteriocin against diverse microbial targets in the gut. In _V. cholerae_, colibactin killing is cell contact dependent (Fig. 1E and ref. 21), and this may result from the highly unstable nature of colibactin or contact requirements for colibactin secretion or delivery. Production of colibactin in other _iks_ _E. coli_ is affected by a number of genetic and environmental cues (15), including iron and carbon sources. We found that EMS1 _clbB_ gene expression and colibactin killing was activated when EMS1 was grown in low-nutrient medium with glucose microaerobically (Fig. 4), but no killing effect was detected in rich LB medium or aerobically (shaking). Such differences again may reflect the unstable and contact-dependent nature of colibactin. Colibactin cross-links interstrain DNA and induces DNA DSBs due to a unique structure that has two electrophilic cyclopropane warheads at both ends (48, 49). EMS1 colibactin caused DNA damage responses in HeLa cells and in hemo-lymph cells of wax moth (SI Appendix, Fig. S5), as expected due to its known role in damaging eukaryotic DNA. EMS1 colibactin also damaged _V. cholerae_ DNA, activated the SOS response, and induced intracellular ROS accumulation (Fig. 4), suggesting that a possible mechanism of colibactin-mediated bacterial killing is through DSB-caused cell death. Interestingly, many bacteria, including _E. coli_, are resistant to colibactin killing due to production of a cyclopropane hydrolase _Col_ that directly inactivates colibactin (50, 51). The discovery that ectopic expression of _recG_, an _E. coli_ DNA helicase gene that repairs DNA DSBs (52), can suppress ROS accumulation and protect _Vc_ from killing by colibactin (Fig. 5) further supports a role for colibactin in inducing DSBs to kill bacteria. We speculate that bacteria possessing either _clbS_ or a robust DNA repair system may be more resistant to _iks_ _E. coli_ antagonism.

**Colibactin and the Gut Microbiota.** In addition to an inhibitory effect toward both _V. cholerae_ and other gut-associated bacteria in vitro, EMS1 directly inhibited _V. cholerae_ colonization and affected gut microbiome composition by driving lower levels of _B. fragilis_ in an adult mouse model, which was confirmed in direct in vitro killing assays. These data provide direct evidence that _E. coli_–derived colibactin plays a role in shaping the enteric microbiome, thereby potentially modulating the metabolic functions of the gut commensal communities and thus interactions with invading pathogens. An analysis of the metagenomic data of household contacts of cholera patients
showed a relatively lower abundance of pks islands in infected SYM contacts as opposed to those infected ASYM or UNI household contacts, suggesting that the effects of colibactin may be associated with cholera outcomes in human populations. Using antibiotic-treated adult mice, we identified potential species that respond to colibactin, observing depletion especially of B. fragilis and Enterococcus sp. but enrichment of Aerococcus sp., C. paraputrificum, Actinomycetales sp., and Coprococcus sp. These findings were mirrored in the human gut microbiome data, where we found that B. fragilis and E. hirae were decreased in communities containing the clb cluster, results that were confirmed with B. fragilis using in vitro colibactin-dependent killing assays. B. fragilis is a common enteric commensal that is able to modulate the maturation of the immune system (53), although toxigenic strains are responsible for anaerobic infections in several body sites (54) and for infectious diarrhea in Bangladesh (55, 56). An association of B. fragilis with colibactin-producing E. coli in interspecies biofilms exists in colorectal cancer patients (57), with an interaction between colibactin+ E. coli and toxigenic B. fragilis leading to modulation of tumorigenesis in susceptible animals. However, previous work has not identified a role for colibactin-driven killing of either toxigenic or commensal B. fragilis, no significant changes in the levels of B. fragilis and pks+ E. coli were noted upon cocolonization. Other genotoxins have also been associated with microbiome changes in mice, including the cytotoxicid distending toxin (CDT) from C. jejuni (58). C. jejuni was enriched in communities containing clb in UNI individuals. While specific microbial CDT targeting is not described, these findings highlight the possibility of specific interactions between microbial genotoxins in modifying complex gut communities. In contrast to prior studies, we demonstrated the growth-inhibiting effects on V. cholerae of colibactin derived from a native mouse isolate, expanding the functional range of colibactin to include antibacterial functions in addition to activity against eukaryotes.

Serving as a disruptive and competitive factor, colibactin can affect the species composition and thus the biochemical environment of host environments encountered by pathogenic bacteria. Colibactin-dependent effects on commensal and pathogenic bacteria may work via different mechanisms, whether by direct contact- or proximity-dependent killing for V. cholerae or indirectly through modulation of the fitness or metabolic activities of commensals that produce necessary metabolites for other members of the community. Bactericidal function may also be modulated by other environmental factors besides proximity; in studies of colorectal cancer tissues (57), an association with pks+ E. coli and enterotoxigenic B. fragilis was noted, although no colonicization differences as a function of cocolonization were observed. Our findings also highlight the strain specificity of commensal–pathogen interactions and the limits of 16S amplicon sequencing approaches in identifying these. Previous studies showed that the presence of some murine commensal E. coli was able to promote virulence (59); highly strain-specific associations of E. coli with infection outcomes are likely in human populations, especially given that phyotype B2 group E. coli are variably distributed in humans (60) and that the observed intragenus variance in the ability of colibactin to kill both pathogenic and commensal gut-associated bacteria. The regulation of colibactin production by nutrient condition further complicates our understanding of how this factor integrates into interspecies dynamics in complex microbiomes; rapid changes in the nutritional environment of the gut can be driven by host diet and commensal microbial metabolism. Further experimentation will be required to elucidate the complex potential interactions between specific commensal genotoxin targets and their subsequent effects on gut microbiome structure.

Materials and Methods

Strains, Plasmids, and Culture Conditions. V. cholerae El Tor C6706 was used as the primary wild-type strain in this study. The E. coli strain EMS1 was isolated from the small intestine of a CD-1 adult mouse. Both V. cholerae and E. coli were propagated in LB Miller medium with appropriate antibiotics at 30°C and 37°C, respectively, unless otherwise noted. All other bacterial strains used in this study are described in SI Appendix, Tables S1 and S2. The detailed description of constructs of deletion strains and transcriptional reporters is provided in the SI Appendix, Supplemental Methods and Materials.

V. cholerae Inhibitory Assays by EMS1. Unless otherwise indicated, 105 CFU/mL V. cholerae from overnight cultures was inoculated alone or with 106 CFU/mL EMS1 or EMS1-derived mutants into M9-glucose medium (10 mM HEPES, pH 7.4, was added to buffer pH). The cultures were incubated at 37°C microaerobically (no shaking) for 24 h. Viable V. cholerae cells were determined by serial dilution and plating on selective LB plates (usually containing streptomycin [100 µg/mL] and polymyxin B [6 µg/mL]).

Screens of EMS1 Mutants that Lost Anti-Vibrio Activity. Approximately 3,000 Tin5 insertional mutants of EMS1 were inoculated into 96-well plates containing M9 medium with C6706 (pBAD-lux) and incubated at 37°C for 24 h. LB medium containing 0.1% arabinose (to induce pBAD-lux) and streptomycin (to inhibit EMS1 growth) was added, and the plates were incubated at 37°C with shaking for 2 h. Luminescence was then measured. Transposon mutants that were unable to inhibit V. cholerae growth, as identified by high luminescence wells, were then purified, and transposon insertion was determined using arbitrary PCR (61) and sequencing.

Identification of Colibactin-Resistant Factors. The E. coli MG1655 genomic library plasmids were introduced into V. cholerae by conjugal transfer. The V. cholerae library was then incubated with EMS1 (pMal-2x) in M9 medium microaerobically for 24 h at 37°C. The recovered V. cholerae cells were incubated with EMS1 for another round of selection. The resistant V. cholerae isolates were purified, and the plasmid was extracted for sequencing analysis.

Human Metagenomic Data Analysis. The Bangladesh human metagenomic data were obtained from Levade et al. (62), accession number PRJNA608678. The results from a total of 82 individuals, for individuals that had multi-time point samples, were summed up. To test the presence of clb, the human metagenomic reads were aligned to the clb cluster (clbA-S) using blasr, with the E value cutoff of 0.00001. MetaPhlAn3 (63) was then used to profile the composition of the gut microbial community of each individual at day 2. To obtain species that might respond to colibactin, edgeR (64) was used to calculate the fold change and significance of intergroup differences. Species reaching a maximum relative abundance of at least 1% in at least one sample were retained for further analysis.

Data Availability. The complete genome sequence of E. coli EMS1 is deposited in the National Center for Biotechnology Information (NCBI) GenBank (accession no. CP072942). The 16S sequence data have been deposited in NCBI under BioProject number PRJNA768438. All other study data are included in the article and/or supporting information.

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