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Identification of the Colicin V Bacteriocin Gene Cluster by Functional Screening of a Human Microbiome Metagenomic Library

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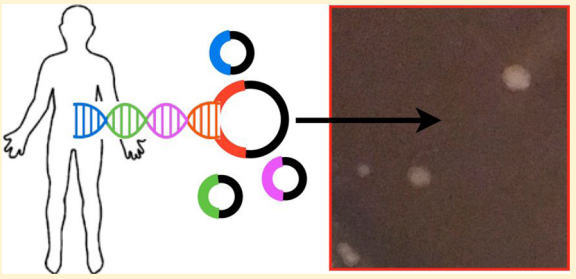
ABSTRACT: The forces that shape human microbial ecology are complex. It is likely that human microbiota, similarly to other microbiomes, use antibiotics as one way to establish an ecological niche. In this study, we use functional metagenomics to identify human microbial gene clusters that encode for antibiotic functions. Screening of a metagenomic library prepared from a healthy patient stool sample led to the identification of a family of clones with inserts that are 99% identical to a region of a virulence plasmid found in avian pathogenic *Escherichia coli*. Characterization of the metagenomic DNA sequence identified a colicin V biosynthetic cluster as being responsible for the observed antibiotic effect of the metagenomic clone against *E. coli*. This study presents a scalable method to recover antibiotic gene clusters from humans using functional metagenomics and highlights a strategy to study bacteriocins in the human microbiome which can provide a resource for therapeutic discovery.



KEYWORDS: *microbiome, metagenome, bacteriocin, eDNA, antibiotic*

The human microbiome is home to hundreds of unique bacterial species whose collective function is believed to have a role in human health and disease.[1,2] The mechanisms through which diverse bacterial species are able to form a stable ecosystem in humans are poorly understood.[3] It is likely, however, that microbiota-produced antibiotics play an important role in the ecology of the human microbiome, as has been seen in other ecosystems, and that these antibiotics may represent a resource for therapeutic discovery.[4,5] The inability to culture many bacterial species found in the human microbiome limits the utility of traditional culture-based methods for identifying the antibiotics they produce. Functional metagenomic screening methods circumvent the culturing requirement and provide a culture-independent screening approach to characterize bioactivities encoded in the collective human microbial metagenome. Such studies rely on cloning large fragments of DNA extracted directly from environmental samples (environmental DNA, eDNA) into a model bacterial host and screening the resulting eDNA clones for phenotypes associated with a desired trait.[6] Here, we used functional metagenomics to identify an antibacterially active clone in a cosmid library of DNA extracted from the stool of a healthy patient.

A single stool sample was collected from a healthy patient, defined as a patient having no known bowel disease, bowel surgery, antibiotic use in the previous 6 months, or symptoms suggestive of an intestinal disease. A metagenomic library was created using an established method for constructing cosmid-based metagenomic libraries from human stool.[7] Briefly, 6 g of fresh stool was washed twice with a sodium chloride solution (0.9%). The washed stool was collected by centrifugation and resuspended in the same sodium chloride solution. Approximately 15% of this sample was partitioned by Nycodenz gradient centrifugation. The upper layer containing commensal bacteria was removed, and bacteria were pelleted by centrifugation. The pellet was resuspended in lysis buffer and incubated at 70 °C for 2 h. eDNA was isopropanol precipitated from the resulting bacterial lysate, washed with 70% ethanol, and resuspended in TE (Tris-EDTA) buffer. This crude eDNA extract was then separated by preparative agarose gel electrophoresis and high molecular weight (~40 kb) DNA was electroeluted from the DNA compression band at the top of the gel. Purified high molecular weight DNA was blunt ended, ligated with the broad host-range cosmid vector pJWC1, packaged into lambda phage, and transfected into *E. coli* EC100, and transformants were selected using tetracycline (15 μg/mL).[8] Titers obtained from the initial library plating step indicated the stool metagenomic DNA library contained in excess of 1×10^6 unique cosmid clones. The final metagenomic library was washed from the selection plates with 10% glycerol and archived at −80 °C as one combined glycerol stock.

While the metagenomic library we constructed contains in excess of 1×10^6 unique cosmid clones, deep sequencing

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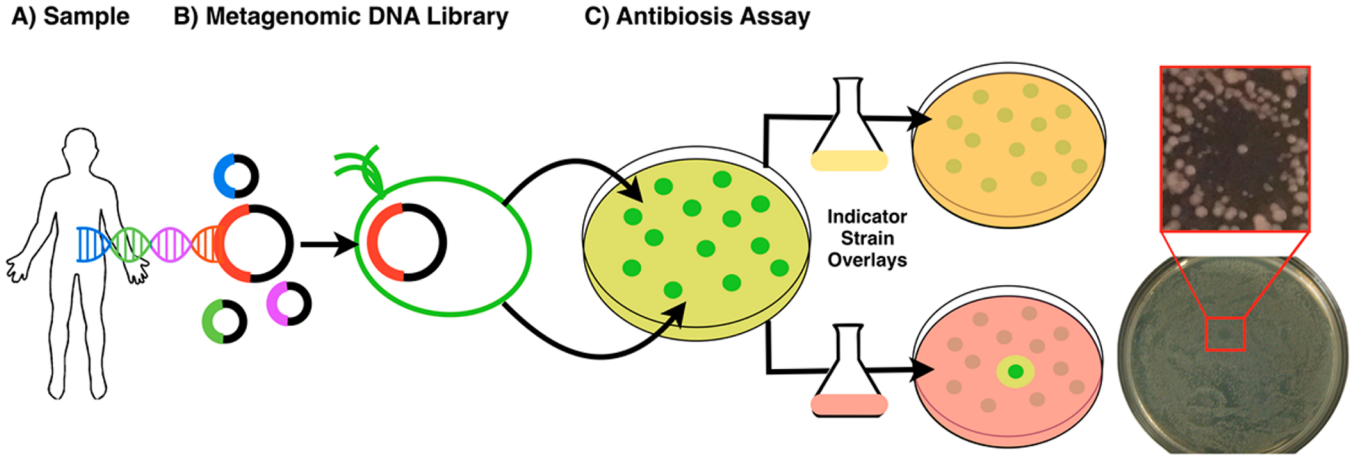

Figure 1. Functional metagenomic screen for antibiotics. (A) Samples of metagenomic DNA can be isolated from any part of the human microbiome and cloned into a cosmid DNA library (B). Cosmids containing metagenomic DNA are introduced into heterologous bacterial hosts, where foreign metagenomic DNA is expressed. (C) Metagenomic clones are plated on agar and allowed to produce potential antibiotics encoded on the piece of metagenomic DNA. Indicator strains are overlaid on top of the metagenomic clones, and active metagenomic clones are identified by inhibition of growth of the indicator strain, producing a zone of clearing. Pictured is an active metagenomic clone inhibiting the growth of an *E. coli* indicator strain.

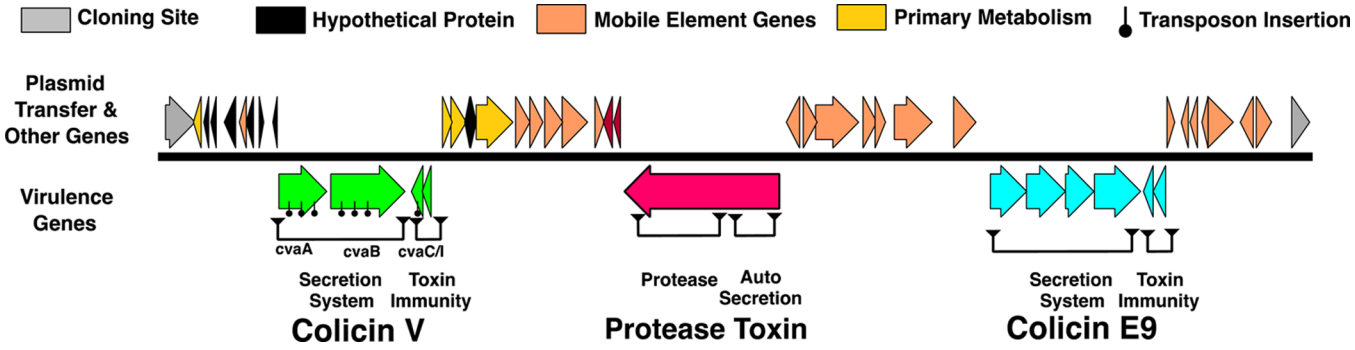

Figure 2. Active metagenomic clone DNA sequence. The metagenomic DNA insert contains three gene clusters with predicted virulence functions including colicin V, colicin E9, and a protease toxin. The flanking regions of the DNA contain mobile element genes. Transposon insertion sites in the genes of the colicin V cluster are pictured.

studies of human microbiomes suggest that a significant portion of the gene diversity in the microbiota can be captured in as little as 1 GB of sequenced DNA.[9] 1 GB of DNA corresponds to ~30 000 cosmid clones (35 kb average insert × 30 000 = 1.05 GB), indicating that only a small fraction must be screened to explore a significant portion of the gene diversity present in this healthy patient's microbiome. To screen for antibacterially active clones, the library was plated on LB agar (15 μg/mL tetracycline) at a density of ~1000 colony forming units (CFU) per 150 mm plate. Colonies were allowed to mature at 30 °C for 5 days and overlaid with top agar containing either *E. coli* or *Bacillus subtilis* as indicator strains (Figure 1). Indicator strains were allowed to grow for 24 h at 37 °C, and antibiotic producing clones were identified by the appearance of zones of growth inhibition in the emergent lawn of the indicator bacteria. In total, 30 000 library members were screened using each indicator strain. No clones were found to produce zones of growth inhibition against *B. subtilis*; however, two metagenomic clones were observed to inhibit the growth of *E. coli* (Figure 1). Because the initial small-scale assay successfully identified antibacterial activities against *E. coli*, we expanded the search for *E. coli* active clones by looking for clones that inhibited the growth of other library members when plating at a much higher density (~45 000 CFU per plate, ~350 000 clones in total). In this larger scale screen, the antibiosis hit rate remained approximately 1 out of every 30 000 library members. In total, we identified 12 clones that showed reproducible antibacterial activity against *E. coli*.

To determine whether a secreted product mediated the observed antibacterial activities, cultures of each active metagenomic clone were passed through a 0.22 μM filter to remove viable bacteria and each sterile, spent culture broth filtrate was assayed for antibiosis against *E. coli*. Consistent with the production of a secreted antibiotic, the culture broth filtrate from each clone was antibacterially active. Ethyl acetate extracts were then created from the culture broth of each antibacterially active clone and assayed for antibiosis against *E. coli*. These extracts failed to show any antibiotic activity indicating that the activity was not the result of the production of an organic extractable small molecule. As water-soluble antibiotics can be quite challenging to characterize, we proceeded to study each clone through full sequencing and bioinformatic analysis.

Restriction mapping of cosmids isolated from an active clone showed a subset of conserved restriction fragments present in all cosmids suggesting that they represented a collection of overlapping metagenomic DNA sequences. One representative antibacterially active clone (human stool metagenome clone 1, HSM-C1) was fully sequenced (Figure 2). The terminal regions

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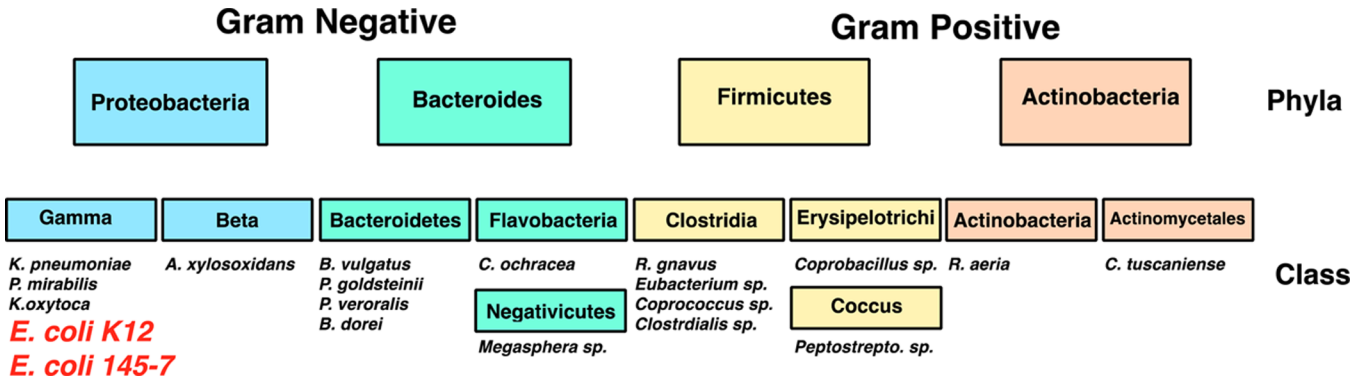

Figure 3. Spectrum of activity. *E. coli* expressing the colicin V gene cluster was plated on LYH-BHI agar media and overlaid with indicator strains of 20 human commensal bacteria from the four common phyla present in the human microbiome. Inhibition of the indicator strain was only observed for *E. coli* strains K12 and MS145-7, a strain of adherent invasive *E. coli* (AIEC), both indicated in red. There was no inhibition of any strains by *E. coli* carrying the empty vector.

of the metagenomic DNA insert from the other 11 clones were sequenced using vector specific primers and confirmed to map to portions of the fully sequenced clone metagenomic DNA insert suggesting overlapping metagenomic DNA inserts. HSM-C1, which contains a 31 kB metagenomic DNA insert, was used for all subsequent analyses.

Bioinformatic analysis of the metagenomic DNA insert in HSM-C1 identified two predicted bacteriocin gene clusters (Colicin V and Colicin E9) and a predicted protease with similarity to the *tsh* gene in *E. coli* (Figure 2).[10] The function of this class of protease is still unclear, but it has conserved regions similar to serine proteases with activity against human IgA, hemoglobin, or coagulation factor V (Figure 2). The full 31 kB metagenomic insert was searched against the NCBI nr data set and found to be 99% identical to regions from three virulence plasmids isolated from avian pathogenic *E. coli* (APEC).[11] A blastN search of reference genomes from human microbiome isolates, including 31 *E. coli* isolates (Human Microbiome Project, www.hmpdacc.org), failed to identify an identical 31 kB region.

To identify the specific genetic elements responsible for encoding the observed antibacterial activity, the fully sequence cosmid was transposon mutagenized using the EZ-Tn5 system (Epicentre). The transposon reaction was electroporated into *E. coli*, and successful transposon mutants were identified by selection on LB agar containing kanamycin (50 μg/mL). 100 transposon mutants were screened for antibacterial activity against the original *E. coli* indicator strain, and 8 mutants were found to no longer inhibit *E. coli* growth. Cosmid DNA isolated from each antibiosis knockout mutant was Sanger sequenced using primers designed to recognize the ends of the transposon. The eight knockout transposons inserted into three different genes that are predicted to be part of a colicin V gene cluster: *cvaA*, *cvaB*, and *cvaC* (Figure 2). *cvaC* encodes the colicin V precursor peptide, while *cvaA* and *cvaB* encode MDR-like transport proteins that shuttle colicin V across the inner membrane.[12,13] Secretion of colicin V across the outer bacterial membrane is dependent on *tolC*-like transporters that are generally encoded chromosomally. We did not observe a transposon insertion into the predicted immunity gene *cvaI*, which is not unexpected as this would likely be lethal. To confirm that the colicin V gene cluster was sufficient for antibiosis, we subcloned the gene cluster and repeated the antibiosis overlay assay with *E. coli* containing this new construct. The subcloned colicin V gene cluster was sufficient to inhibit the growth of the *E. coli* indicator strain (zone of inhibition 6.8 ± 1.1 mm [mean ± SD]). In an overlay assay, the activity of HSM-C1 against *E. coli* is slightly less than that of a representative *E. coli* isolate (ATCC 14763) that is known to natively produce colicin V (zone of inhibition 8.9 ± 0.49 mm [mean ± SD]).

Colicin V is known to be active against *E. coli* with a reported MIC of 0.1 nM, but to the best of our knowledge, the activity of colicin V against a broad collection of human commensal bacteria has never been explored.[14−19] The bactericidal activity of colicin V is mediated by importing the toxin across the outer membrane of the target bacteria by tonB type transporters and then binding sdaC on the inner membrane, which leads to disruption of the inner membrane potential and cell death.[20] The human microbiome is known to contain *E. coli* and related *Enterobacteriaceae* against which colicin V is known to be active.[21] We used an overlay assay to test HSM-C1 for activity against a panel of Gram-negative and Gram-positive bacteria from the human microbiome. Among the aerobic and anaerobic bacteria screened, colicin V was only active against *E. coli* (Figure 3). Even closely related, commensal Proteobacterial species including species of *Proteus* and *Klebsiella* were resistant to colicin V activity. Previous studies have characterized the effect of narrow spectrum bacteriocins on *E. coli* pathogens, as well as pathobionts like adherent invasive *E. coli* (AIEC), which is associated with Crohn's disease.[19,22] The *E. coli* containing the subcloned colicin V gene cluster was assayed for activity against *E. coli* MS-145-7, an AIEC isolate from the colon of a patient with Crohn's disease.[23] In overlay assays, AIEC MS-145-7 was susceptible to *E. coli* expression of colicin V but not *E. coli* containing an empty vector control (zone of inhibition 7.7 ± 0.53 mm [mean ± SD]).

The entire 31 kB metagenomic DNA insert including the colicin V gene cluster in HSM-C1 is 99% identical to a virulence plasmid found in APEC, suggesting the transfer of virulence genes between avian *E. coli* reservoirs in the environment and human associated *E. coli*. APEC strains are the etiological agent of avian colibacillosis, a devastating problem in the poultry industry and a major reason for the use of *E. coli* active antibiotics in animal husbandry. General concern has been raised about how excessive antibiotic use in poultry may increase the rise of antibiotic resistance and virulence in human bacterial strains.[24] Studies that have looked specifically at APEC to understand its relationship to human pathogenic *E. coli* have identified shared virulence genes with

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E. coli isolates from human urinary tract infections (uropathogenic *E. coli*, UPEC).[25,26] These discoveries suggest a shared ancestry but not a direct transmission of *E. coli* virulence genes to human associated bacteria, as the genes seen in APEC and UPEC are similar but not identical. The transfer of virulence plasmids has been observed in a farm setting where workers have direct, daily contact with poultry; however, the patient associated with the metagenomic library we screened in this study lived in an urban environment without close or extensive contact with live poultry.[27] The discovery of these virulence genes from the microbiome of an individual living in New York City suggests potential dissemination of these genes away from the site of contact between humans and poultry.

A query of data from a deep sequencing study of metagenomic DNA from 124 patient stool samples revealed one patient sample in which genes from every portion of the metagenomic DNA clone discovered in this study could be identified and 9 samples where individual genes from the metagenomic DNA clone could be identified.[9] These findings all suggest that virulence genes not only are able to enter the human reservoir temporarily at sites of contact with environmental pathogens but also can also persist and disseminate in the collective human microbiome. Future studies will be needed to better understand whether the presence of APEC virulence genes in the human microbiome affects the pathogenicity of any human microbiota.

It was not surprising that our study identified a bacteriocin gene cluster as they are some of the most common and diverse biosynthetic gene clusters among human microbiota.[28,29] In fact, bacteriocins are common to microbiota that inhabit almost every niche of the human microbiome.[29,30] Two studies of human microbial biosynthetic gene clusters suggest bacteriocin gene clusters (including ribosomally synthesized post translationally modified peptides [RIPPs]) are common in the human microbiome with almost 5000 unique gene clusters.[28,29] Despite the abundance of this biosynthetic gene family in the human microbiome, few studies have functionally characterized bacteriocins isolated from human microbiota. Examples of bacteriocins (RIPPs) characterized from human microbiota include the lantibiotics epidermin/epilancin, salivaricin, cytolysin, and ruminoccin A as well as the thiopeptide lactocillin.[31−34] Class II bacteriocins have been isolated from nonpathogenic human associated *E. coli* strains. Functional analysis of *E. coli* strains isolated from humans suggested >40% were colicinogenic strains and that these strains may be enriched in patients with Inflammatory Bowel Disease.[35,36]

Additional studies will be needed to determine whether bacteriocin gene clusters in the human microbiome encode functions that shape human microbial ecology and/or affect host physiology *in vivo*. Functional metagenomics screening methods are likely to facilitate the study of human microbial bacteriocins as these gene clusters are physically small in size, facilitating their capture and heterologous expression. There is continued interest in the therapeutic development of bacteriocins due to their varied mechanisms of action, diverse spectrum of activity, and a biosynthetic scheme that is easy to manipulate and express in host microbes.[37] For example, the narrow spectrum of activity we observed for colicin V among commensal bacteria might allow for targeting pathobionts like AIEC while preventing disruption of other beneficial commensals. The high density of bacteriocin genes in the human microbiome suggests that these antibiotics have an important role in ecology of human-associated bacteria. The large-scale application of functional metagenomics to eDNA libraries derived from diverse patient samples should provide a systematic means of identifying additional biomedically and ecologically relevant bacteriocins encoded within the panhuman microbiota.

METHODS

A patient was recruited at Mount Sinai Hospital (New York, NY) under IRB approved consents (#11-0716 Mount Sinai). 6 g of stool from one healthy control was resuspended in 0.9% NaCl to a total volume of 40 mL and then centrifuged (800*g*, 5 min, 4 °C). The resulting pellet was resuspended in 40 mL of 0.9% NaCl and pelleted once again (3200*g*, 30 min, 4 °C). Washed stool samples were resuspended in 5 mL of 0.9% NaCl. 750 μL of this sample was layered on 500 μL of Nycodenz in a 1.5 mL Eppendorf tube. After centrifugation (21 130*g*, 10 min, room temperature) the top layer was removed and mixed with 500 μL of 0.9% NaCl in a 15 mL conical tube, and bacteria were collected by centrifugation (5800*g*, 10 min, room temperature). The bacterial pellet was resuspended in 8 mL of lysis buffer (100 mM Tris-HCl, 100 mM Na EDTA, 1.5 M NaCl, 1% (w/v) CTAB, 2% (w/v) SDS, pH 8.0) and incubated at 70 °C for 2 h. Crude eDNA was precipitated by addition of 0.7 volumes of isopropanol, collected by centrifugation (4000*g* × 30 min), washed with 70% ethanol, and then resuspended in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM Na EDTA, pH 8.0). eDNA was separated by preparative agarose (0.7% agarose) gel electrophoresis (3 h, 100 V), and high molecular weight DNA was excised from the gel and collected by electroelution (100 V, 2 h). Purified high molecular weight eDNA was blunt ended (End-It; Epicentre), ligated into ScaI digested pJWC1 cosmid vector packaged into lambda phage *in vitro* (MaxPlax Packaging Extracts; Epicentre), and transfected into *E. coli* EC100. Titers were determined for packaging reaction, and the library was expanded until it contained ∼1.5 million clones. Clone colonies were selected by plating on LB with 15 μg/mL tetracycline. Colonies were washed from the plates and stored as concentrated glycerol stocks without further expansion.

A scraping of the library glycerol stock was diluted in fresh LB and plated on LB agar at an average density of 1000 colonies per 150 mm agar plate. Thirty metagenomic clone plates were made for each indicator strain tested, and each plate was placed at 30 °C for 5 days. *E. coli* EC100 and *B. subtilus 168* 1A1 indicator strains were grown overnight in LB (37 °C with shaking 200 rpm) and in the morning the culture was diluted 1:1000 into sterile LB half agar cooled to 55 °C. Half agar containing the indicator strain was then poured on top of the metagenomic clone plates and placed at 37 °C for 24 h. Plates were then inspected for zones of inhibition around metagenomic clone colonies which suggest antibiotic properties. Clones that exhibited antibiotic properties were selected and streaked again on LB, and the experiment was repeated to confirm antibiosis. After confirmation of inhibition against *E. coli*, the original library was plated again at high density (45 000 clones per 150 mM plate) without an overlay to determine whether the antibiotic clones were active against other library clones. The same inhibition phenotype was identified with 1 clone per 30 000 library clones exhibiting the antibiotic phenotype. From these high-density library plating experiments, 12 clones were selected for further evaluation.

Twelve active clones were selected with antibiotic properties, and cosmid DNA was isolated (Qiagen, QIAprep Spin

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Miniprep). DNA was restriction digested using *Eco*RI and BamI. After subtraction of cosmid bands, there were clear patterns that indicated a conserved metagenomic DNA region suggesting all 12 clones contained overlapping DNA sequences. DNA from 1 clone was fully sequenced by PGM IonTorrent. IonTorrent DNA reads were assembled single large contig by Newbler which was then annotated using CloVR.[38] Each of the 11 remaining clones were Sanger sequenced from the end of the metagenomic insert and aligned to the fully assembled metagenomic clone (MacVector) to confirm overlapping metagenomic DNA inserts. A representative clone, which contained all predicted virulence genes, was selected and transposon mutagenized using the EZ Tn-5 Kan Transposon (Epicentre). Transposon mutants were selected using kanamaycin (50 μg/mL). 100 transposon mutants were then selected, and the antibiosis overlay assay was repeated to look for loss of antibiotic phenotype. Eight transposon mutants failed to inhibit the growth of *E. coli* and were sent for Sanger sequencing from the transposon insertion site. All clones were confirmed to have interruptions in a biosynthetic gene cluster for producing colicin V. The colicin V gene cluster was then subcloned to confirm the activity. Forward primer 5′-catgagagctccattaatccagataaacaac and reverse primer 5′-cgaacgagctctatcatgtcgatgacgggg were used to PCR amplify the colicin V gene cluster. The PCR product was gel purified, digested with SacI, blunt ended (End-It, Epibio), and ligated into ScaI digested pJWC1 vector. Subclones were selected by plating on LB agar with 15 μg/mL tetracycline, and cloning was confirmed by PCR amplification of the cloned gene cluster. Subclones were then subjected to the same antibiosis overlay assay against *E. coli* and confirmed to exhibit the antibiotic phenotype. For all antibiosis assays, *E. coli* with the empty pJWC1 vector was used as a negative control to confirm no antibiotic properties in the host *E. coli*.

The nonredundant gene set was downloaded for each of the 124 patient samples from the MetaHIT data set.[9] The three virulence gene clusters (Figure 2) along with each of the surrounding metagenomic regions were individually searched (blastN) against the nonredundant gene set for each patient sample. Genes with 99% nucleotide identity over the full gene length were identified and recorded for each patient sample. In one patient sample, there was at least one gene present in that sample from all three virulence gene clusters and each of the metagenomic regions around the virulence gene clusters.

All indicator bacteria were ordered from BEI resources. The colicin V producing *E. coli* strain 14763 was obtained from ATCC. Bacteria were assayed by overlay on top of *E. coli* expressing the colicin V gene cluster in the same method as the original library screening. All bacteria were grown in LY-BHI media [brain−heart infusion medium supplemented with 0.5% yeast extract (Difco), 5 mg/L hemin (Sigma), 1 mg/mL cellobiose (Sigma), 1 mg/mL maltose (Sigma), 0.5 mg/mL cysteine (Sigma)]. *E. coli* expressing colicin V was plated on LY-BHI agar, and the indicator strain was inoculated into LY-BHI half agar. Bacteria were grown anaerobically when indicated, and in those cases, the *E. coli* was inoculated onto the LY-BHI agar aerobically and then placed into the anaerobic chamber to equilibrate for 4 h prior to the anaerobic indicator strain overlay. Plate images were analyzed with ImageJ, and zones of inhibition were averaged from 5 measurements in triplicate experiments.

50 mL cultures of metagenomic clones with antibiotic activity and *E. coli* with the empty pJWC vector (negative control) were grown at 30 °C with shaking at 200 rpm for 5 days. The culture broth was centrifuged (200*g* × 10 min), and the supernatant then passed through a 0.22 μM filter (Millipore). 100 μL of sterile culture broth from either the colicin V producing clone or the negative control were added to wells of a 96 well microplate (Grenier, clear bottom) along with 50 μL of fresh LB media. To this was added a 1/10 000 dilution of an overnight culture of the *E. coli* indicator strain. OD600 was measured at baseline and then at 3 h intervals to 12 h. At 12 h, there was growth inhibition in wells with the colicin V sterile supernatant. To determine whether antibiosis was due to an organically extractable molecule, 50 mL of culture broth from the active metagenomic clones and the negative control was extracted 1:1 with ethyl acetate and the organic layer was dried by vacuum centrifugation. Organic extracts were then assayed at 10 and 100 μg/mL final concentrations for antibiotic properties in the same growth inhibition assay as well as by plating on a lawn of indicator *E. coli*. In both experiments, there were no observed antibiotic effects of the crude organic extracts.

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Author Contributions

L.J.C. and S.F.B. designed the research; L.J.C., S.H., and Y.-H.H. performed the research; L.J.C. and S.H. analyzed the data; L.J.C. and S.F.B. wrote the paper.

Notes

The authors declare no competing financial interest.

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