The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens

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GOS scaffolds, finding 46 MpnS and 20 HepD homologs, using a protein basic local alignment search tool (BLASTP) cutoff value of 10^{-10} (table S2). No HppE homologs were observed. None of the HepD homologs were identified when *N. maritimus* MpnS was used as the query sequence; likewise, none of the MpnS homologs were identified when HepD was used as a query. Thus, BLASTP clearly differentiates between the two homologous groups, supporting the assignment of the recovered sequences as MpnS and HepD proteins, respectively. To independently support these functional assignments, we constructed maximum-likelihood phylogenetic trees including biochemically validated MpnS, HepD, and HppE proteins (Fig. 3A and fig. S9). We also used a hierarchical clustering method to examine all putative and validated MpnS, HepD, and HppE proteins (fig. S10). In both cases, robust support for the functional assignments was obtained. Thus, we conclude that the recovered GOS MpnS homologs are likely to be methylphosphonate synthases.

Additional support for the function of the MpnS homologs was revealed by analysis of neighboring genes found in GOS DNA scaffolds (Fig. 3B and table S3). Many of the nearby open reading frames are homologous to those found in the *N. maritimus* gene cluster, including the phosphonate biosynthetic genes *ppm*, *ppd*, and *pdh*, as well as homologs of the sulfatases and nucleotidyl transferase genes, suggesting that the GOS scaffolds encode genes for the synthesis of similar MPn esters. Several other genes found on the scaffolds provide evidence for the identity of the organisms in which they are found. One of the scaffolds includes a 23*S* ribosomal RNA gene that can be confidently placed within the SAR11 clade between *Pelagibacter* species (fig. S11), whereas two of the *manC* genes are nearly identical to ones found in *Pelagibacter sp.* HTCC7211. Although the *mpnS* gene is absent in sequenced *Pelagibacter* genomes, these data strongly support the conclusion that some members of this genus have the capacity to synthesize MPn.

Relatives of *Nitrosopumilus* and *Pelagibacter* are among the most abundant organisms in the sea, with global populations estimated at 10^{28} for both ammonia-oxidizing Thaumarchaeota (*14*) and members of the SAR11 clade (*22*). Thus, the observation of *mpnS* in some members of these genera is consistent with the idea that MPn synthesis is prevalent in marine systems. To provide direct support for this notion, we measured the abundance of the *mpnS* gene relative to the abundance of typical single-copy genes as previously described (*23*). We also quantified the occurrence of the *ppm* gene to provide an estimate of the relative occurrence of phosphonate synthesis in general (table S4). Based on these data, we estimate that ~16% of marine microbes are capable of phosphonate biosynthesis, whereas 0.6% have the capacity to synthesize MPn. Because the GOS samples are confined to the upper few meters of the ocean, extrapolation of this analysis to the deeper ocean should be viewed with some skepticism. Nevertheless, the upper 200 m of the world's oceans are thought to contain ~3.6×10^{28} microbial cells, with an average generation time of ~2 weeks (*24*). Thus, even with the relatively modest abundance of MPn biosynthesis suggested by our data, it seems quite possible that these cells could provide sufficient amounts of MPn precursor to account for the observed methane production in the aerobic ocean via the C-P lyase–dependent scenario suggested by Karl *et al*. (*2*).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1104/DC1
Materials and Methods
Figs. S1 to S11
Tables S1 to S4
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The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens

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Soil microbiota represent one of the ancient evolutionary origins of antibiotic resistance and have been proposed as a reservoir of resistance genes available for exchange with clinical pathogens. Using a high-throughput functional metagenomic approach in conjunction with a pipeline for the de novo assembly of short-read sequence data from functional selections (termed PARFuMS), we provide evidence for recent exchange of antibiotic resistance genes between environmental bacteria and clinical pathogens. We describe multidrug-resistant soil bacteria containing resistance cassettes against five classes of antibiotics (β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) that have perfect nucleotide identity to genes from diverse human pathogens. This identity encompasses noncoding regions as well as multiple mobilization sequences, offering not only evidence of lateral exchange but also a mechanism by which antibiotic resistance disseminates.

The continued evolution and widespread dissemination of antibiotic resistance genes in human pathogens is a preeminent clinical challenge (*1*). Environmental reservoirs have long been implicated as a source of resistance found in human pathogens (*2*). However, apart from certain opportunistic bacterial pathogens, among which the same species can be found in the environment or infecting humans (*3*), examples of resistance genes from environmental bacteria with high identity to those of pathogens are rare (*4*, *5*). The two documented examples are of *Kluyvera* and *Shewanella* isolates, which are found free-living in environmental settings (*5*, *6*) yet have resistance genes (CTX-M β-lactamase and qnrA genes, respectively) with high identity (100% identity in clinical *Kluyvera* isolates) to those of pathogens (*4*, *5*). The limited examples of resistance genes shared between environmental microbes and human pathogens raise questions regarding the clinical impact of environmental resistance. For instance, whether shared resistance

is confined to genes of particular mechanisms (such as enzymatic β-lactam cleavage) or applies to many genes with diverse mechanisms of resistance is unknown. Additionally, whether a single horizontal gene transfer (HGT) event between environment and clinic can result in the de novo acquisition of a multidrug-resistant phenotype is unclear. The two previous reports of high-identity resistance genes shared between environmental and pathogenic bacteria did not find evidence of colocalized resistance genes or of syntenic mobilization elements (*4*, *5*), hallmarks of transferable multidrug resistance (*7*, *8*). Determining the clinical impact of environmental resistance requires a deeper profiling of environmental reservoirs for the organisms and genotypes most likely to exchange resistance with human pathogens.

Soil, one of the largest and most diverse microbial habitats on earth, is increasingly recognized as a vast repository of antibiotic resistance genes (*9*–*13*). Not only does soil come into direct contact with antibiotics used extensively in rearing livestock (*14*) and plant agriculture (*15*), but it is also a natural habitat for the Actinomycete genus *Streptomyces*, whose species account for the majority of all naturally produced antibiotics (*16*). Despite numerous studies demonstrating that soil contains resistance genes with biochemical mechanisms similar to those in common pathogens (*3*, *11*–*13*), the sequence identities of these genes diverge from those of pathogens (*17*), providing little evidence that these resistomes have more than an evolutionary relationship. Therefore, whether soil has recently contributed to or acquired resistance genes from the pathogenic resistome remains an open question, and accordingly, the role of soil in the current global exchange of antibiotic resistance remains poorly defined.

To examine the capacity of nonpathogenic, soil-dwelling organisms to exchange antibiotic resistance with human pathogens, we sought to select for organisms prone to this exchange. Because many major clinical pathogens are Proteobacterial (*18*), we cultured multidrug-resistant Proteobacteria from the soil (*19*), with the aim of enriching for resistance genes shared between soil and human pathogens. We interrogated the resistome of the resulting culture collection using functional metagenomic selections, which are ideally suited to characterize acquirable resistance because they identify any gene sufficient to confer resistance to a new host (such as a pathogen) (*20*). To facilitate the rapid and efficient functional characterization of metagenomic libraries, we developed a massively parallel, multiplexed functional selection platform that enables simultaneous sequencing, de novo assembly, and functional annotation of hundreds of resistance fragments from many independent selections (termed PARFuMS: Parallel Annotation and Re-assembly of Functional Metagenomic Selections) (fig. S1) (*19*).

We applied PARFuMS to a collection of 95 soil-derived cultures ("AB95"), representing bacteria with high-level resistance to various antibiotics. Cultures were obtained from 11 U.S. soils (table S1), passaged serially through minimal and rich media containing one of 18 antibiotics at 1000 mg/L (tables S2 and S3) (*21*), and subjected to 16*S* ribosomal DNA (rDNA) profiling (*19*). We confirmed that the culture collection was enriched for Proteobacteria and dominated by traditional soil-dwelling organisms (such as *Pseudomonas* and *Pandoraea*) (fig. S2). Equal proportions of the 95 cultures were pooled, and bulk genomic DNA was extracted. One- to 3-kb fragments of this metagenomic DNA were cloned into an expression vector and transformed into *Escherichia coli*. The resulting 2.57-Gb metagenomic library was selected on solid culture medium containing 1 of 12 antibiotics representing amino acid derivatives, aminoglycosides, amphenicols, β-lactams, and tetracyclines, at concentrations to which the host-strain was susceptible (table S4). Resistance was detected against all 12 antibiotics, and resistance-conferring fragments were sequenced, assembled, and annotated by using PARFuMS, yielding 161 contigs (N50 > 1.7 kb). Of the 252 open reading frames (ORFs) identified, 110 (44%) could confidently be annotated as antibiotic resistance genes (by similarity to a known resistance gene, which was consistent with functional selection), whereas another 62 (25%) were categorized as resistance-related (Fig. 1, A to C, and table S5).

Of the 110 resistance genes, 18 had 100% amino acid identity to entries in GenBank, and another 32 were highly similar (≥90% identity). Thus, although we recovered several genes previously identified, most of the resistance genes discovered (54%) were formerly unknown (Fig. 1D). For instance, we identified a gene conferring D-cycloserine resistance from an AB95 isolate (most closely related to *Serratia ficara*) for which sequence alone could not predict resistance function (*19*). The ORF was 92% identical to a protein of unknown function from *Serratia proteamaculans* 568 (CP000826) (Fig. 2A) and enabled *E. coli* to tolerate high concentrations of D-cycloserine (128 μg/mL) (Fig. 2B). The D-cycloserine resistance protein had low-level identity to a drug/metabolite transporter (46% identity over 91% of the sequence; YP_001583420), indicating that the gene may have efflux-related function, which is consistent with known mechanisms of D-cycloserine tolerance (*22*).

Of the 110 AB95 resistance genes, 55 were β-lactamases. The majority of these sequences clustered with class C β-lactamases and were dissimilar to entries currently in GenBank (fig. S3), which is a common result from metagenomic experiments (*11*, *20*, *23*). AB95 β-lactamases were highly divergent from those of the antibiotic-

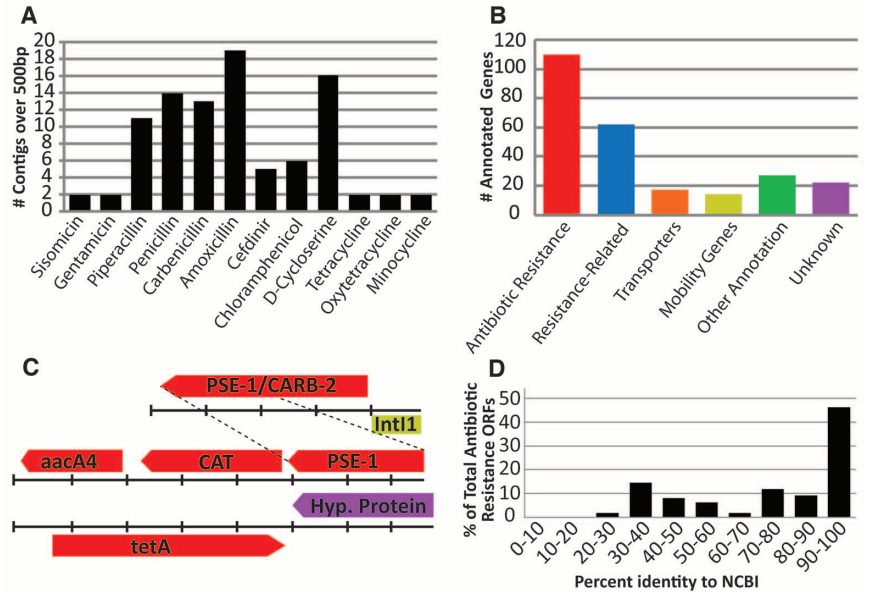

Fig. 1. Functional selection of the AB95 soil metagenomic library with 12 antibiotics (*19*). (**A**) Bar chart depicting the number of distinct contigs over 500 base pairs (bp) recovered from selection with each of the 12 antibiotics. (**B**) Functional classification of ORFs predicted by PARFuMS, across all selections. (**C**) Three representative metagenomic fragments; colors match catergorizations depicted in (B). The distance between tick marks is 300 bp, and dashed lines indicate common sequence on two distinct fragments. (**D**) Amino acid identity between antibiotic-resistance ORFs and the closest hit from GenBank, across all selections.

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producing *Streptomyces*, indicating ancient evolutionary relationships (fig. S3 and table S6). However, several β-lactamases with >99% identity to sequences from both soil and enteric organisms were recovered (fig. S3).

We identified 16 sequences, from 10 selections, with 100% nucleotide identity to antibiotic resistance genes previously sequenced from clinical isolates of many common human pathogens (Table 1). A bacterium was considered pathogenic only if it was isolated from an infection in a diseased human host. The 16 sequences represent seven different genes, conferring resistance to five classes of antibiotics (β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) (Table 1). We discovered multiple examples of syntenic, soil-derived resistance genes shared with many common pathogens. For example, a chloramphenicol-acetyltransferase with 99.7% identity to *K. pneumoniae* clinical isolates was adjacent to both an aminoglycoside-acetyltransferase and a β-lactamase identical to genes found in many pathogens (JX009248). Additionally, an insert from two selections contained *aadB* (an aminoglycoside-adenyltransferase) adjacent to *qacEΔ1* (an efflux pump conferring antiseptic resistance) and *sul1* (a dihydropteroate synthase conferring sulfonamide resistance) in a class 1 integron-like structure (JX009286). All three genes and much of the surrounding integron (>2 kb) are 100% identical to numerous clinical pathogens. The seven soil-derived resistance genes (Table 1) are globally distributed amongst human pathogens: Clinical isolates from many countries and all major continents contain genes with perfect nucleotide identity to genes from this set (fig. S4).

To identify soil isolates from the AB95 culture collection harboring the aforementioned resistance genes, we performed polymerase chain reactions using primers specific to the boundaries of the predicted ORFs (*19*). We identified two organisms isolated from farmland soil containing six of the resistance genes identical to pathogens, as well as two additional genes with over 99% identity to those in pathogens (tables S7 and S8) (*19*). We confirmed that seven genes were present in an organism most closely related to *Pseudomonas sp.* K94.23 [a member of the *P. fluorescens* complex (*24*)], three originated from a strain most similar to *Ochrobactrum anthropi*, and two were in both genomes (*19*). *P. fluorescens* is not believed to cause human infection (*25*), and there are only limited examples of *O. anthropi* subgroups known to infect humans (*26*). Rather, these two organisms are predominantly found in environmental settings (*25*, *27*). The substantial phylogenetic divergence between these traditionally nonpathogenic soil isolates and numerous human pathogens (table S9) contrasts with the 100% identity of numerous resistance genes found in both groups, confirming that these genes moved between species via HGT.

Three ORFs from *O. anthropi* and *P. fluorescens*, conferring β-lactam, aminoglycoside, and amphenicol resistance and representing one gene shared by both organisms and one specific to each, were cloned from their genomic DNA, expressed in *E. coli*, and verified for resistance to seven antibiotics (*19*). In all cases, the ORFs conferred resistance at concentrations 16-fold greater than that of an empty-vector control and enabled growth in a minimum of 128 μg/mL (and up to 2048 μg/mL) of antibiotic (Table 2). These results mirror the minimum inhibitory concentrations of the source soil strains (Table 2), demonstrating that the resistance genes retain functionality even when removed from all native genomic context, emphasizing their broad host-range compatibility.

Perfect nucleotide identity between full-length resistance genes from distinct species implies that recent HGT has occurred between these organisms (*28*)—evidence that has not been previously reported between a nonpathogenic soil-dwelling organism and human pathogens. The seven resistance genes we discovered encompass all major mechanistic classes of antibiotic resistance (*29*) and are identical to genes found in diverse human pathogens, representing both Gram-negative and -positive bacteria. Moreover, for five of the soil-derived contigs that share resistance genes with pathogens, at least 80% of the contig is identical to sequence from a clinical isolate, encompassing coding and noncoding regions alike (the maximum span of identity is 2.28 kb) (table S10). In support of recent mobilization, we found 11 distinct sequences annotated as either an integrase or transposase from six antibiotic selections. Two *intI1* integrases were adjacent to resistance genes from both our organisms and pathogens, indicating a shared mechanism of HGT between soil and pathogenic bacteria. Four of the contigs assembled from our set are over 99% identical to a large span of sequence, found in numerous pathogens, that contains a high density of resistance genes and is flanked by multiple mobility elements (Fig. 3). This cluster of resistance genes exhibits extensive modularity; many combinations of the individual resistance elements are present in a multitude of clinical pathogens.

The closest homologs to each AB95 resistance gene include pathogenic resistance genes that are chromosomal as well as plasmid-borne, implying a diverse genetic organization of these

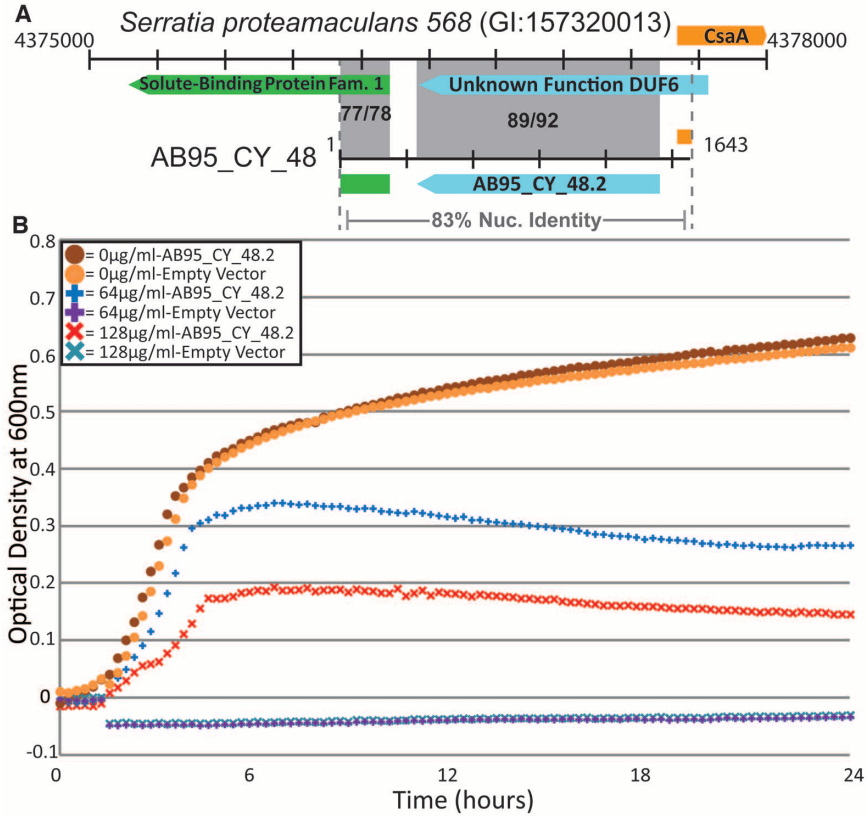

Fig. 2. A gene conferring resistance to D-cycloserine was captured for which sequence was unable to predict resistance function. (**A**) Resistance-conferring fragment AB95_CY_48 compared with its closest hit from the National Center for Biotechnology Information (NCBI) nucleotide collection. ORFs of the same color indicate homologous sequence; both nucleotide and amino acid percent identities are given in shaded regions (nucleotide/amino acid). Base-pair coordinates flank sequences, and the distance between each tick mark is 300 bp. (**B**) Measurements of absorbance at 600 nm, taken every 15 min, depict growth of *E. coli*, containing either AB95_CY_48.2 or an empty vector at clinically relevant concentrations of D-cycloserine. Measurements are corrected for background absorbance from media-only controls and are averages of three trials (*19*).

genes. Four of the pathogen-identical genes from *P. fluorescens*, conferring resistance to the aminoglycosides, tetracyclines, amphenicols, and sulfonamides, were identified in a plasmid preparation, implicating conjugation or transformation as potential mechanisms of HGT (table S11). Additionally, we discovered nine integrases/transposases proximal to resistance genes not yet identified in pathogens, indicating that additional resistance genes from these soil bacteria may be available for HGT with pathogens.

Table 1. Nonredundant antibiotic resistance genes with 100% identity to known human pathogens.

Gene name	GenBank ID	Number of selections*	Antibiotic class	Annotation [mechanism]	Pathogens hit (GI number)
AB95_PI_68.1	JX009363	4	β-lactam	blaP1 [enzymatic degradation]	*A. baumannii* (94960156), *K. pneumoniae* (114147191), *P. aeruginosa* (117321883), *S. typhimurium* (12719011), *P. mirabilis* (157674381)†
AB95_CH_13.1	JX009364	1	Amphenicol	Chloramphenicol efflux [efflux]	*A. baumannii* (169147133), *P. aeruginosa* (260677483)
AB95_TE_2.2	JX009366	3	Tetracycline	tetA(G) [efflux]	*A. baumannii* (169147133), *S. typhimurium* (12719011)
AB95_TE_1.1	JX009365	3	Tetracycline	tetA [efflux]	A. baumannii (169147133), *E. coli* (312949035), *K. pneumoniae* (290792160), *S. typhimurium* (37962716)†
AB95_GE_3.3	JX009367 JX009373	2	Aminoglycoside	aadB [covalent modification]	*E. cloacae* (71361871), *K. pneumoniae* (206731403), *P. aeruginosa* (37955767), *S. typhimurium* (17383994)†
AB95_GE_3.1	JX009368 JX009374	2	Sulfonamide	sul1 [target modification]	*C. diptheriae* (323714042) *E. cloacae* (71361871), *K. pneumoniae* (206731403), *P. aeruginosa* (37955767), *S. typhimurium* (17383994), *Yersinia pestis* (165913934)†
AB95_CH_21.1	JX009369	1	Aminoglycoside	aacA4 [covalent modification]	*A. baumannii* (164449567), *K. pneumoniae* (238865601), *P. aeruginosa* (219872982), *S. typhi* (34014739)†

*Number of selections in which the entirety of a given gene was captured. †More pathogens exist for which 100% nucleotide identity was observed than listed

Given the extensive interspecific transfer of antibiotic resistance, and our data suggesting recent exchange between soil bacteria and clinical pathogens, we sought to identify routes of dissemination between these reservoirs. Possibilities include direct exchange between soil microbes and human pathogens or indirect transfer via reservoirs such as the human intestinal microbiota. Many resistance genes from the intestinal microbiota are identical to those found in diverse human pathogens (*20*), and accordingly, we compared the AB95 resistance genes with a set of resistance genes from cultured intestinal isolates (*20*), a collection of 128 representative gut organisms (table S12), and resistance genes from fecal metagenomes (*19*, *20*). Most AB95 resistance genes were dissimilar to sequences from any intestinal data set, with the average amino acid identity ranging from 30.2 to 45.5% (fig. S5). However, the two cultured data sets contained perfect matches to distinct AB95 resistance genes (table S13). One such AB95 gene (JX009365) was not only identical to *tetA* from an intestinal isolate, but also to numerous pathogens, including *A. baumannii*, *E. coli*, *K. pneumoniae*, and *S. typhimurium*, indicating potential interconnections between the resistomes of the human gastrointestinal tract, soil, and clinical pathogens.

The exchange of resistance between soil and pathogens emphasizes the clinical importance of the soil resistome, regardless of whether resistance genes are moving from soil to the clinic, or vice versa. Transmission from soil to clinic establishes soil as a direct source of pathogenic resistance genes. Movement of resistance from pathogens into soil means pathogens can transfer resistance

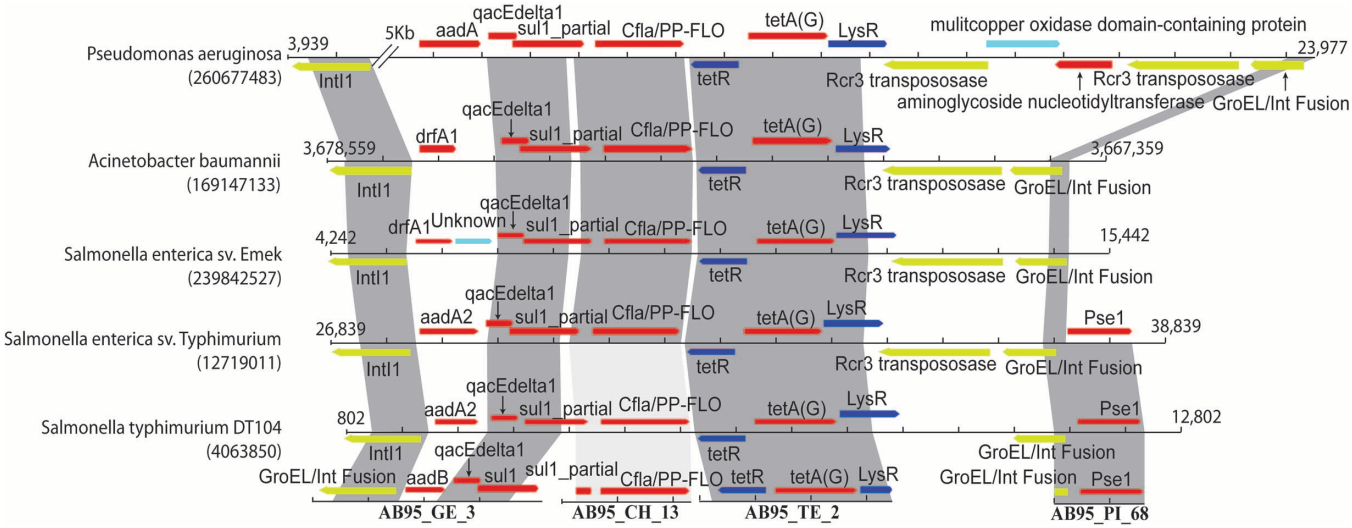

Fig. 3. Comparison of four AB95-derived resistance fragments to five human pathogenic isolates. The four fragments are depicted along the bottom, and shading indicates high nucleotide identity between the fragments and pathogens (NCBI GenInfo numbers identify each pathogenic isolate). Dark gray shading indicates >99% identity; light gray shading indicates ~88% identity. Base-pair coordinates flank pathogenic sequences, and the distance between each tick mark is 800 bp. Red ORFs represent resistance genes, yellow represents mobility elements, dark blue represents resistance-associated regulatory elements, and light blue represents other functions.

Table 2. Minimum inhibitory concentrations of various antibiotics toward both multidrug resistant soil isolates and *E. coli* clones expressing selected resistance genes (all concentrations are μg/mL). AX, amoxicillin; CA, carbenicillin; PE, penicillin; PI, piperacillin; CF, cefdinir; CH, chloramphenicol; SI, sisomicin; GE, gentamicin; MN, minocycline; OX, oxytetracycline; TE, tetracycline; and blank cells indicate inhibitory concentrations were not determined.

	AX	CA	PE	PI	CF	CH	SI	GE	MN	OX	TE
Ochrobactrum soil isolate	>2048	>2048	>2048	>2048	<16	512	512	512	<4	256	64
Pseudomonas soil isolate	>2048	>2048	>2048	>2048	>1024	1024	>1024	>1024	8	128	32
AB95_PI_68.1	>2048	>2048	2048	2048							
AB95_CH_33.1						256					
AB95_GE_3.3							>1024	>1024			
E. coli + empty vector control	<16	<32	64	16	<8	<8	<8	<8	<8	8	4

to soil organisms, of which many can cause nosocomial infection and may emerge as pathogens, akin to the rise of *A. baumannii*.

Powered by PARFuMS, a method for characterizing functional selections at <1% of the cost of traditional approaches (*19*), we describe antibiotic resistance genes found in nonpathogenic soil-dwelling bacteria and of all major mechanistic classes (*29*) with perfect nucleotide identity to many diverse human pathogens. We also show that multiple resistance genes are colocalized within long stretches of perfect nucleotide identity and are flanked by mobile DNA elements. These findings not only provide evidence for recent HGT of multidrug resistance cassettes between soil and clinic, but also a mechanism through which this exchange may have occurred.

The *Ochrobactrum* and *Pseudomonas* isolates originated from farmland soils fertilized with manure from antibiotic-treated livestock. However, our current study design did not enable a statistically significant association of pathogen-identical resistance genes to specific soils. Rather, our results highlight the fact that soil and pathogenic resistomes are not distinct, emphasizing the clinical importance of environmental resistance. Our new method provides the increased throughput required to power future studies to identify soil (*11*), aquatic (*5*), and other (*20*) environments prone to resistance exchange with human pathogens and to understand how specific anthropogenic practices influence the likelihood of this dissemination (*3*, *23*).

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Supplementary Materials

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TLR13 Recognizes Bacterial 23*S* rRNA Devoid of Erythromycin Resistance–Forming Modification

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Host protection from infection relies on the recognition of pathogens by innate pattern-recognition receptors such as Toll-like receptors (TLRs). Here, we show that the orphan receptor TLR13 in mice recognizes a conserved 23*S* ribosomal RNA (rRNA) sequence that is the binding site of macrolide, lincosamide, and streptogramin group (MLS) antibiotics (including erythromycin) in bacteria. Notably, 23*S* rRNA from clinical isolates of erythromycin-resistant *Staphylococcus aureus* and synthetic oligoribonucleotides carrying methylated adenosine or a guanosine mimicking a MLS resistance–causing modification failed to stimulate TLR13. Thus, our results reveal both a natural TLR13 ligand and specific mechanisms of antibiotic resistance as potent bacterial immune evasion strategy, avoiding recognition via TLR13.

Toll-like receptor 2 (TLR2), TLR4, and TLR9 are major host sensors of Gram-negative bacteria, and TLR2 is thought to be the central detector of Gram-positive bacteria, whereas other pattern-recognition receptors (PRRs) such as TLR7 contribute to bacteria sensing as well (*1*–*7*). However, the high sensitivity of mice lacking expression of these TLRs to Gram-positive