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Uncultured soil bacteria are a reservoir of new antibiotic resistance genes

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Summary

Antibiotic resistance genes are typically isolated by cloning from cultured bacteria or by polymerase chain reaction (PCR) amplification from environmental samples. These methods do not access the potential reservoir of undiscovered antibiotic resistance genes harboured by soil bacteria because most soil bacteria are not cultured readily, and PCR detection of antibiotic resistance genes depends on primers that are based on known genes. To explore this reservoir, we isolated DNA directly from soil samples, cloned the DNA and selected for clones that expressed antibiotic resistance in *Escherichia coli*. We constructed four libraries that collectively contain 4.1 gigabases of cloned soil DNA. From these and two previously reported libraries, we identified nine clones expressing resistance to aminoglycoside antibiotics and one expressing tetracycline resistance. Based on the predicted amino acid sequences of the resistance genes, the resistance mechanisms include efflux of tetracycline and inactivation of aminoglycoside antibiotics by phosphorylation and acetylation. With one exception, all the sequences are considerably different from previously reported sequences. The results indicate that soil bacteria are a reservoir of antibiotic resistance genes with greater genetic diversity than previously accounted for, and that the diversity can be surveyed by a culture-independent method.

Introduction

Antibiotic resistance is a concern for the management of disease in humans, animals and plants. The intense research efforts to elucidate mechanisms of resistance have focused on genes derived from a narrow range of environments. Most of the known resistance determinants have been discovered in clinical and veterinary bacterial isolates, whereas other environmental reservoirs of antibiotic resistance are not well characterized (Nwosu, 2001; Séveno *et al*., 2002).

Cultured microorganisms have been the source of almost all characterized antibiotic resistance genes; therefore, most previous studies have ignored the potential reservoir of antibiotic resistance genes in uncultured bacteria. The majority of bacteria are not readily cultured on standard laboratory media (Giovannoni *et al*., 1990; Ward *et al*., 1990; Amann *et al*., 1995; Suzuki *et al*., 1997; Hugenholtz *et al*., 1998), and the diversity of the uncultured majority is vast (Head *et al*., 1998; Torsvik *et al*., 1998; Whitman *et al*., 1998; Béjà *et al*., 2002). Despite recent progress in culturing methods (Connon and Giovannoni, 2002; Janssen *et al*., 2002; Kaeberlein *et al*., 2002), culture-independent techniques are required to access the genetic diversity of most bacteria.

The polymerase chain reaction (PCR) is currently used for culture-independent isolation of antibiotic resistance genes from environmental samples (Waters and Davies, 1997; Smalla *et al*., 2000; Aminov *et al*., 2001; Frana *et al*., 2001; Stokes *et al*., 2001), but only accesses genes that are similar to known sequences and often does not recover complete genes. Because of the possibility of undiscovered gene families and undiscovered genetic diversity within known gene families, we circumvented the limitations of both culturing and PCR-based methods by extracting and cloning DNA directly from soil samples, thus constructing libraries that include the genes of uncultured soil bacteria. Such libraries containing environmental DNA (Stein *et al*., 1996), or 'metagenomic libraries' (Rondon *et al*., 2000), have been used previously to identify clones expressing various enzymes (Henne *et al*., 2000; Rondon *et al*., 2000; Knietsch *et al*., 2003) and antimicrobial activities (Brady and Clardy, 2000; Brady *et al*., 2001; Gillespie *et al*., 2002; Courtois *et al*., 2003). Diaz-Torres and colleagues (2003) recently constructed metagenomic libraries from the human oral cavity and identified a novel tetracycline resistance gene.

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Table 1. Characteristics of soil metagenomic libraries.

Library name	Vector	Enzyme used for cloning	No. of clones	Average insert size (kb)	Amount of cloned DNA (gigabases)	Reference
SL1	pBeloBAC11	*Hin*dIII	3 600	27.0	0.1	Rondon *et al.* (2000)
SL2	pBeloBAC11	*Hin*dIII	24 600	45.0	1.1	Rondon *et al.* (2000)
EL1	pJN105	*Pst*I	200 000	4.1	0.8	This work
EL2	pJN105	*Eco*RI	58 000	2.7	0.2	This work
EL3	pJN105	*Pst*I	250 000	3.5	0.9	This work
EL4	pCF430	*Pst*I	650 000	3.5	2.3	This work

Here, we describe the use of metagenomic libraries to isolate and identify antibiotic resistance genes from soil. We constructed libraries that contain fragments of cloned soil DNA and selected for clones expressing antibiotic resistance in *Escherichia coli*.

Results

Isolation of clones expressing antibiotic resistance

We selected antibiotic-resistant clones from two bacterial artificial chromosome (BAC) libraries and four small-insert libraries that collectively contain 5.4 gigabases of cloned soil DNA (Table 1). In a preliminary screen, we detected 10 unique clones expressing resistance to aminoglycoside antibiotics or tetracycline (Table 2), two from the BAC library, SL2, and eight from the small-insert libraries. No nalidixic acid-resistant clones were detected.

Antibiotic susceptibility testing

We measured the minimum inhibitory concentrations (MICs) of five different aminoglycosides on the nine clones that were selected on media containing aminoglycoside antibiotics (Table 3). Clones 85C1, 171D10, CR6 and CR10 are more resistant to amikacin, butirosin, kanamycin and tobramycin than strains that contain a vector with no insert. Clones CR5 and CR11 have the same resistance profile as 85C1, 171D10, CR6 and CR10, but the increase in amikacin resistance is less than fourfold compared with the vector-only control. CR7 is more resistant to butirosin and kanamycin than the vector-only control. Of the selected antibiotics, CR8 is only resistant to apramycin. CR9 displays increased resistance to all the selected aminoglycoside antibiotics. The MIC of tetracycline was fourfold higher for clone CR4 than for the vector-only control (data not shown).

Table 2. Library clones expressing antibiotic resistance.

Clone name	Vector	Insert size (kb)	Antibiotic used for selection
85C1	pBeloBAC11	65	Kanamycin
171D10	pBeloBAC11	48	Kanamycin
CR4	pJN105	5.2	Tetracycline
CR5	pJN105	7.1	Kanamycin
CR6	pJN105	4.1	Kanamycin
CR7	pJN105	7.2	Kanamycin
CR8	pCF430	2.2	Apramycin
CR9	pCF430	2.1	Butirosin
CR10	pCF430	3.7	Tobramycin
CR11	pCF430	1.9	Sisomycin

Table 3. Sensitivity of clones to selected aminoglycoside antibiotics.

		Minimum inhibitory concentration (μg ml^{-1})[a]				
Strain (plasmid)	Clone	AMI	APR	BUT	KAN	TOB
DH10B (pBeloBAC11)	Vector only	3.1	12.5	3.1	3.1	0.8
	85C1	**12.5**	12.5	**25**	**25**	**6.3**
	171D10	**25**	12.5	**100**	**>200**	**25**
DH10B (pJN105)	Vector only	6.3	12.5	6.3	6.3	3.1
	CR5	12.5	12.5	**25**	**25**	**12.5**
	CR6	**50**	12.5	**100**	**200**	**50**
	CR7	6.3	25	**25**	**>200**	3.1
DH5α (pCF430)	Vector only	0.8	1.6	0.8	1.6	0.4
	CR8	0.8	**12.5**	0.8	1.6	0.8
	CR9	**6.3**	**12.5**	**6.3**	**12.5**	**3.1**
	CR10	**12.5**	3.1	**12.5**	**100**	**12.5**
	CR11	1.6	3.1	**12.5**	**12.5**	**6.3**

a. Measurements were made in LB broth containing: amikacin (AMI); apramycin (APR); butirosin (BUT); kanamycin (KAN) or tobramycin (TOB). The table shows the median value of three independent experiments. MICs that differ from the vector-only controls by more than twofold are shown in bold.

Identification of antibiotic resistance genes

The sequences contain open reading frames (ORFs) that resemble enzymes that confer resistance by inactivation of aminoglycoside antibiotics by phosphorylation, 3-N-acetylation and 6′-N-acetylation and efflux of tetracycline (Fig. 1). Six of the nine aminoglycoside-resistant clones contain sequences that resemble 6′-N-aminoglycoside acetyltransferase [AAC(6′)] enzymes. The resistance mechanism of clone CR9 was not obvious based on the sequence.

All but one of the resistance genes that we identified encode deduced amino acid sequences that are considerably different (<60% identity) from any previously reported sequence. These include the predicted gene products of clones CR7 [41% identical to a protein in the sequenced genome of *Deinococcus radiodurans* (GenBank accession number NC_001263)] and CR8 [55% identical to a protein in the sequenced genome of *Sinorhizobium meliloti* (NC_003047)] as well as the six aac(6′) gene products. In contrast, the predicted gene product of clone CR4 is nearly identical (96% identity) to a probable membrane transport protein in the sequenced genome of *Mesorhizobium loti* (NC_002678).

Sequence analysis of the 6′-N-aminoglycoside acetyltransferases

The AAC(6′) sequences contain conserved amino acids such as the active-site residues Glu-72, His-74, Leu-76 and Tyr-147 (Draker and Wright, 2004) thought to be involved in acetyl-CoA positioning and substrate specificity (Shmara *et al*., 2001), including a GCN5-related N-acetyltransferase (GNAT) motif that is characteristic of AAC(6′) enzymes (Wybenga-Groot *et al*., 1999).

Three phylogenetically distinct clades of AAC(6′) enzymes have been described (Hannecart-Pokorni *et al*., 1997; Salipante and Hall, 2003). A phylogenetic analysis indicates that all six of the AAC(6′) enzymes belong to a monophyletic lineage (posterior probability of 0.98) within clade B (Fig. 2). The other members of this clade were discovered in clinical isolates (Tenover *et al*., 1988; Costa *et al*., 1993; Hannecart-Pokorni *et al*., 1997; Centron and Roy, 1998). The apparent sequence divergence and the types of genes adjoining the aac(6′) ORFs (Fig. 1) suggest that all the genes are derived from different genomic contexts, except for CR5 and CR6, which carry similar flanking genes, possibly suggesting a common or closely related origin.

Discussion

We designed a metagenomic analysis to isolate antibiotic resistance genes from soil. Metagenomic analysis has advantages over cultivation or PCR-based methods for isolating antibiotic resistance genes in that it: (i) provides access to uncultured microorganisms; (ii) does not require prior knowledge of gene sequences; and (iii) recovers complete genes. We identified clones expressing antibiotic resistance in *E. coli* from soil metagenomic libraries. With one exception, all the resistance proteins that we identified have deduced amino acid sequences with <60% identity to previously published sequences, suggesting that soil microorganisms harbour antibiotic resistance genes with considerably more genetic diversity than has been accounted for by previous work.

The aminoglycoside antibiotics are structurally related inhibitors of bacterial protein synthesis. The sequence diversity of aminoglycoside acetyltransferase enzymes makes it difficult to detect new aminoglycoside resistance genes using PCR with primers based only on sequences of known genes (Fluit *et al*., 2001). Given the difficulty of isolating aminoglycoside resistance genes by PCR, functional metagenomic analysis is an appealing alternative. To our knowledge, only one other aminoglycoside resistance gene, a kanamycin 3-N-acetyltransferase, has been isolated and sequenced from cloned soil DNA (Courtois *et al*., 2003).

We identified nine aminoglycoside resistance genes, six of which encode 6′-N-acetyltransferases. The predicted amino acid sequences differ considerably from previously described aminoglycoside acetyltransferases. Further work will be required to determine how the sequence divergence relates to the enzymatic activity and protein structure. The low resistance levels for some clones may be explained by poor expression in *E. coli*. These levels were measured under one set of conditions and may vary with several factors including temperature, plasmid copy number and media.

Identifying antibiotic resistance genes from uncultured soil bacteria may contribute to the search for new antibiotics in two ways. First, studies of the genetic diversity, enzymology and structure of bacterial antibiotic resistance proteins may eventually lead to the design of compounds that inhibit resistance mechanisms, thus extending the useful lifetime of currently available antibiotics. Boehr and colleagues (2003) and Sarno and colleagues (2003) recently described progress towards developing compounds that inhibit aminoglycoside resistance mechanisms. To have sustained effectiveness, inhibitor design should consider the diversity of aminoglycoside resistance genes, including those of the uncultured soil bacteria. Secondly, as genes for antibiotic resistance are often clustered with genes for antibiotic biosynthesis (Benveniste and Davies, 1973), antibiotic resistance studies may lead to the discovery of biosynthetic pathways encoding potentially novel antibiotics (Anderson *et al*., 2002). Although we did not observe a direct link between genes for antibi-

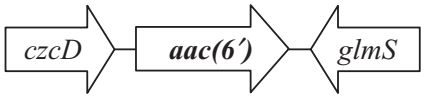
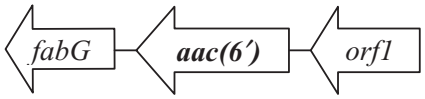
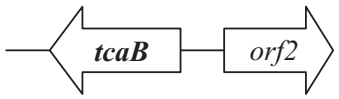
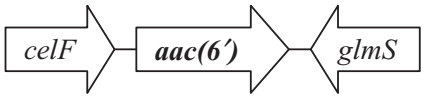
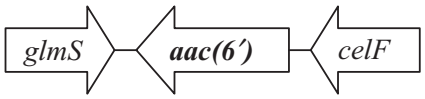
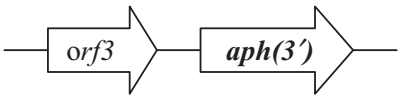
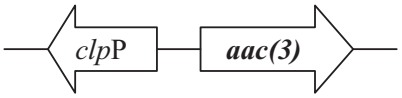
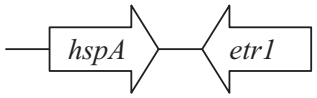
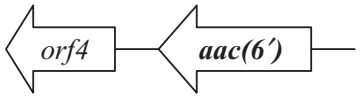
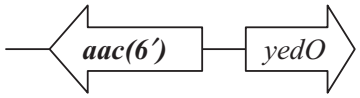
Clone	Sequence length (kb)	Schematic diagram	Deduced resistance protein	GenBank accession #
85C1	1.2		6′-N-acetyltransferase	AY566820
171D10	1.8		6′-N-acetyltransferase	AY566821
CR4	1.9		Tetracycline efflux protein	AY566822
CR5	1.1		6′-N-acetyltransferase	AY566823
CR6	1.3		6′-N-acetyltransferase	AY566824
CR7	1.4		3′-O-phosphotransferase	AY566825
CR8	2.1		3-N-acetyltransferase	AY566826
CR9	1.9		?	AY566827
CR10	1.6		6′-N-acetyltransferase	AY566828
CR11	1.9		6′-N-acetyltransferase	AY566829

Fig. 1. Schematic diagrams based on sequence analysis of the clones listed in Table 2. Antibiotic resistance genes are indicated in bold. The open reading frames are indicated by arrows (not drawn to scale): ***aac*(*6′*)** (6′-N-acetyltransferase); ***tcaB*** (drug resistance transporter, Bcr/CflA family); ***aph*(*3′*)** (3′-O-phosphotransferase); ***aac*(*3*)** (3-N-acetyltransferase); *czcD* (cation efflux family protein); *glmS* (glucosamine-fructose-6-phosphate aminotransferase); *fabG* (3-oxoacyl-(acyl-carrier protein) reductase); *celF* (6-phospho-beta-glucosidase); *orf2* (transcriptional regulator, TetR family); *clpP* [ATP-dependent Clp protease (proteolytic subunit)]; *hspA* (low-molecular-weight heat shock protein); *etr1* (ethylene response regulator); *yedO* (1-aminocyclopropane-1-carboxylate deaminase); *orf4* is a putative transposase; *orf1* and *orf3* have unknown functions. Sequence data are available under the listed accession numbers.

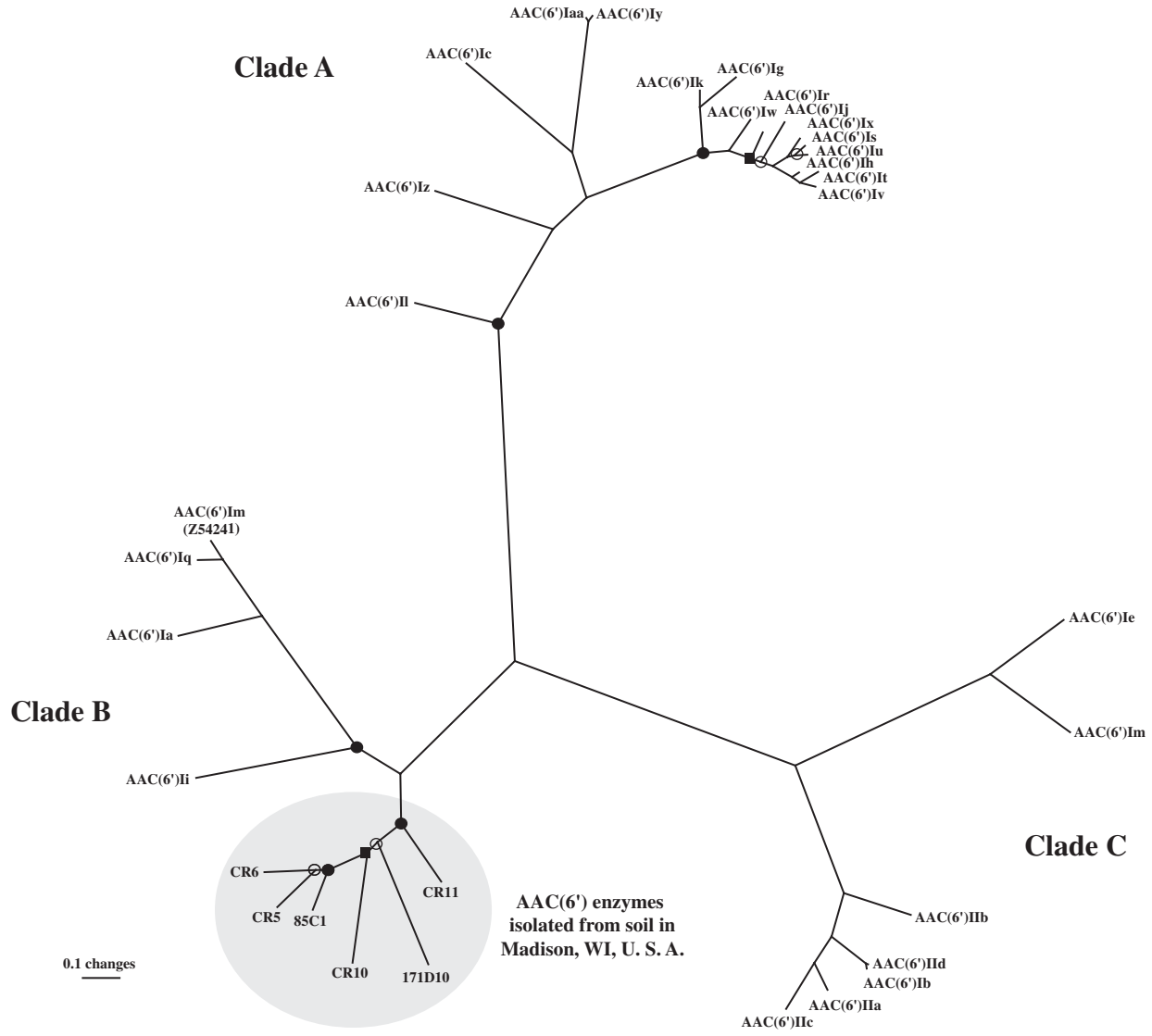


Fig. 2. Bayesian phylogenetic tree of AAC(6′) enzymes. Gene abbreviations and clade definition according to Salipante and Hall (2003). Solid circles represent nodes that had a posterior probability between 0.80 and 0.90, open circles represent posterior probabilities between 0.70 and 0.80, and solid squares represent posterior probabilities between 0.50 and 0.70. All other nodes had a posterior probability >0.90.

otic resistance and antibiotic biosynthesis in this study, this may be observed in future studies.

Our sequence data suggest that the antibiotic resistance genes are probably not derived from actinomycetes. With one exception, the G+C content of the aminoglycoside resistance ORFs ranged from 48.5% to 61.7%, which is far lower than would be expected for genes of actinomycete origin (≈ 70%). The exception was an ORF encoding a (putative) 3-N-acetyltransferase, which has a G+C content of 68.3%.

One clone (CR9) conferred low-level resistance to all the selected aminoglycoside antibiotics by a resistance mechanism that was not obvious based on the sequence. The sequence contains an ORF that resembles a low-molecular-weight heat shock protein. Aminoglycoside antibiotics bind to bacterial ribosomes and cause error-prone translation, an accumulation of misfolded protein and, ultimately, cell death (Vakulenko and Mobashery, 2003). As expression of heat shock proteins and increased resistance to antibiotics have been correlated (Hallett *et al.*, 1990; Powell and Young, 1991; Qoronfleh *et al.*, 1998; Frees and Ingmer, 1999; Thomsen *et al.*, 2002), we speculate that expression of the low-molecular-weight heat shock protein protects *E. coli* from the misfolded protein, thus conferring aminoglycoside resistance.

The results also demonstrate the power of selections in the functional analysis of metagenomic libraries. The high microbial diversity in soil necessitates building libraries containing large numbers of clones. Screening such libraries is laborious. In this study, selecting for clones express-

ing antibiotic resistance in a collection of over one million clones was extremely rapid.

Studies of diverse phenotypes indicate that clones with the desired phenotype are identified at a low frequency from metagenomic libraries (Henne *et al*., 2000; Rondon *et al*., 2000; Majernik *et al*., 2001). In this study, the frequency of clones expressing detectable antibiotic resistance averaged two clones per gigabase of cloned DNA. Numerous factors probably contribute to the low abundance of functionally detectable clones, including poor heterologous gene expression and the inability to detect recessive traits. For example, the absence of clones expressing nalidixic acid resistance in our libraries is not surprising given that a common mechanism of nalidixic acid resistance is a mutation of *gyrA*, which is recessive to the wild-type *gyrA* (Hane and Wood, 1969), and plasmid-mediated resistance is rare (Martínez-Martínez *et al*., 1998; Jacoby *et al*., 2003). Although it will be difficult to devise strategies to detect recessive traits, barriers to heterologous gene expression will be readily examined.

Barriers to heterologous gene expression include both promoter recognition and translation of rare codons. In subsequent studies, we will attempt to expand the range of genes that are expressed in *E. coli* by supplementing the host with alternative sigma factors and extra tRNAs for rare codons. Future work will also explore the use of alternative host species, including those from phyla other than Proteobacteria.

Extensions of this work will include analysis of soils from diverse geographical locations and resistance to other antibiotics. The results of these studies will provide insight into the significance of the vast uncultured community of soil bacteria as a reservoir for antibiotic resistance genes. It is not known whether antibiotic resistance genes move readily from environmental reservoirs to clinical settings, but future work should consider the potential contributions of soil bacteria to the problem of antibiotic resistance.

Experimental procedures

Soil description

The source of soil for this work was a remnant oak savannah site at the West Madison Agricultural Research Station (Madison, WI, USA). To our knowledge, antibiotics have never been applied to the site. The soil type is a Plano silt loam containing 61% sand, 23% silt and 16% clay with 1.7% organic matter (Bintrim *et al*., 1997). In previous studies, we characterized the microbial diversity of the soil by constructing and sequencing libraries of 16S rRNA genes (Bintrim *et al*., 1997; Liles *et al*., 2003). The studies indicate that the soil contains a diverse bacterial flora, which includes members of several bacterial divisions with few or no cultured representatives.

Bacterial strains

The *E. coli* strains DH10B (F^- *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 D(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G) and DH5α (F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-,m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) (Life Technologies) were the host strains for maintaining libraries.

Libraries of cloned soil DNA

We reported previously metagenomic libraries SL1 and SL2 (Table 1), which contain large fragments (average 27 and 45 kb respectively) of soil DNA cloned into pBeloBAC11 (New England Biolabs) with clones arrayed in 96-well format.

We constructed four new libraries (Table 1) by cloning soil DNA fragments into the broad-host-range expression vectors pJN105 (encoding gentamicin resistance) and pCF430 (encoding tetracycline resistance) (Newman and Fuqua, 1999). Strains were grown in Luria–Bertani (LB) medium with the appropriate antibiotic to select for plasmid maintenance (30 μg ml^{-1} gentamicin or 5 μg ml^{-1} tetracycline). DNA was isolated from soil core samples using a FastDNA® Spin kit (BIO 101) and then extracted with hexadecyltrimethylammonium bromide (CTAB) (Ausubel *et al*., 1995). Soil DNA was digested with the restriction enzymes *Eco*RI or *Pst*I, and fragments of 1.5 kb to 10 kb were gel purified by electroelution and ligated into pJN105. The vector was prepared by digestion with the same enzymes, treatment with shrimp alkaline phosphatase and gel purification by electroelution. Libraries were transformed into ElectroMAX™ DH10B cells (Gibco BRL) by electroporation [Gene Pulser™ (Bio-Rad) 2.5 kV, 25 μF, 200 Ω)]. Another library (EL4) was prepared as described above except that a Qiaex® II gel extraction kit (Qiagen) was used for gel purification of *Pst*I-digested soil DNA, fragments were cloned into pCF430, and the resulting library was transformed into electrocompetent DH5α cells. This library is ideal for aminoglycoside resistance studies because the host genotype does not include the *rpsL* mutation (streptomycin resistance) and the selection for the vector is tetracycline rather than gentamicin resistance. Enzymes were purchased from Promega and used according to the manufacturer's instructions. To determine the average insert size for each library, the plasmids from 24 colonies were isolated, digested with the same enzyme used for cloning and sized by gel electrophoresis. Pools of library clones were stored at −80°C.

Isolation of clones expressing antibiotic resistance

Pools of clones from libraries SL1, SL2, EL1, EL2 and EL3 (Table 1) were plated on to LB medium containing inhibitory concentrations of kanamycin (10 μg ml^{-1}), tetracycline (5 μg ml^{-1}) or nalidixic acid (5 μg ml^{-1}). Selections for aminoglycoside-resistant clones were conducted by plating pools of EL4 clones on to LB medium containing inhibitory concentrations of the aminoglycoside antibiotics amikacin (2.5 μg ml^{-1}), apramycin (5 μg ml^{-1}), butirosin (2.5 μg ml^{-1}), gentamicin (1.25 μg ml^{-1}), kanamycin (2.5 μg ml^{-1}), neomycin (2.5 μg ml^{-1}), sisomycin (1.25 μg ml^{-1}), streptomycin (5 μg ml^{-1}) or tobramycin (2.5 μg ml^{-1}). Plates were incubated

overnight at 37°C. The plasmids of 24 antibiotic-resistant colonies were isolated and digested with the same enzyme used to clone the soil DNA. Plasmids with unique restriction fragment length polymorphism (RFLP) patterns were retransformed into DH10B or DH5α, and the phenotypes were verified by patching cells on to the appropriate media to ensure that the cloned DNA was responsible for the phenotype. Antibiotics were purchased from Sigma-Aldrich.

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using microtitre plate dilution assays in LB broth with about 1×10^5 cells per well. Experiments were done in triplicate.

Identification of antibiotic resistance genes

Resistance genes were identified by subcloning and/or *in vitro* transposon mutagenesis with the Genome Priming System, GPS-1™ (New England Biolabs). Subcloning was accomplished by partial digestion of plasmid DNA with *Sau*3AI, ligation into the *Bam*HI site of pUC19 (encoding ampicillin resistance) and selection for subclones on LB media with the appropriate antibiotics. Subclones were sized by gel electrophoresis after digestion with *Pvu*II. *In vitro* transposon mutagenesis inserts transposons randomly into plasmid DNA, and antibiotic-susceptible mutants were identified by patching cells on to media containing the appropriate antibiotic. Sequencing reactions, using M13 forward and M13 reverse primers or with primers specific for the transposon ends, were performed on both DNA strands with BigDye™ (Applied Biosystems) and analysed at the University of Wisconsin-Madison Biotechnology Center. Sequences were assembled with SEQMAN™ software (DNAStar). Putative ORFs were identified with ORF FIND and BLAST (http://www.ncbi.nlm.nih.gov). Sequence data are available under the listed GenBank accession numbers (Fig. 1).

Phylogenetic analysis

The amino acid sequences of six AAC(6′) enzymes identified in this study, the 26 AAC(6′) enzymes reviewed by Salipante and Hall (2003) and one AAC(6′) sequence identified by Hannecart-Pokorni and colleagues (1997) (GenBank accession no. Z54241) were aligned in CLUSTALW using the default parameters. For the two sequences (M18086 and AF453998) that encode AAC(6′) enzymes fused to other proteins, only the AAC(6′) portion was included in the alignment. As described by Salipante and Hall (2003), a Bayesian phylogenetic tree was constructed using MRBAYES (Huelsenbeck and Ronquist, 2001). A total of 3 000 000 generations were performed, and 3000 trees were saved. A consensus tree was constructed after removing the first 100 trees as burn-in.

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