Applied and Environmental Microbiology, June 2010, p. 3753–3757 Vol. 76, No. 11
0099-2240/10/$12.00 doi:10.1128/AEM.03080-09

Identification of Diverse Antimicrobial Resistance Determinants Carried on Bacterial, Plasmid, or Viral Metagenomes from an Activated Sludge Microbial Assemblage▽

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Received 21 December 2009/Accepted 30 March 2010

Using both sequence- and function-based metagenomic approaches, multiple antibiotic resistance determinants were identified within metagenomic libraries constructed from DNA extracted from bacterial chromosomes, plasmids, or viruses within an activated sludge microbial assemblage. Metagenomic clones and a plasmid that in *Escherichia coli* expressed resistance to chloramphenicol, ampicillin, or kanamycin were isolated, with many cloned DNA sequences lacking any significant homology to known antibiotic resistance determinants.

Activated sludge in wastewater treatment plants is an open system with a dynamic and phylogenetically diverse microbial community (2, 3, 6, 7, 10, 11). Since the activated sludge process promotes cellular interactions among diverse microorganisms, there is great potential for the lateral transfer of antibiotic resistance genes between microbes in activated sludge and in downstream environments. Several studies have previously identified antibiotic resistance determinants from wastewater communities that are carried on bacterial chromosomes (1, 4, 14) and plasmids (9, 12, 13), but to our knowledge, a simultaneous metagenomic survey of antibiotic resistance determinants from all three genetic reservoirs (i.e., chromosomes, plasmids, and viruses) has never been performed within the same environment. To achieve a more comprehensive assessment of antibiotic resistance genes in the activated sludge microbial community, this study used both function- and sequence-based metagenomic approaches to identify antibiotic resistance determinants carried on bacterial chromosomes, plasmids, or viruses within an activated sludge microbial assemblage.

Identification and characterization of chromosomally carried antibiotic resistance genes. Four small insert bacterial metagenomic libraries were constructed in the pSMART-LCKan or LCAmp vector (Lucigen Corp., Middleton, WI), which allowed for cross-selection of antibiotic resistance (i.e., plating the pSMART-LCKan library on LB agar containing ampicillin and vice versa) and required transcription of cloned genes from native or cryptic promoters due to the presence of transcriptional terminators flanking the cloning sites in the pSMART vectors. These libraries, which collectively contained approximately 1.85 Gbp of cloned DNA (ca. 400 *Escherichia coli* genome equivalents), were screened for clones encoding resistance to each of the following 11 antibiotics: ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, polymyxin B, rifampin, spectinomycin, tetracycline, and trimethoprim. Nine unique clones exhibiting resistance to chloramphenicol ($n = 7$), ampicillin ($n = 1$), or kanamycin ($n = 1$) were identified and validated by retransformation of the recombinant clone into a naïve *E. coli* strain and demonstration of the respective antibiotic resistance phenotype. Analysis of the cloned insert DNA sequences revealed that their predicated gene products in some cases ($n = 3$) had significant homology with known antibiotic resistance determinants in the GenBank nonredundant nucleotide (nr/nt) database, but most cloned insert DNA sequences ($n = 6$) did not have any significant homology to a known antibiotic resistance determinant, even though significant GenBank hits in some cases were identified (Table 1).

The clones that expressed resistance to ampicillin and kanamycin contained inserts with sequences similar to those of a class C β-lactamase and an *O*-methyltransferase, respectively. However, six of the seven clones exhibiting resistance to chloramphenicol (Cm) appeared to encode gene products that have not been associated with Cm resistance, such as the chloramphenicol acetyltransferase (CAT) enzyme. Clone A5's insert sequences did not have any significant homology to any predicted gene products in either the nr/nt or ENV GenBank databases.

Characterization of Cm^r clones with no homology to known resistance genes. To identify the genetic loci necessary for Cm^r in these six clones, the clones were mutagenized with a *Tn*5 transposon containing a tetracycline resistance cassette to generate loss-of-function mutants. In two cases, the interrupted open reading frames (ORFs) necessary for Cm^r did not have homology with any sequences in the GenBank nr/nt or ENV databases. Three clones contained interrupted ORFs whose top BLASTx hits indicated hypothetical proteins, including clone B4, which contained an additional interrupted ORF required for Cm^r with similarity to an ABC transporter protein.

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▽ Published ahead of print on 9 April 2010.

TABLE 1. Predicted gene products and phylogeny of putative antibiotic resistance determinants from metagenomic clones and plasmid pAS1 based on BLASTx comparison with the GenBank nr/nt database

Sample	Source	Antibiotic resistance	Top BLASTx result	Nearest neighbor	% identity/ % similarity
A4	Bacterial genome	Cm	PKD-repeating protein	*Cytophaga hutchinsonii*	39/54
A5	Bacterial genome	Cm	No significant hits found	N/A[a]	N/A
A7	Bacterial genome	Cm	Hypothetical protein FB2170	Flavobacteriales bacterium HTCC2170	59/76
A9	Bacterial genome	Cm	Hypothetical protein sce4763	*Sorangium cellulosum*	57/75
A10	Bacterial genome	Cm	Large subunit of *N*,*N*-dimethylformidase	*Hoeflea phototrophica* DFL-43	31/48
A12	Bacterial genome	Cm	Class V aminotransferase	*Hyphomonas neptunium*	33/45
B4	Bacterial genome	Cm	Hypothetical protein Mpe_A2882	*Methylibium petroleiphilum*	67/77
D10	Bacterial genome	Amp	PBP, Class C β-lactamase	*Spirosoma linguale*	66/80
E5	Bacterial genome	Km	*O*-Methyltransferase	*Microscilla* sp.	55/62
pAS1	Plasmid	Km	Aminoglycoside phosphotransferase	*Rhodopseudomonas palustris*	70/83
pAS1	Plasmid	Amp	TEM β-lactamase	*Bacillus* sp. BT-192	95/96
Ph-A8	Phage genome	N/A	Acriflavin resistance protein	*Xanthobacter autotrophicus*	53/74
Ph-C2	Phage genome	N/A	Drug resistance transporter, Bcr/CflA subfamily	*Serratia proteamaculans*	96/98
Ph-C3	Phage genome	N/A	Putative transmembrane multidrug efflux system lipoprotein	*Ralstonia pickettii*	95/97
Ph-D8	Phage genome	N/A	Class A β-lactamase	*Capnocytophaga ochracea*	98/98
Ph-E10	Phage genome	N/A	TetC protein	*Chlamydia suis*	89/92
Ph-G10	Phage genome	N/A	Glyoxalase/bleomycin resistance protein	*Stenotrophomonas maltophilia*	84/89

[a] N/A, not applicable.

BLASTx comparison and multiple alignments of the six Cm^r clones along with known Cm^r determinants (type I, II, and III CATs) revealed that these genes do not share any significant similarity with CATs and may originate from diverse bacterial lineages (data not shown). MIC data obtained for each Cm^r clone indicate a higher level of resistance than what is often seen with this particular antibiotic. While many of the clones showed similar MIC threshold levels (64 μg/ml), two of the clones (A7 and B4) exhibited much higher levels of Cm resistance (256 μg/ml).

To further investigate the resistance mechanism(s) encoded by these six Cm^r clones, clone supernatants were analyzed by high-performance liquid chromatography (HPLC), and the resulting profiles were compared to that of pSU2719, a plasmid that encodes the CAT enzyme. All six of the clones showed comparable peak profiles to that of pSU2719, with some minor peaks differing between the CAT and clone-incubated media (Fig. 1), suggesting that in each of the respective clones, Cm may be enzymatically modified and inactivated. The presence of a CAT-like mechanism was further supported by the results obtained from florfenicol susceptibility testing, in which all six Cm^r clones tested exhibited zones of inhibition to 30 μg florfenicol measuring ≥25 mm and were considered susceptible. Because florfenicol resistance is typically conferred by efflux pumps, which also provide resistance to chloramphenicol (15), the observation of the clones' resistance to chloramphenicol but not florfenicol supports the evidence of enzymatic modification as the mechanism of Cm^r for these clones.

Identification of the multidrug-resistant plasmid pAS1. Community plasmid DNA was isolated from the activated sludge basin and treated with a plasmid-safe exonuclease (Epicentre, Madison, WI) to degrade contaminating chromosomal DNA. The purified plasmid DNA was then either transformed directly into *E. coli* strain DH10B or first mutagenized with the R6Kγ*ori*/KAN-2 transposon (Epicentre, Madison, WI) to rescue plasmids that may be unable to replicate in an *E. coli* host (5). Functional screening against the panel of 11 antibiotics yielded the same 4.2-kb plasmid (pAS1) from both transformation reactions, which conferred both ampicillin and kanamycin resistance to *E. coli.* The resistance phenotypes were then verified by retransformation of pAS1into a naïve *E. coli* strain DH10B and plating onto the respective antibiotic-containing medium. Shotgun subcloning and sequence analysis of pAS1 identified two predicted gene products most likely responsible for the antibiotic resistance phenotypes, with significant homology to gene sequences in the GenBank nr/nt database encoding an aminoglycoside phosphotransferase from the genus *Rhodopseudomonas* (*Alphaproteobacteria*) and a TEM β-lactamase from the genus *Bacillus* (*Firmicutes*), respectively (Table 1).

Identification of putative antimicrobial resistance genes carried by bacteriophages. Functional screening of a viral metagenomic library (8) containing approximately 1.2 Mbp of cloned DNA from activated sludge did not yield any antibiotic-resistant clones. The small average insert size of these clones (662 bp) likely contributed to the inability to isolate any clones with intact resistance determinants. Therefore, a sequence-based approach was employed to identify phage-carried resistance genes from the 1,161 viral metagenomic clones. Six clone inserts were identified that had homology with known antibiotic resistance genes in the GenBank nr/nt database, representing bacterial taxa from the *Bacteroidetes* and *Chlamydiae* phyla and *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* classes (Table 1). Based on BLASTx data and ORF analysis, these clones appear to carry partial genes that may be responsible for resistance to several antibiotics, including tetracycline, ampicillin, acriflavine, and bleomycin, as well as efflux systems that may mediate resistance to additional antibiotics. None of the clones identified by sequence analysis conferred their predicted antibiotic resistance to *E. coli* when plated onto media containing the respective antibiotic, likely due to incomplete ORFs and/or lack of heterologous expression in *E. coli*.

In general, the degree of homology of plasmid- or phage-

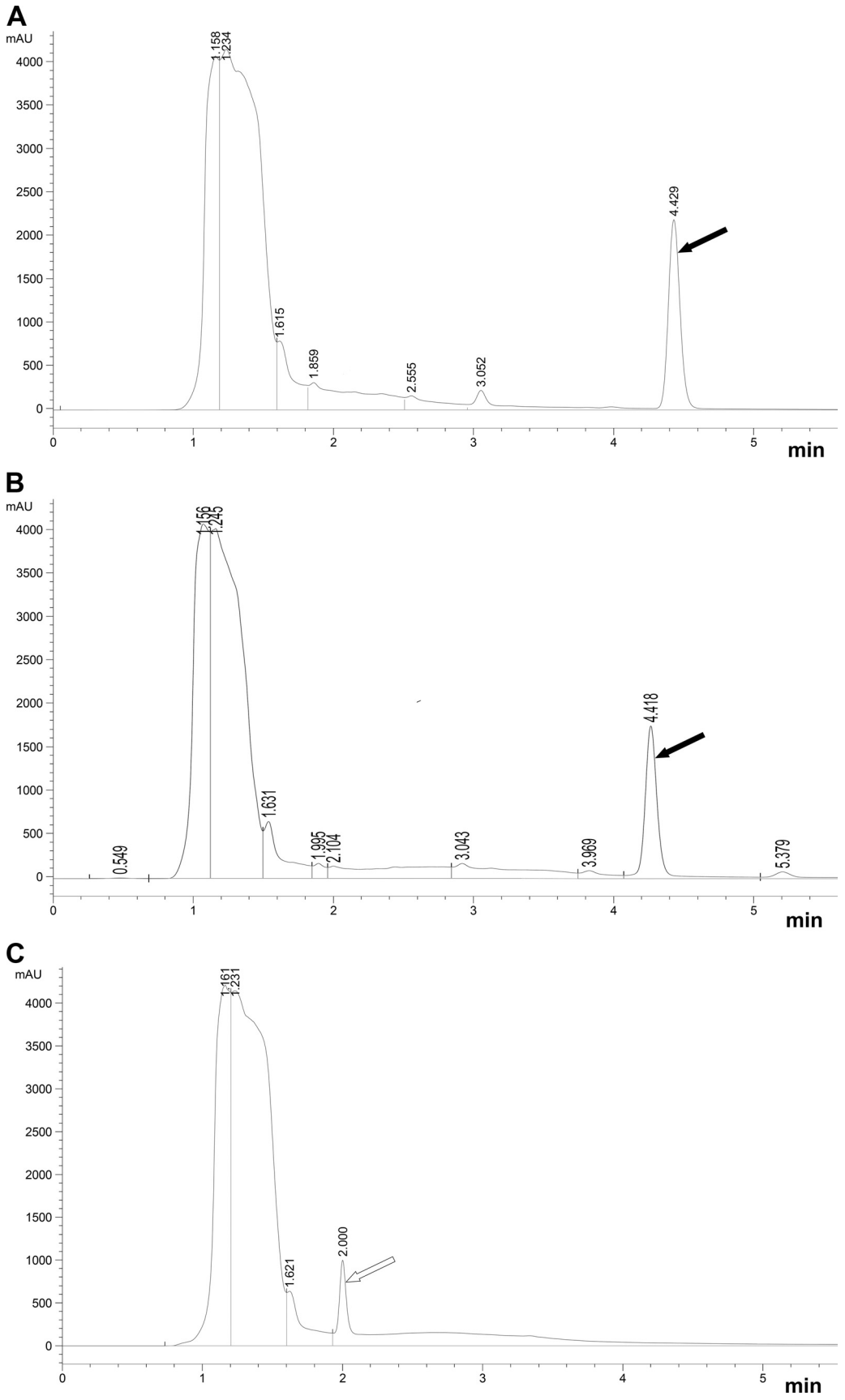

FIG. 1. HPLC profiles of clone A5 (A) and pSU2719 (B) supernatants from cultures grown overnight in the presence of chloramphenicol. (C) Negative control in which chloramphenicol was incubated overnight in cell-free medium. Filled arrows indicate the appearance of a by-product with a peak at 4.4 min, and the empty arrow indicates the native chloramphenicol peak. mAU, milli-absorbance units.

TABLE 2. Predicted gene products and phylogeny of putative mobile genetic elements identified from metagenomic clones and plasmid pAS1

Sample	Source	Top BLASTx result	Nearest neighbor	% identity/ % similarity
E5	Bacterial genome	Transposase	*Acidovorax avenae*	90/93
pAS1	Plasmid	Transposase	*Proteus mirabilis*	100/100
Ph-A11	Phage genome	Transposase protein A	*Methylococcus capsulatus*	50/63
Ph-C11	Phage genome	Transposase	*Nitrobacter* sp. Nb-311A	70/74
Ph-C12	Phage genome	Transposase	*Gluconobacter oxydans*	75/81
Ph-E06	Phage genome	Transposase, IS605 OrfB family	*Burkholderia cenocepacia* MC0-3	33/51
Ph-F11	Phage genome	Transposase of ISAba1, IS4 family	*Acinetobacter baumannii*	99/99
Ph-G04	Phage genome	TniA transposase	*Pseudomonas stutzeri*	34/44

derived antibiotic resistance determinants to entries within the GenBank databases was significantly higher than that observed for chromosomally carried loci. The latter observation indicates that there is a relatively unknown reservoir of antibiotic resistance determinants present within prokaryotic genomes that may potentially be laterally transferred. Further testing of the host range of each antibiotic-resistant clone will help to define the limits of heterologous expression for these loci.

Potential for lateral transfer of antibiotic resistance genes from bacterial chromosomes, plasmids, and bacteriophages. Sequence analysis of DNA from all three metagenomic sources was performed to identify gene products potentially involved in the lateral transfer of antibiotic resistance genes (Table 2). Clone E5, isolated from the bacterial metagenomic library, contains an insert sequence with 90% sequence identity to a gene encoding a transposase from *Acidovorax avenae* (*Betaproteobacteria*). This clone also carries a putative *O*-methyltransferase and confers kanamycin resistance to *E. coli*, yet the nearest neighbor for the predicted *O*-methyltransferase gene (55% sequence identity) is from the genus *Microscilla* (*Bacteroidetes*). The higher percent G+C content of clone E5 (58%) is more similar to that of *Acidovorax* spp. (ca. 65% G+C) than to that of *Microscilla* spp. (ca. 41% G+C). The close proximity of the putative transposase and *O*-methyltransferase genes within the E5 clone insert suggests that the *O*-methyltransferase gene, and therefore kanamycin resistance, may be laterally transferred among bacterial hosts.

The multidrug resistance plasmid pAS1 showed evidence of mediating the transposition of both of the resistance genes into the host genome. A 900-bp fragment present in pAS1 from its original isolation was absent in all plasmid preparations from *E. coli* cultures after a single passage, yet the *E. coli* culture antibiotic resistances persisted (data not shown). Secondary transformations performed with pAS1 lacking the 900-bp fragment did not result in any clones resistant to either of the antibiotics, which supports the hypothesis that these resistance genes may be easily transposed from the plasmid to the host genome. The plasmid also contains genes with 100% homology to a transposase derived from *Gammaproteobacteria* taxa, indicating its potential to shuttle genes between bacterial hosts not only by plasmid transfer but also by transposition of specific regions of DNA into the host genome.

Bacteriophages, which in themselves mediate the transduction of genes between bacterial hosts, also harbored diverse putative transposases identified from the viral metagenomic libraries (Table 2). These transposases may be responsible for the transfer of various genetic loci between diverse bacterial hosts, as evidenced by the number of bacterial divisions represented by the antibiotic resistance gene and mobile genetic element (MGE) sequences from phage-derived metagenomic sequences. Although all of the MGEs identified from the surveys are likely derived from proteobacteria taxa, the observation that they were often linked with antibiotic resistance loci with a high degree of homology to resistance genes potentially derived from other phyla suggests that genes from phylogenetically diverse sources may have been transferred through multiple mechanisms (e.g., conjugation, transduction, and transformation).

The discovery of these resistance genes and MGEs illustrates the advantages of using both function- and sequence-based screening methods, as many of the Cm^r genes likely would not have been identified by sequence-based screening alone; conversely, sequence-based analysis of the viral metagenomic library allowed for the identification of putative antibiotic resistance genes and MGEs. The identification of functionally and phylogenetically diverse antibiotic resistance determinants and MGEs from all three metagenomic reservoirs demonstrates the widespread occurrence and potential movement of antibiotic resistance determinants within activated sludge microbial communities.

Nucleotide accession numbers. Chromosome- and plasmid-carried resistance genes and MGEs are deposited in GenBank under accession numbers GU720994 to GU721005. The viral metagenomic DNA sequences were registered as part of the Wastewater Viral Metagenome project at GenBank (Trace Archives) and are listed under TI reference numbers 2251203077 to 2251204802.

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