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Functional Metagenome Mining of Soil for a Novel Gentamicin Resistance Gene

Hyunjoo Im[1,2], Kyung Mo Kim[3,4], Sang-Heon Lee[3,4], and Choong-Min Ryu[1,2*]

[1]*Molecular Phytobacteriology Laboratory, KRIBB, Daejeon 34113, Republic of Korea*
[2]*Biosystems and Bioengineering Program, University of Science and Technology (UST), Daejeon 34141, Republic of Korea*
[3]*Microbial Resource Center, KRIBB, Jeongeup 56212, Republic of Korea*
[4]*Department of Bioinformatics, University of Science and Technology (UST), Daejeon 34141, Republic of Korea*

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**Corresponding author*
Phone: +82-42-879-8229;
Fax: +82-42-860-4488;
E-mail: cmryu@kribb.re.kr

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Extensive use of antibiotics over recent decades has led to bacterial resistance against antibiotics, including gentamicin, one of the most effective aminoglycosides. The emergence of resistance is problematic for hospitals, since gentamicin is an important broad-spectrum antibiotic for the control of bacterial pathogens in the clinic. Previous study to identify gentamicin resistance genes from environmental samples have been conducted using culture-dependent screening methods. To overcome these limitations, we employed a metagenome-based culture-independent protocol to identify gentamicin resistance genes. Through functional screening of metagenome libraries derived from soil samples, a fosmid clone was selected as it conferred strong gentamicin resistance. To identify a specific functioning gene conferring gentamicin resistance from a selected fosmid clone (35–40 kb), a shot-gun library was constructed and four shot-gun clones (2–3 kb) were selected. Further characterization of these clones revealed that they contained sequences similar to that of the RNA ligase, T4 *rnlA* that is known as a toxin gene. The overexpression of the *rnlA*-like gene in *Escherichia coli* increased gentamicin resistance, indicating that this toxin gene modulates this trait. The results of our metagenome library analysis suggest that the *rnlA*-like gene may represent a new class of gentamicin resistance genes in pathogenic bacteria. In addition, we demonstrate that the soil metagenome can provide an important resource for the identification of antibiotic resistance genes, which are valuable molecular targets in efforts to overcome antibiotic resistance.

Keywords: Gentamicin, RnlA, metagenome, shot-gun library, antibiotic resistance

Introduction

Environmental bacteria represent a huge resource of antibiotic-resistant species awaiting characterization [8]. The majority of antibiotic-resistant bacteria in the environment are derived from clinical and veterinary places, enter rivers from sewage and waste water, and can then be delivered to, and accumulate in, soil [13]. To understand the nature of antibiotic resistance, previous studies have sought to characterize antibiotic resistance genes from soil using agar-based culture-dependent protocols under laboratory conditions [1, 35]. For example, artificial agar media containing target antibiotics have been used to isolate and characterize specific bacterial species that are resistant to particular antibiotics, including streptomycin-resistant *Pseudomonas fluorescens*, penicillin-resistant *P. fluorescens* and *Burkholderia cepacia*, and chloramphenicol-resistant *Flavobacterium* sp. and *Streptomyces venezuelae* [1, 35]. However, a number of recent studies indicate that only 0.1 to 1% of bacteria from environmental samples, such as soil, are culturable [3]. These data suggest that many antibiotic resistance genes remain undiscovered and have not yet been characterized, providing a potential source of information relevant to human and animal health.

To overcome the limitations of culture-dependent methods, a next-generation sequencing-based metagenomic approach

has been employed and evaluated for its ability to identify unknown antibiotic resistance genes [40]. Beyond a sequencing-based metagenome approach, function-based (functional) metagenome analysis allowed metagenome clones to be selected and characterized by biological validation [2, 10, 17, 46, 47]. Clearly, unknown antibiotic resistance genes cannot be detected through database searches; therefore, functional metagenomic techniques have been developed to address this problem. Functional metagenome libraries are generally constructed in a surrogate host (usually *Escherichia coli*) to facilitate expression of fragmented environmental DNA [6, 30]. Screening of functional metagenome libraries has led to the identification of a number of novel antibiotic resistance genes from soil, including beta-lactamases, which mediate resistance to carbenicillin and amoxicillin [11], acetyltransferases for resistance to kanamycin [38], drug resistance transporters for florfenicol and chloramphenicol [23, 44], and tetracycline efflux proteins mediating resistance to tetracycline [40]. Despite the fact that gentamicin is a powerful broad-spectrum antibiotic, gentamicin resistance genes are poorly represented in previous metagenome mining reports [14, 27]. Aminoglycoside (including gentamicin) resistance genes play critical roles in attenuating antibiotic uptake or decreasing cell permeability, altering ribosomal-binding sites and production of aminoglycoside-modifying enzymes, resulting in poor ribosome binding [9, 42]. Enzymatic modification, such as ATP-dependent *O*-phosphorylation by phosphotransferases (APH), ATP-dependent *O*-adenylation by nucleotidyltransferases, and acetyl CoA-dependent *N*-acetylation by acetyltransferases, is the most common type of aminoglycoside resistance [43].

Here, we applied a functional metagenomic approach to isolate unknown gentamicin resistance genes from environmental samples. We employed a metagenome library from soil bacteria communities into *E. coli* and isolated four clones conferring gentamicin resistance. All four clones encoded a putative RNA ligase that was similar to T4 *rnlA*, a known bacterial toxin [29, 32]. We then characterized the molecular mechanism underlying the antibiotic resistance mediated by this gene. To the best of our knowledge, this is the first study to demonstrate that toxin-like genes from a soil metagenome can confer gentamicin resistance.

Materials and Methods

Bacterial Strains and Culture Conditions

E. coli strain EPI300 was used for metagenome library construction and strain DH5α was used for shot-gun library construction. *E. coli* cells were routinely cultured in Luria-Bertani (LB) medium (Affymetrix, Cleveland OH, USA) at 37°C, and plating media contained 2% agar. When appropriate, media were supplemented with 20 μg/ml chloramphenicol and 100 μg/ml ampicillin for strain and plasmid maintenance, respectively. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Metagenome Library Construction

Metagenome libraries were obtained from the Metagenome Bank of the Microbial Genomics Frontier Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB) [24, 25]. Briefly, the libraries were constructed from various soil samples that included 38 spots in South Korea, such as mud flat, compost, paddy soils, upland soils, and rhizosphere soils. The fosmid libraries consisted of extracted DNA fragments from each soil source (35–40 kb), which were cloned into the vector pCC1fos. Fosmid clones were deposited into single wells of 96-well microtiter plates (a single pool per well) with an average of 1,000 clones per single pool. The number of total clones were mathematically 5.76×10^5.

Screening for Gentamicin-Resistant Clones

The fosmid libraries were pooled and spread on LB agar containing 10, 20, and 40 μg/ml gentamicin for isolating different capacities of antibiotic resistance. These concentrations of gentamicin were chosen because they inhibit the growth of the host *E. coli* strain EPI300. The library was plated in triplicates and plates were incubated aerobically at 37°C for 24–48 h. Fosmid clones pA1, pA3, pC1, and pG1 that were resistant on three replicate plates were selected, their resistance phenotype was verified by re-streaking onto agar containing 20 μg/ml chloramphenicol (pCC1fos vector marker gene), and stocks were prepared for further analysis. To screen for genes conferring gentamicin resistance, high-molecular-weight DNA from fosmid clone pA1 isolated from paddy soils from Asan-si, Gongju-si, and Suncheon-si was sheared [39] and a shot-gun library (2–3 kb) was constructed in the pUC118 vector by a commercial company (Genotech, Daejeon, Korea). The shot-gun library fragments were transformed into DH5α competent cells (Enzynomics, Daejeon, Korea). The shot-gun library was plated onto LB agar with 10, 20, and 40 μg/ml gentamicin and incubated aerobically at 37°C for 24–48 h. Shot-gun clones pA1b, pA1g, pA1h, and pA1j that were resistant in three replicate plates were selected, their resistance phenotype was verified by re-streaking onto agar containing 100 μg/ml ampicillin (pUC118 vector marker gene), and stocks were prepared for further analysis. Plasmid DNA was isolated from individual resistant clones and used for repeat transformation of *E. coli* DH5α cells to confirm that the plasmid was responsible for the resistance phenotype.

Spot Assays

Cultures containing fosmid clones, vector control (pCC1fos), and positive control (pDONR207: containing a known gentamicin

resistance gene, acetyltransferases, *acc*) were grown to an OD_{600} of 0.3 in LB containing 20 μg/ml chloramphenicol at 37°C and allowed to grow for some time in the presence of 100 μM IPTG to induce expression of the cloned gene product. Ten microliters of 10-fold serial dilutions (1:10 to 1:10^4) of each bacterial culture was spotted onto LB agar plates containing 0, 1.25, 2.5, 5, 10, 20, and 40 μg/ml gentamicin. The plates were incubated aerobically at 37°C for 24 h.

Minimum Inhibitory Concentration Assays

Minimum inhibitory concentrations (MICs) of antibiotics for shot-gun clones were determined by serial dilution assay of the relevant antibiotic in LB broth. *E. coli* DH5α cultures containing the pUC118 vector were grown to an OD_{600} of 0.3 in LB containing 100 μg/ml ampicillin with shaking at 37°C. After inoculation of cultures into LB broth (0.5%), the suspensions were serially diluted from the suspensions containing 50 μg/ml gentamicin [49]. Positive (broth +inoculum only) and negative (broth + 50 μg/ml gentamicin) controls were included in each assay. Tubes were incubated aerobically at 37°C for 24 h on a shaker at 200 rpm. MICs were measured and compared with that of the vector control (pUC118) to determine the relative increase in resistance. For comparison, spot assays were conducted for each population of clones. Ten microliters of 10-fold serial dilutions of resistant cells grown in the presence of 3 or 12 μg/ml gentamicin was applied to LB agar plates containing the same concentrations of gentamicin. The MIC_{50} and MIC_{90} values were calculated by probit analysis [12, 37]. Determination of log10 gentamicin concentration values were taken with the probit points 5.0000 (MIC_{50}) and 6.2816 (MIC_{90}).

Sequencing of Fosmid and Shot-Gun Clones

Plasmids were extracted from bacterial cultures using the QIAprep Spin Mini Prep kit (Qiagen, Sussex, UK) and sequenced by a commercial company (Genotech, Korea). Protein-coding genes were identified using Prodigal v2.6.1 [18]. Predicted coding sequences (CDSs) were BLAST-searched against UniProt [51], Pfam [4], and Clusters of Orthologous Groups (COG) [45] databases to gain insight into the molecular functions and family classifications of predicted genes. Signal peptides and transmembrane helices were predicted using SignalP v4.1 [34] and TMHMM v2.0 [20]. rRNA, tRNA, and other miscellaneous features were predicted using RNAmmer v1.2 [22], tRNAscan-SE v1.21 [26], and Rfam v12.0 [16]. The graphic circular map of the genome was constructed and visualized using Circos v0.67 [21]. Automatic detection of clustered regularly interspaced short palindromic repeats (CRISPR) was performed using MinCED v0.2.0 [5]. Shot-gun clones were sequenced using M13 forward and reverse primers and data analyzed using NCBI BLAST and NCBI ORF finder to identify resistance genes. Both nucleotide and amino acid sequence alignments were calculated using BLAST (http://blast.ncbi.nlm.nih.gov), and putative protein functions were annotated based on similarities to sequences in BLAST2go v3.2 [7] and Prodigal [18].

Metagenome Fragment Analyses

The organism identification of the pA1 fosmid clone was predicted using PhylopythiaS, a bioinformatics search engine [28]. (http://binning.bioinf.mpi-inf.mpg.de/).

Results

Soil Metagenome Screening for Gentamicin-Resistant Fosmid Clones

Screening of a 576-fosmid-clone metagenome library constructed from soil DNA led to the selection of four clones stably expressing a resistance phenotype on LB plates containing 10 and 20 μg/ml gentamicin. The MICs of gentamicin were determined for the four fosmid clones, pA1, pA3, pC1, and pG1 (Fig. 1); all showed stable growth on LB agar plates containing 20 μg/ml gentamicin, whereas the pCC1fos vector control was susceptible at 20 μg/ml gentamicin. Clones pA1, pA3, pC1, and pG1, therefore, demonstrated an 8-fold higher resistance to gentamicin than the pCC1fos fosmid vector control. *E. coli* cells with plasmid pDONR207 (containing a known gentamicin resistance gene, *acc*) were used as a positive control as they conferred 16-fold greater gentamicin resistance than the pCC1fos negative control (Fig. 1C). We chose clone pA1 for further characterization, due to its consistent phenotype occurrence.

Screening of Shot-Gun Clones to Identify Gentamicin Resistance Genes

To identify specific genes associated with gentamicin resistance, the pA1 fosmid clone (38 kb) was fragmented and a shot-gun library of the resulting fragments constructed. The average size of the cloned DNA fragments was 2–3 kb. The MICs of gentamicin were determined for four shot-gun clones originating from fosmid pA1; pA1b, pA1g, pA1h, and pA1j (Fig. 2A). All four clones grew in the presence of 12–25 μg/ml gentamicin, representing a concentration 4- to 8-fold higher than that in which the vector control grew. This indicated that the resistance to gentamicin conferred by the shot-gun clones was similar to that conferred by the fosmid clone, whereas cultures containing the pUC118 vector control were susceptible to 3 μg/ml gentamicin (Fig. 2A). Consistent with the results, all four shot-gun clones showed resistance to both 3 and 12 μg/ml gentamicin when grown on LB agar, whereas the control vector grew on plates containing 3 μg/ml but not 12 μg/ml gentamicin (Figs. 2B and 2C). Likewise, pA1 showed resistance to both 3 and 12 μg/ml gentamicin, whereas the fosmid vector control pCC1fos showed growth inhibition

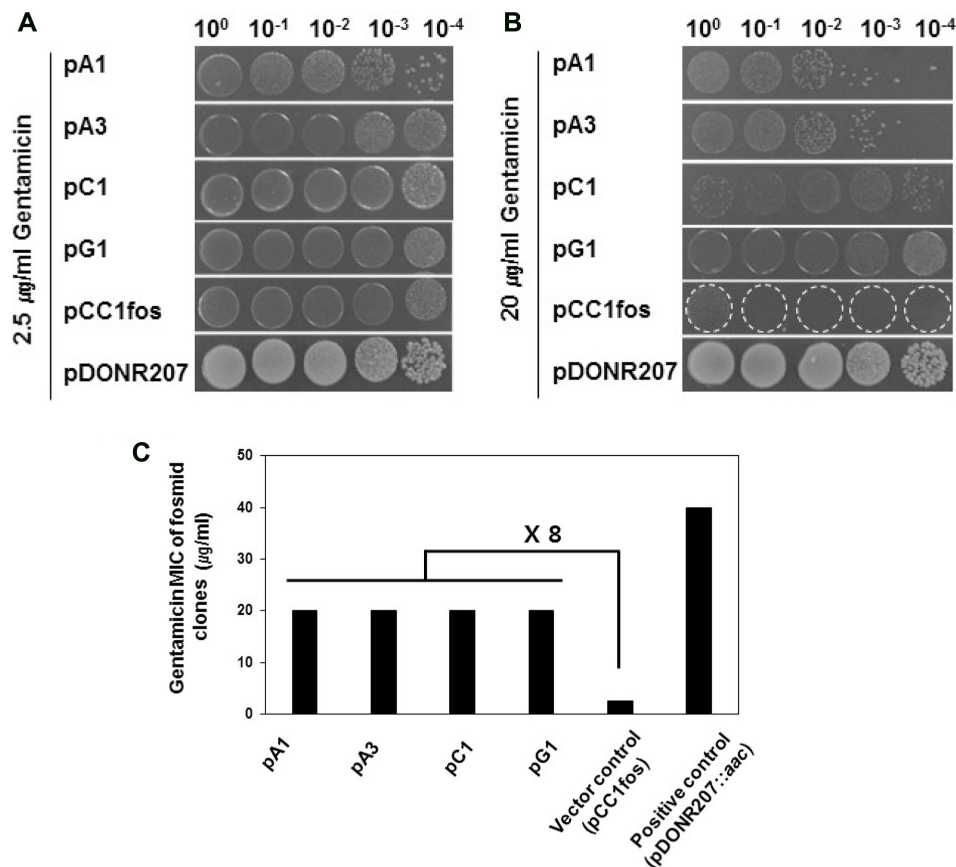

Fig. 1. Assessment of the gentamicin resistance of fosmid clones.

Spot assay of four selected fosmid clones. Ten-fold serial dilutions of a log-phase culture were spotted onto LB agar media containing 0, 1.25, 2.5, 5, 10, 20, or 40 µg/ml gentamicin. Representative (**A**) 2.5 and (**B**) 20 µg/ml gentamicin plates. (**C**) Data plotted as a graph. pCC1fos and pDONR207 were used as negative and positive controls for gentamicin resistance, respectively.

at 12 µg/ml gentamicin (Figs. 2B and 2C). MICs for 50% (MIC_{50}) and 90% (MIC_{90}) of shot-gun and fosmid clones were calculated by log-probit analysis (Table 1). The MIC_{50} values were 0.2, 1.1, 0.9, and 1.0 µg/ml for pA1b, pA1g, pA1h, and pA1j, respectively; the MIC_{90} values were 1.3, 1.6, 1.4, and 1.4 µg/ml for pA1b, pA1g, pA1h, and pA1j, respectively. The MIC_{50} value of pA1b (0.2) out of the four selected clones was the lowest, whereas the MIC_{90} value of pA1b (1.3) was not much different than that of the other clones (1.4 and 1.6) (Table 1). The MIC_{50} and MIC_{90} of the fosmid clone pA1 were close to pA1b.

Resistance of the Shot-Gun Clones to Various Antibiotics

To characterize the resistance profile of the shot-gun clones, their susceptibility to a wide range of antibiotics classes, including cephalosporin (cephalothin), aminoglycosides (kanamycin and streptomycin), MLSK (spectinomycin), tetracycline, lipopeptides (colistin), quinolones (ciprofloxacin), fluoroquinolones (lomefloxacin), and trimethoprim, was examined. The MIC of each antibiotic for cultures containing the pUC118 control vector was first determined and shot-gun clones were subsequently tested for susceptibility. The shot-gun clones were resistant to streptomycin, kanamycin, and spectinomycin (Figs. 2D, 2E, and 2F); however, their susceptibility to all other antibiotics was similar to that of the vector control (data not shown). Streptomycin, kanamycin, and gentamicin are all aminoglycosides, which inhibit protein synthesis and, although not of the same class (Figs. 2D and 2E), spectinomycin also works by the same mechanism (Fig. 2F). These results strongly suggest that a gene sequence in the shot-gun clones plays an important role in blocking protein synthesis inhibition.

Sequencing of the Full-Length pA1 Fosmid Sequence

The general features of the pA1 fosmid clone sequence are shown in Table 2 and Fig. 3A. The complete sequence of

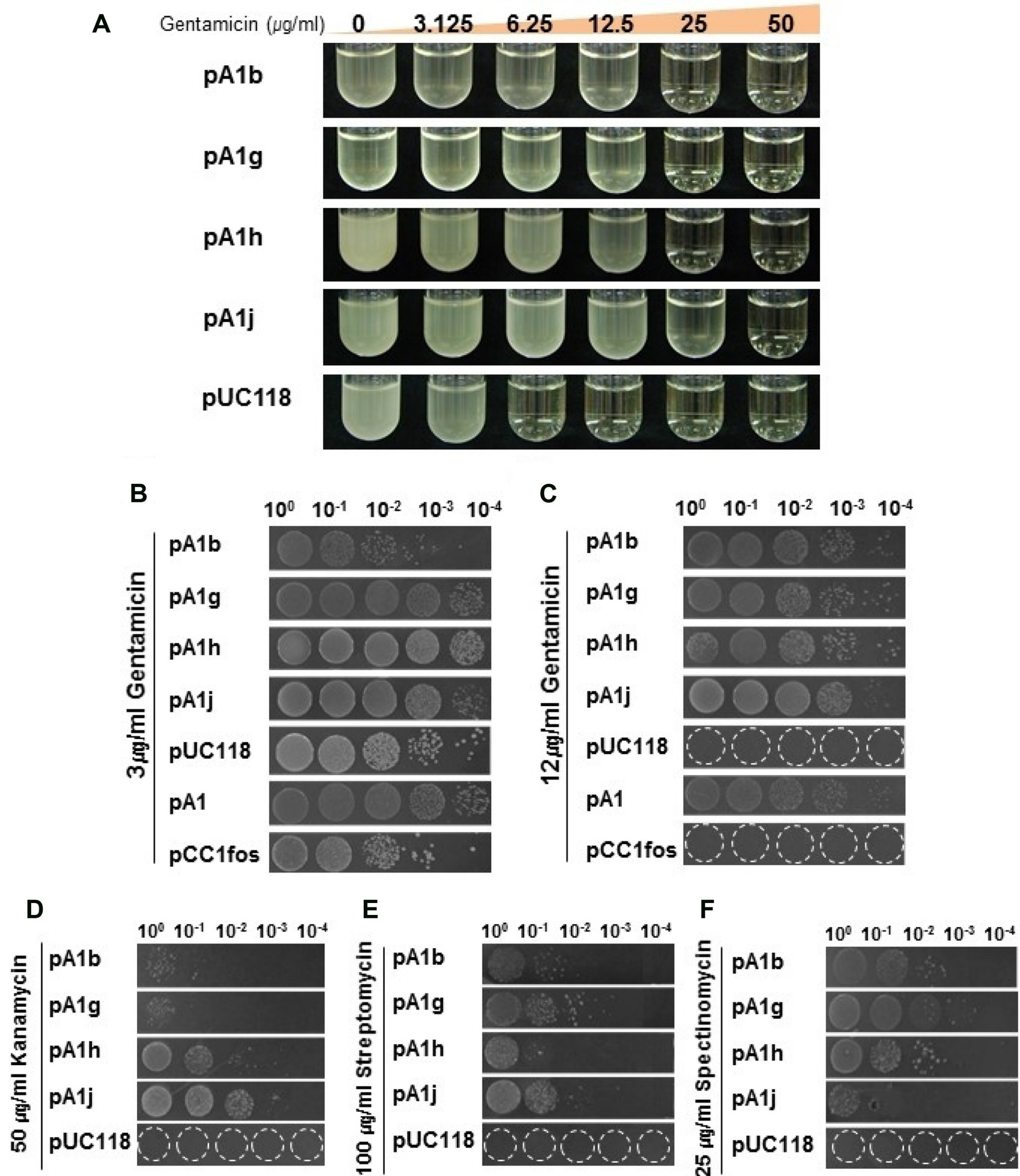

Fig. 2. Characterization of gentamicin-resistant shot gun clones.

(**A**) Minimum inhibitory concentrations of gentamicin for each of the four shot-gun clones were determined in liquid culture using 10-fold serial dilution assays. Cultures were incubated aerobically at 37°C for 24 h, and pUC118 was used as a vector control. Spot assay of four shot-gun clones and fosmid clone pA1. Ten-fold serial dilutions of each culture grown in gentamicin were spotted onto LB agar media containing the same concentrations, (**B**) 3 and (**C**) 12 μg/ml of gentamicin. pUC118 and pCC1fos were used as vector controls for shot-gun and fosmid clones, respectively.Variation in the resistance profiles of the shot-gun clones. Spot assays of four shot-gun clones and the pUC118 (vector control). Ten-fold serial dilutions of a log-phase culture were spotted onto LB agar media containing (**D**) 50 μg/ml kanamycin, (**E**) 100 μg/ml streptomycin, and (**F**) 25 μg/ml spectinomycin.

the fosmid insert comprised 38,400 bp with a G+C content of 67.50% (Table 2). The coding regions cover 86.74% of the fosmid (33,309 bp) and encode 43 proteins (Table 2). Of these, 22 protein-coding genes (51.16%) were assigned to COGs (Fig. 3A). The most abundant COG categories were “nucleotide transport and metabolism” (six proteins), followed by “Amino acid transport and metabolism” (three proteins), “Lipid transport and metabolism” (three proteins),

Table 1. MIC_{50} and MIC_{90} values of shot-gun clones and a fosmid clone.

Clones	MIC (μg/ml)[a]	
	50%	90%
Shot-gun clones		
pA1b	0.2	1.3
pA1g	1.1	1.6
pA1h	0.9	1.4
pA1j	1.0	1.4
Fosmid clone		
pA1	0.5	1.7

[a]MICs were calculated by log-probit analysis.

Table 2. General features of the pA1 fosmid.

Component of plasmid	Property
Total size (bp)	38,400
G+C content	67.50%
Number of contigs	1
Number of total genes	43
Protein coding genes	43
Coding region (bp)	33,309
Genes with function prediction	15
Genes assigned to COGs	22
Genes with Pfam domains	24
Genes with signal peptides	2
Genes with transmembrane helices	10

and "Replication, recombination, and repair" (two proteins). Signal peptides and transmembrane helices were found in 2 and 10 protein-coding genes, respectively (Table 2). The accession number of pA1 is KU240005.

Sequencing of Shot-Gun Clones

To determine genes involved in gentamicin resistance, the isolated resistant shot-gun clones were sequenced. The genetic organization, sizes, and similarities of the clone sequences are summarized in Table 3. The inserts of resistant clones were 2–3 kb in size. The nucleotide sequences and encoded proteins of the clones did not show similarity to any known gentamicin resistance genes (*acc*, *ant*, and *aph*) when the sequences were compared using NCBI BLAST. Surprisingly, all the clones showed greatest similarity to T4 RnlA, with 33% amino acid sequence identity (Table 3). *rnlA* encodes a type II toxin and is, therefore, likely to be expressed in response to stress. These results suggest that the resistance conferred by these clones is due to an *rnlA*-like gene, although the mechanism of action is unknown.

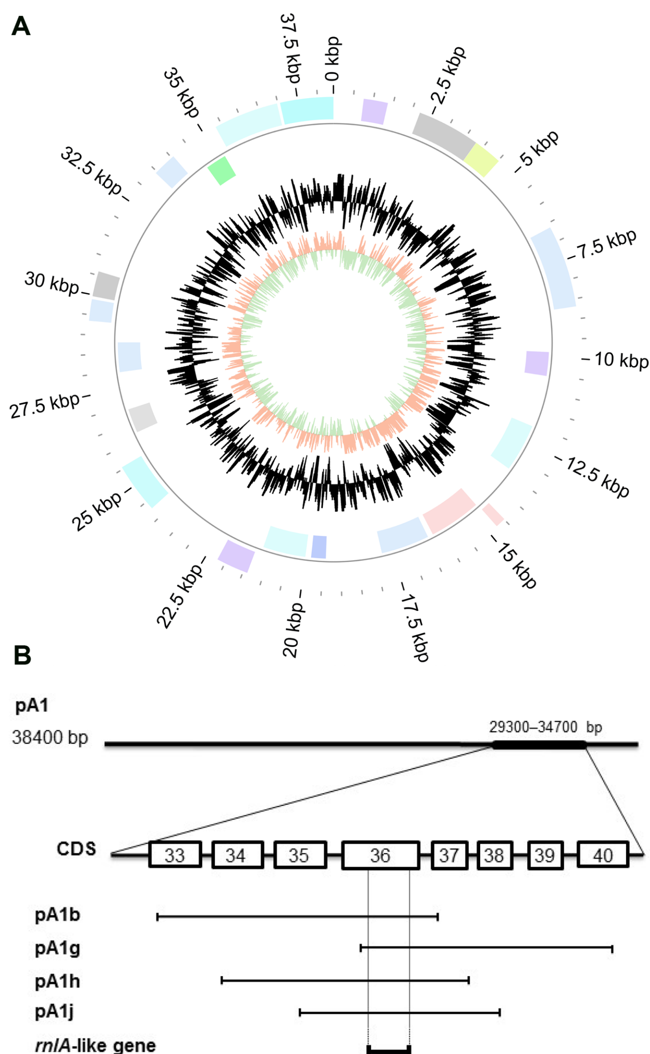

Fig. 3. Profiling of pA1 and the *rnlA*-like gene.
(**A**) Graphical circular map of the pA1 fosmid. From outside to center: Genes on the forward strand (color coded according to COG categories); genes on the reverse strand (color coded according to COG categories); GC content (black); and GC skew (light green/orange). (**B**) Schematic diagram of shot-gun clone sequences compared with that of fosmid pA1. Alignment of pA1 and four shot-gun clones using NCBI BLAST. Among the 43 CDS identified in pA1, eight (the 33–40th in the region 29,300–34,700 bp) are present in the four shot-gun clones. The numbers indicate individual similar proteins as follows. 33: Ham1 family protein (3.50E-68, 68.15%); 34: n-methyl-D-aspartate receptor NMDAR2C subunit (3.29E-60, 58.35%); 38: glycine-rich protein family protein (2.96E-26, 57.90%); 40: uridine kinase (7.75E-38, 51.05%); 35, 37, and 39: hypothetical proteins.

The putative gene responsible for the resistance phenotype was identified within the full-length pA1 fosmid insert by alignment of shot gun clone sequences using Prodigal. We

Table 3. Predicted ORFs from the gentamicin-resistant shot-gun clones.

Clones	Sizes	GenBank Accession No.	ORFs	Most similar protein	Number of amino acids	E-value	Identity/ Similarity (%)
pA1b	2.8 kb	WP_007412515.1	1	Hypothetical protein [*Pedosphaera parvula*]	210	4e-43	50/64
		WP_009629790.1	2	RNA ligase, T4 RnlA [*Pseudanabaena biceps*]	350	8e-33	33/51
pA1g	2.3 kb	EKD49389.1	1	Hypothetical protein [uncultured bacterium]	102	2e-13	37/55
		WP_009629790.1	2	RNA ligase, T4 RnlA [*Pseudanabaena biceps*]	350	6e-33	33/51
pA1h	2.2 kb	WP_009629790.1	1	RNA ligase, T4 RnlA [*Pseudanabaena biceps*]	350	6e-33	33/51
pA1j	2 kb	WP_009629790.1	1	RNA ligase, T4 RnlA [*Pseudanabaena biceps*]	350	3e-33	33/51
		KKW41617.1	2	Hypothetical protein [Parcubacteria (*Magasanikbacteria*) bacterium]	99	4e-21	52/59

found that among the 43 CDSs in pA1, CDS 36 was the only sequence common to all four shot-gun clones. Strikingly, CDS no. 36 encoded an *rnlA*-like gene (Fig. 3B). We attempted to clone the specific region but failed to construct the specific area conferring the *rnlA*-like gene from CDS 36. The possible explanation can be overexpression of the toxin gene resulting in loss of robustness in a single cell and due to high G+C contents.

Genus Identification of the pA1 Fosmid Clones

To determine the source organism of the pA1 fosmid clone containing the *rnlA*-like gene, we applied a bioinformatics search engine, PhylopythiaS. The full-length sequence was similar to that derived from a single genus, *Slackia* (data not shown).

Discussion

In this study, we used functional metagenomics to identify gentamicin resistance genes in soil. A metagenome library containing soil DNA in surrogate host *E. coli* cells was screened, and all four selected gentamicin resistance clones encoded a protein with a deduced amino acid sequence with 33% identity to the RNA ligase, T4 RnlA.

RnlA is a toxin of the type II toxin-anti-toxin (TA) system; both toxin and anti-toxin are proteins and the anti-toxin neutralizes the toxin by direct interaction [15]. For many years, toxins belonging to the TA system were thought to cause persistent cell formation, leading to antibiotic resistance. HipA was the first toxin found to be involved in persistence in *E. coli* [29]; later, it was shown to be neutralized by the HipB anti-toxin [41]. Although *rnlA*-mediated cell persistence has not been extensively studied, *rnlA* overexpression in *E. coli* results in persistent cell formation and assists survival under antibiotic selection pressure [33]. These data led us to hypothesize that the mechanism underlying the gentamicin resistance of shot-gun clones pA1b, pA1, pA1h, and pA1j is similar to that conferred by *rnlA*. However, further experiments demonstrated that the *rnlA*-like gene, which confers resistance to gentamicin in *E. coli*, did not restore the RnlA activity of an *E. coli rnlA* mutant (data not shown). Alternatively, an unknown protein in *E. coli* may act as an "adapter anti-toxin" that undergoes protein-protein interaction with the metagenome library-derived *rnlA*-like gene because no *rnlB*-like gene exists in the pA1 fosmid clone near the *rnlA*-like gene [50].

PhylopythiaS can predict the organism of origin from short metagenomic DNA fragments; no previously available method can do this [28]. Our results indicate that the fosmid clone pA1 is likely derived from an organism belonging to the genus *Slackia*. Genome data (GenBank Accession Numbers NZ_ACUX00000000, NZ_ADMD00000000, and NC_013165) for *Slackia exigua*, *S. piriformis*, and *S. heliotrinireducens* are currently available [36]. To validate the existence of the *rnlA*-like gene, we attempted to search for it in the *Slackia* genomes, but failed to find any, indicting that no homologous gene exists or different *Slackia* species contained the homologous gene. *Slackia* is a genus of family Coriobacteriaceae within the Actinobacteria phylum, and its members are gram-positive, non-motile, obligate anaerobes [48]. *Slackia* may accumulate in soil contaminated by sewage, since *Slackia* species are generally isolated from

human feces, wound infections, and abscesses [19, 31]. *Slackia* species are resistant only to methoxazole-trimethoprim at >32 μg/ml; [19] however, the clone containing the *rnlA*-like gene is susceptible to 20 μg/ml trimethoprim. Studies of gentamicin resistance have yet to be performed in *Slackia*. Therefore, our data indicating that *Slackia* may be resistant to gentamicin is important, since *Slackia* species are known to be pathogenic to humans [19].

Our results presented herein identify an *rnlA*-like gene-mediated gentamicin resistance mechanism, using functional metagenome screening, which may be a novel mechanism of antibiotic resistance mediated by a protein with a function previously unassociated with this trait. These data contribute to our knowledge about the presence and diversity of antibiotic resistance genes in environmental bacteria. Future work will involve characterization of the mechanism by which the *rnlA*-like gene confers resistance to gentamicin and examination of its role in resistance to other antibiotics.

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