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NOTE

Fosmid Cloning, Nucleotide Sequence, and Characterization of a Beta-Lactamase Gene from Subsurface Isolates

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A beta-lactamase gene was isolated for the first time from a terrestrial subsurface environment using a combined cultivation and direct cloning strategy. The gene, discovered from 24 m below land surface in Hawaii, was most similar to the penicillinase from *Bacillus licheniformis*. The resistance gene was confirmed via subcloning and its minimum inhibitory concentration values were measured against several test beta-lactam antibiotics. This study extends the knowledge on resistance to antimicrobials, which may help the efforts to minimize their future threat.

Keywords: beta-lactamase, terrestrial subsurface, antibiotic resistance, direct cloning

The emergence and spread of antibiotic resistance among environmental bacteria may become a global threat in our near future (Hawkey and Jones, 2009; Allen *et al.*, 2010; Wright, 2010). Knowledge on novel antibiotic resistance genes from natural environments is necessary in order to minimize this problem. An extreme increase of antibiotic resistance genes has been observed in various natural environments (Allen *et al.*, 2010), but terrestrial subsurface microbial life has received relatively little attention (Brown and Balkwill, 2009). Although microbial communities in the subsurface environments are exposed to more extreme conditions compared to modern surface soils and sediments, their populations and activities are still considerable (Parkes and Wellsbury, 2004). The aim of this study was to attain more insight into the frequency and diversity of antibiotic resistance genes present in the terrestrial subsurface environment using direct cloning tools (Entcheva *et al.*, 2001).

Soil/crushed rock samples were collected from 24 m below land surface in Ewa, Hawaii on July, 2008. Coring was performed using a rotary drill rig with some of the recommended cautions to minimize external contamination (Phelps *et al.*, 1989). Samples that had been in contact with drilling hardware were discarded and sampling was done using sterilized scoops and bags. Sample surfaces were scraped to remove additional contamination. In order to avoid loss of microbial diversity, collected samples were frozen immediately on site using dry ice and later stored at -80°C in the laboratory. Due to the expected decreased bacterial populations 24 m below surface, no visible amount of DNA could be directly extracted for the library construction; therefore, the soil/rock particles were placed on agar plates with sterile complex medium (Maeda *et al.*, 2007). Plates were incubated at 25°C for three to five days until bacterial growth was visible. Three different colored/shaped isolates were picked and re-grown on liquid complex medium and genomic DNA was extracted by the use of a Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol.

A 16S rRNA gene library was constructed to evaluate the existing bacterial community present in the isolates. For this purpose, universal bacterial 16S rRNA gene-specific reverse primer 1492R1 (5′-TGA CTG ACT GAG GYT ACC TTG TTA CGM YTT-3′) and forward primer 530F (5′-TGA CTG ACT GAG TGC CAG CMG CCG CGG-3′) were employed. The resulting PCR products were cloned into an *Eco*RV digested pBluescript vector and transformed into *Escherichia coli* DH5α cells. A total of 10 plasmid DNAs containing inserts were randomly selected and sequenced with universal primers T7 and M13-reverse using standard protocols. The bacterial ribosomal gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) function at the National Centre for Biotechnology and Information (NCBI) website using the available DNA databases. It was found that the microbes present in this bacterial community were closely related to genus *Bacillus* spp. The nucleotide sequences generated for the 16S rRNA genes were deposited in GenBank under accession numbers JN937581, JN937582, JN937583, JN937584, JN937585, and JN937586.

The three different isolates were then tested for growth with antibiotics rifampicin (100 μg/ml), neomycin (50 μg/ml), kanamycin (100 μg/ml), ampicillin (100 μg/ml), or tetracycline (10 μg/ml) at 37°C for 24 h. The first isolate grew only with ampicillin, the second isolate grew with neomycin, kanamycin, and ampicillin, and the third isolate did not grow on any antibiotics medium, the same as the negative control, *E. coli* EPI300. Four different fosmid DNA libraries were constructed using total genomic DNA from isolates grown

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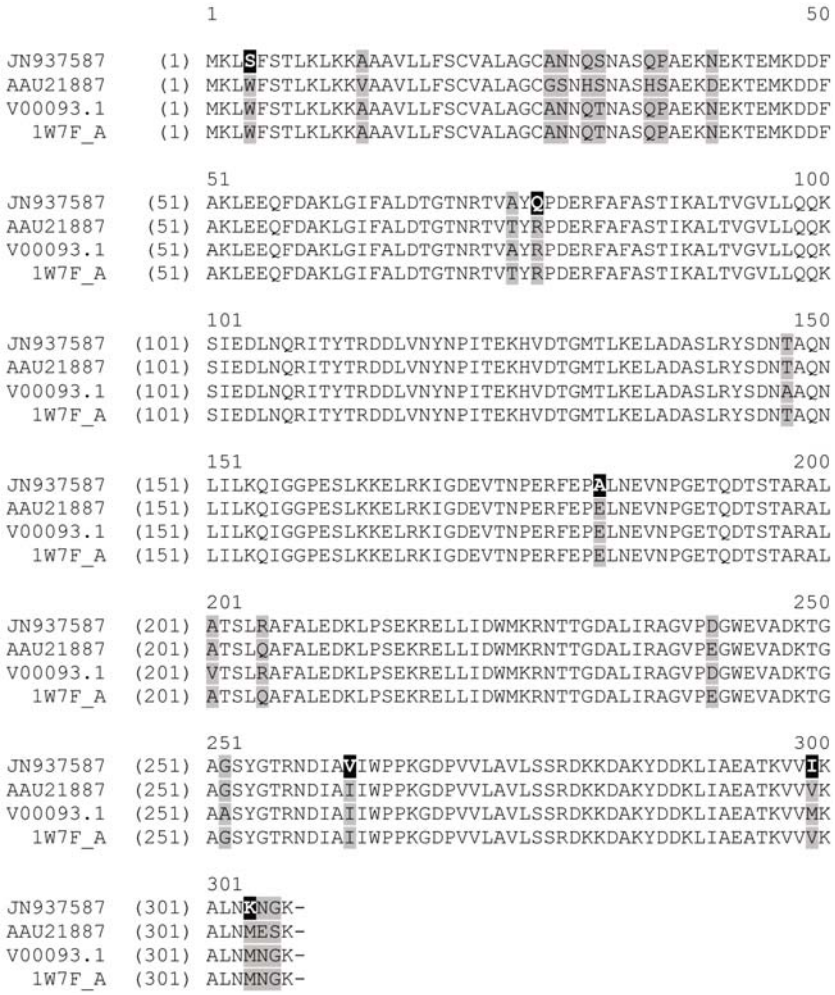

Fig. 1. Amino acid sequence comparison for beta-lactamase isolated from subsurface isolates (GenBank ID: JN937587) and the three most closely related sequences; beta-lactamase precursor of *B. licheniformis* ATCC 14580 (GenBank ID: AAU21887) (Rey *et al.*, 2004), penicillinase of *B. licheniformis* 749/C (GenBank ID: V00093.1) (Neugebauer *et al.*, 1981), and beta-lactamase of *B. licheniformis* BS3 (GenBank ID: 1W7F_A) (Fonze *et al.*, 2002). Multiple sequence alignments were carried out using Vector NTI. Amino acid sequence positions are indicated with numbers, and different amino acids in one of the four sequences are highlighted in grey. White bold letters on a black background indicate amino acid positions that are novel in the isolated beta-lactamase.

with neomycin, kanamycin, and ampicillin. Genomic DNA was isolated by the use of a Wizard® Genomic DNA Purification Kit (Promega) and the library was constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre® Biotechnologies, USA) according to the manufacturer's instructions. Phage T1-resistant TransforMax EPI300-T1R *E. coli* was used as the host for transformation. Transformants were selected on the basis of their ability to grow on Luria-Bertani (LB) agar plates containing 12.5 μg/ml chloramphenicol and another appropriate antibiotic (neomycin: 50 μg/ml, kanamycin: 100 μg/ml, or ampicillin: 100 μg/ml), on which the host *E. coli* alone could not grow. After 18–36 h at 37°C, a total of 12 colonies having resistance to ampicillin were obtained. Selection for neomycin and kanamycin resistance yielded no colonies. Restriction analysis with *Kpn*I, *Nco*I, and *Xba*I showed that all clones had an insert and the estimated average size was around 33 kb. The partial sequences of the DNA inserts were obtained by primer-walking and the resulting sequences were analyzed using Vector NTI software (InfoxMax, Inc., USA) and compared with the sequence database at the NCBI using BLAST. All twelve fosmid clones were found to have the same nucleotide base sequence for the gene conferring ampicillin resistance and in the same orientation.

Subcloning was performed to confirm that the predicted beta-lactamase (*bla)* gene was responsible for the ampicillin resistance, using primer DS *Sal*I forward (5′-CGAAGGTC GACGATTTTGATG-3′), which generated a unique *Sal*I site upstream of the *bla* start codon and primer DS *Eco*RI reverse (5′-TGACGGAATTCATCGCAATG-3′), which located the *Eco*RI site downstream of the *bla* stop codon. A PCR program consisting of 30 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2.15 min with final extension at 72°C for 7 min was used. The resulting PCR product was then cloned into the multiple cloning site in pBS(Kan) (Canada *et al.*, 2002) after double digestion with *Sal*I and *Eco*RI to create pBS(Kan)Bla (5060 bp). The resulting plasmid library was electroporated into *E. coli* TG1 competent cells by using a GenePulser (Bio-Rad) at 15 kV/cm, 25 μF, and 200 Ω. Transformants were selected on the basis of their ability to grow on LB agar plates containing 100 μg/ml kanamycin and 100 μg/ml ampicillin. The correct plasmid was verified by restriction digestion and sequencing using universal primers M13-forward and M13-reverse. The nucleotide sequence generated for the *bla* gene which was confirmed to be responsible for the ampicillin resistance was deposited

Table 1. MIC values (μg/ml) of the subclone (pBS(Kan)Bla) and the vector only control (pBS(Kan)) against seven beta-lactam antibiotics

Antibiotics	MIC values (μg/ml)[a]	
	TG1/pBS(Kan)Bla	TG1/pBS(Kan)
Amoxicillin	32768 (4096)	8
Ampicillin	16384 (2048)	8
Cefaclor	2048 (512)	4
Cefixime	512 (256)	2
Cefuroxime	1024 (128)	8
Cephalexin	512 (16)	32
Piperacillin	4096 (2048)	2

[a] Measurements were made in *E. coli* TG1 host cells growing in LB broth supplemented with the corresponding antibiotic at 37°C for 18 h. Number in parenthesis indicates the fold difference with respect to empty vector MIC values in the same host cells.

in GenBank under accession number JN937587.

It was found that the isolated beta-lactamase sequence comprises 307 amino acids (GenBank ID: JN937587) and its closest relatives in the database are from *B. licheniformis* strains ATCC14580 (GenBank ID: AAU21887) (Rey *et al.*, 2004), 749/C (GenBank ID: V00093.1) (Neugebauer *et al.*, 1981), and BS3 (GenBank ID: 1W7F_A) (Fonze *et al.*, 2002) with over 94% identity (Fig. 1). The sequence of the isolated *bla* and the three most closely related genes differ in 22 amino acids located at positions 4, 13, 28, 29, 31, 32, 36, 37, 41, 75, 77, 147, 182, 201, 205, 241, 252, 262, 299, 304, 305, and 306. Among all, positions Bla-S4, Q77, A182, V262, I299, and K304 are found to be novel in the *bla* gene isolated from subsurface sediments. Position I299, corresponding to L287 in the BS3 structure (PDB ID: 1I2S), is in the α/β-domain and is known to be involved in hydrophobic clusters (Fonze *et al.*, 2002). Residue V262 (I249 in BS3) is completely buried inside the protein, whereas Q77 (R61 in BS3) and A182 (E168 in BS3) locate on the surface. Position 182 locates nearby, but points away from the active site; therefore, replacement of glutamic acid with alanine is unlikely to cause any significant effect. However, substitution of an isoleucine in position 262 with a smaller valine residue and mutation at position 299 might have an effect on the protein activity profile. The role of these positions could be examined through site directed mutagenesis in the future.

Minimum inhibitory concentrations (MICs) of seven different beta-lactam antibiotics including amoxicillin, ampicillin, cefaclor, cefixime, cefuroxime, cephalexin, and piperacillin were determined at two fold concentration increases using the subclone (pBS(Kan)Bla) according to a published protocol (Andrews, 2001) (Table 1). Experiments were done in triplicates using serial tube dilution assays of appropriate beta-lactam antibiotics in LB broth, with approximately 10^5 cells/ml. Growth was measured using turbidity at 600 nm after incubation for 18 h at 37°C. Intermediate growth was not considered and the lowest values were reported for the subclone. Cells without the beta-lactam antibiotics were used as the positive control for growth. The subclone conferred high-level resistance to all the selected beta-lactam antibiotics compared to the vector only control (pBS(Kan)). The MIC for *E. coli* TG1 carrying the subclone was 32768 μg/ml of amoxicillin, 16384 μg/ml of ampicillin, 2048 μg/ml of cefaclor, 512 μg/ml of cefixime, 1024 μg/ml of cefuroxime, 512 μg/ml of cephalexin, and 4096 μg/ml of piperacillin. These values were at least 16 to 4096 fold higher than the values observed for the host *E. coli* TG1 carrying the empty vector (<2–32 μg/ml) (Table 1). All antibiotics were purchased from Sigma-Aldrich (USA).

In conclusion, a beta-lactamase antibiotic resistance gene was isolated from 24 m below land surface by using a combined cultivation and direct cloning approach (Entcheva *et al.*, 2001). To our knowledge, a beta-lactamase gene has never been cloned, sequenced, and characterized from a terrestrial subsurface environment before, including from a Hawaiian region. Our results agree with the previous findings that antibiotic resistance is common in subsurface bacteria (Brown and Balkwill, 2009) and beta-lactamase resistance genes are frequently detected in environmental samples (Allen *et al.*, 2009). This study presents another beta-lactamase determinant and extends the knowledge on antibiotic resistance genes from natural environments, which may help the efforts to slow down their spread.

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