

Identification of *Streptomyces* sp. AMLK-335 Producing Antibiotic Substance Inhibitory to Vancomycin-Resistant Enterococci

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Abstract The actinomycete strain AMLK-335 was antagonistic to vancomycin-resistant enterococci (VRE). Based on the diaminopimelic acid (DAP) type, and morphological and physiological characteristics revealed by scanning electron microscopy (SEM), AMLK-335 was confirmed to belong to the genus *Streptomyces*. Analysis of the 16S rDNA nucleotide sequences found AMLK-335 to have a relationship with *Streptomyces platensis*. The production of antibiotic from this strain was most favorable when cultured on glucose, polypeptone, yeast extract (PY) medium for 6 days at 27°C. The antibiotic was identified as cyclo(L-phenylalanyl-L-prolyl) by comparing it with the reported MS and NMR spectral data. Cyclo(phe-pro) from the PY cultures of AMLK-335 was most effective against the VRE strains *E. faecium* (K-98-637) and *E. faecalis* (K-98-258). Furthermore, cyclo(phe-pro) had antimicrobial activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae*, but it was ineffective against *Candida albicans*, *Streptomyces murinus*, and *Aspergillus niger*.

Key words: Vancomycin-resistant enterococci (VRE), 16S rDNA, *Streptomyces* sp. AMLK-335, Cyclo(L-phenylalanyl-L-prolyl)

Enterococci are a major cause of many significant infections, including intra-abdominal sepsis, urinary tract infections, bacteremias, and endocarditis. These Gram-positive organisms are inherently resistant to multiple antibiotics, including polymyxins, lincosamides, and trimetoprim-sulfamethoxazole, which reduce susceptibility to cell wall-active agents such as β -lactams and vancomycin [8, 12, 20]. This relative tolerance is overcome by their use in combination with aminoglycosides, which have a synergistic effect [10, 12]. However, during the last 15 years, a high level resistance

to aminoglycosides has become more common among enterococci [22].

Vancomycin-resistant *Enterococcus faecium* infections sometimes cannot be treated by the current antibiotic or antibiotic combinations available [5, 7, 14]. More recently, glycopeptide resistance has become a major worldwide concern [7, 21, 25]. Vancomycin-resistant *E. faecium* and *E. faecalis* account for up to 14% of the enterococcal isolates in U.S. Hospitals [1]. In the U.S., vancomycin resistance was first noted in metropolitan hospitals on the eastern seaboard, and has recently spread over the country [5]. In addition, vancomycin resistance strains have been reported in many European countries, and there is evidence that resistant isolates are becoming more common [13, 19]. They have subsequently spread throughout the world and now pose a serious health problem in hospitals [3]. Since VRE strains are usually resistant to many antibiotics, they are extremely difficult to eradicate. Although many anti-enterococcal agents have been developed, very few can treat VRE infections [10, 22]. Therefore, it is essential to find new drugs with anti-VRE activity. In a course of our screening program, the *Streptomyces* sp. strain AMLK-335, which produces cyclo(L-phenylalanyl-L-prolyl), was isolated from soil.

In the present study, AMLK-335, which has an anti-VRE activity, was phylogenetically classified by the 16S rDNA sequencing process and both the anti-VRE activity and some pathogenic microorganisms that produce cyclo(phe-pro) were evaluated.

MATERIALS AND METHODS

Anti-VRE Antibiotic Producing Actinomycete Strain AMLK-335

The actinomycete strain AMLK-335, which produced an anti-VRE antibiotic, was isolated from a medium (S medium: 1% glycerin, 1% soluble starch, 0.03% casein, 0.2%

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KNO₃, 0.2% K₂HPO₄, 0.2% NaCl, 0.005% MgSO₄ · 7H₂O, 0.001% FeSO₄ · 7H₂O, 0.001% thiamine · HCl, 0.9% agar, adjusted to pH 7.0–7.2. AV medium: 0.1% glucose, 0.1% glycerol, 0.03% K₂HPO₄, 0.03% MgSO₄ · 7H₂O, 0.03% NaCl, 0.03% L-asparagine, 0.1% trace salt solution, 1% vitamine solution, 1% antibiotic solution, 0.9% agar, adjust to pH 6.4) used to isolate actinomycete from a soil sample collected in Suwon, Korea. The organism was grown at 28°C on a modified Bennett's agar [9] slant and stored at 4°C.

Identification of Actinomycete Strain AMLK-335

To determine the genus of actinomycete strain AMLK-335, the type of 2,6-diaminopimelic acid (DAP), one of the cell wall components of actinomycete mycelia, was analyzed by the methods of the International *Streptomyces* Project (ISP) suggested by Shirling and Gottlieb [18], and Bergey's Manual of Systematic Bacteriology [23]. AMLK-335 was cultured on a tryptic soy broth (17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean meal, 5.0 g sodium chloride, 2.5 g dipotassium phosphate, 2.5 g dextrose, and 1-l H₂O, adjusted to pH 7.3 before autoclaving) for 7 days at 28°C using a rotary-shaking incubator. The cultured broth was filtered with a Whatman No. 1 filter paper, washed with sterilized distilled water, and freeze-dried. The dried cells (20 mg) were placed into a captube (13 × 100 mm) containing 5 ml 6 N HCl, sealed tightly, and hydrolyzed by heating the tube in a boiling water bath for 18 h. The hydrolysate was filtered with a Whatman No. 1 filter paper and evaporated to dryness to remove the residual HCl. This residue was then dissolved in 1 ml of distilled water and loaded onto a TLC plate (10 × 10 cm, HFTLC Cellulose, Merck CO.). Five µl of 0.01 M DL-DAP (Sigma) containing both the meso- and LL-DAP isomers and some other amino acids (alanine, glycine, and glutamate) were also loaded on plates as a standard [26].

To examine the spore chain morphology, the AMLK-335 was incubated for 14 days on a yeast extract-malt extract agar (ISP medium 2) (4.0 g yeast extract, 10.0 g malt extract, 4.0 g dextrose, 20.0 g agar, and 1-l H₂O, adjusted to pH 7.3 before autoclaving). The spore chain morphology of the strain AMLK-335 was examined using light (×400 magnification) and scanning electron microscopy (SEM) (Model S-800, Hitachi, Japan). The SEM specimen was

prepared according to the method of Williams and Davies [24]. Among the morphological categories suggested by Pridham *et al.* [16], two categories of *Rectiflexibles* and *Spirales* were employed for evaluating the spore chain morphology.

The Genus *Streptomyces* for 16S rDNA Sequencing

The genus *Streptomyces* investigated in this study were *S. hygrosopicus* KCTC (Korean Collection for Type Culture) 9030, *S. avermitilis* KCTC 9056, *S. setoni* KCTC 9144, *S. tendae* KCTC 9167, *S. turgidiscabies* ATCC (American Type Culture Collection) 700248, *S. bottropensis* ATCC 25435, and *Streptomyces* sp. AMLK-335. All strains were cultured in a tryptic soy broth [9].

Primers and Extraction of Genomic DNA

For PCR amplification and sequencing of the AMLK-335 rDNA, two sets of PCR primers and two kinds of sequencing primers were designed (Table 1). First, 20 rDNA sequences of *Streptomyces* sp. were collected from GenBank and multiple alignments were performed by using the Clustal X program [6]. Several conserved and variable regions were detected and the conserved regions were used for the design sequencing and PCR primers. The streptomycete strains were grown to the late exponential phase in TSB at 27°C, and washed twice with Tris/HCl-EDTA. The chromosomal DNA was isolated using the GENERELEASER kit (Bio Ventures, Inc., Murfreesboro, TN, U.S.A.) according to the manufacturer's instructions. An aliquot of the reaction mixture was used for PCR amplification and sequencing.

Polymerase Chain Reaction (PCR), Sequencing, and Phylogenetic Analysis

The gene-released mixtures were amplified directly using TaKaRa Ex Taq™ (Takara Shuzo, Japan) according to the manufacturer's protocol. The PCR amplification reaction was performed using the primers (set 1: Stf1-Str1, set 2: Stf2-Str2) for 40 cycles in the following three steps; denaturation at 94°C for 30 seconds, annealing at 50°C for 1 min, and elongation at 68°C for 2 min. Finally, one cycle for final extension at 68°C for 7 min was performed. Two PCR amplified DNA bands were eluted from the agarose

Table 1. PCR and sequencing primers used in this study.

Primers		Sequence	Location*
Set 1	Stf1	5'-GGCGTGCTTAACACATGCAAGTC-3'	34–56
	Str1	5'-CCAGAGATCCGCCTTCGC-3'	690–707
	Stsq1	5'-GTGTCTCAGTCCCAGTGTGG-3'	296–315
	Stf2	5'-GTGTAGCGGTGAAATGCGCA-3'	648–667
Set 2	Str2	5'-TACCTTGTTACGACTTCGTCCCAA-3'	1460–1483
	Stsq2	5'-ATAAGGGGCATGATGACTTG-3'	1167–1186

*The number of the location is the number of conserved 16S rRNA sequence of *Streptomyces hygrosopicus* (GenBank accession number X79853).

gel following electrophoresis. These templates were read by PCR sequencing, using a BIG Dye Terminator (PE applied biosystems, U.S.A.) and the sequencing primers Stsq1 for set 1 and Stsq2 for set 2 (Table 1) at an annealing temperature of 45°C, while other parameters were set according to the manufacturer's instructions. The sequence was read by an ABI377 auto sequencer (PE applied biosystems, U.S.A.) that was analyzed directly using the BLAST search program that was maintained at the National Center for Biotechnology Information. In addition, dendrograms were constructed using the neighbor-joining method [17] from a distance matrix calculated with the Clustal X software [6]. The levels of sequence similarity were calculated and the distances derived were used to infer the phylogenetic relationships.

Extraction and Identification of anti-VRE Antibiotics

The anti-VRE antibiotic in the culture fluid (35-l) was applied to a Diaion HP-20 column. After washing with water and 25% methanol, it was eluted with absolute methanol. The elute was concentrated *in vacuo* to a small volume and extracted with ethyl acetate (5-l) at pH 7.0. The ethyl acetate layer was then concentrated to a small volume and again extracted with diethyl ether (2-l). The ether layer was then evaporated, yielding a residue. The residue was then dissolved in a small volume of methanol and the solution was applied to a Sephadex LH-20 column (2.0×90 cm). The column was eluted with absolute methanol. The active fractions were obtained by vacuum evaporation and further purified by preparative TLC (silica gel 60 GF 254, Merck), using the following solvent system; Chloroform:methanol:acetic acid=80:15:5. High performance liquid chromatography (HPLC, Hewlett-Packard 1100) was performed using a diode-array detection system equipped with a μ -bondapak C18 column (10 μ m, 150 mm×3.9 mm, Waters) at room temperature. The system was operated at a flow rate of 0.5 ml/min with a methanol:water (70:30) solvent mixture. The purified compound was identified by GC-MS, ¹H- and ¹³C-NMR, UV, and FT-IR data.

Medium for Anti-VRE Antibiotic Production

The medium used for the AMLK-335 preculture (for 2 days) was PC II (10 g dextrose, 2 g polypeptone, 1 g yeast extract, 1 g meat extract, 0.5 g asparagines, 0.1 g thiamine · HCl, and 1-l H₂O, adjusted to pH 7.0 before autoclaving), and the medium used for the main culture (for 4 days) was PY [5 g dextrose, 3 g polypeptone, 2 g yeast extract, 5 g meat extract, 10 g soluble starch, 10 g glycerol, 1 g casein (from milk), 2 g CaCO₃, 0.01 g thiamine · HCl, and 1-l H₂O, adjusted to pH 7.0 before autoclaving].

The two clinical VREs used for measuring the anti-VRE activity were *E. faecalis* (K-99-258) and *E. faecium* (K-98-637) strains, which were isolated from Korea University hospital during the last two years (1998-1999). Furthermore,

Aspergillus niger ATCC 9642, *Bacillus subtilis* IAM 1069, *Candida albicans* IFO 6258, *Micrococcus luteus* JCM 1464, *Saccharomyces cerevisiae* IFO 1008, *Streptomyces murinus* JCM 4333, and *Staphylococcus aureus* TK 784 were also evaluated in this study. The medium used for the VRE was a brain heart infusion agar (Difco, U.S.A.) and glucose bouillon (GB), and potato dextrose agar (PDA) (Difco, U.S.A.) medium was used for the other organisms. The minimal inhibitory concentration (MIC) was determined by the conventional agar dilution method, which was defined as the lowest concentration to show no visible microbial growth after 48 h of incubation. Assays were performed in triplicate for each experiment.

RESULTS AND DISCUSSION

Analysis of Diaminopimelic Acid (DAP) Type and Morphological Characteristics

The AMLK-335 cell wall hydrolysates were resolved on a cellulose TLC plate, and diaminopimelic acid (DAP) present in the cell wall was found to be LL-DAP (Fig. 2). The AMLK-335 spore chains formed a rectiflexible type, as observed by light microscopy. Under SEM, the AMLK-335 had long and straight spores of cylindrical forms on aerial mycelia. The spore surface ornamentation was a nodule shape that did not belong to one of the five groups classified by Dietz and Mathews [4] (Fig. 1). A special structure such as a zoospore or a sporangium was found in AMLK-335. Based on the DAP type of cell wall and the



Fig. 1. Scanning electron microphotograph of the spore surface of the strain AMLK-335.

Medium: Yeast extract-malt extract agar. Cultivation: 28°C for 14 days.

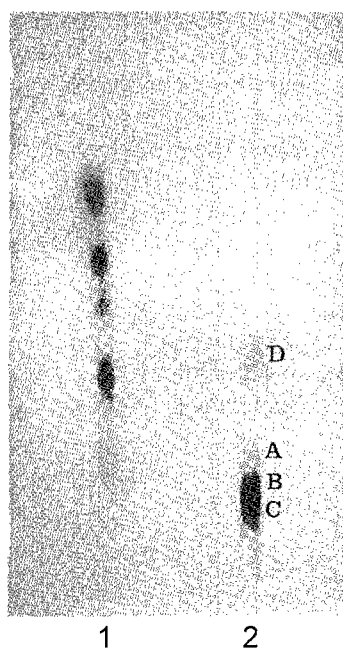


Fig. 2. Cellulose thin layer chromatogram of DAP isomers and glycine.

Lane 1, whole cell hydrolysate of the strain AMLK-335; Lane 2, DAP isomers and glycine; A, LL-DAP; B, meso-DAP; C, 3-OH DAP; D, glycine.

morphological characteristics, AMLK-335 was concluded to belong to the genus *Streptomyces*.

Phylogenetic Analysis of the Strain AMLK-335 Using the 16S rDNA Sequence

The 16S rDNA of the genus *Streptomyces* are highly conserved and several variable regions were detected. Therefore, a comparison of the variable region of AMLK-335 (set 1, 2 sequences) was compared with other *Streptomyces*. A PCR reaction using the two primer sets produced two amplified DNA bands at 670 bp, 840 bp, and 1,500 bp (data not shown). Five other strains showed the same results when the same primer sets were used. Those DNA bands were purified from the agarose gel and read as described in Materials and Methods. When the GenBank search was performed, the sequence from set 1 showed less than 97% similarity with the 16S rDNA sequence of *Streptomyces galbus* (Fig. 3, GenBank accession number X79825) and the sequence from set 2 showed a 98% similarity with *Streptomyces platensis* (Fig. 3, GenBank accession number AB045882). As a control, all the same processes were applied to the type strain of *Streptomyces avermitilis* KCTC 9056, whose sequence resulted in 100% homology with that of *Streptomyces avermitilis* AF145223. This confirmed that AMLK-335 was indeed a member of the genus *Streptomyces*. A dendrogram on the basis of 16S rDNA sequences showing the phylogenetic relationship of *Streptomyces platensis* is shown in Fig. 4.

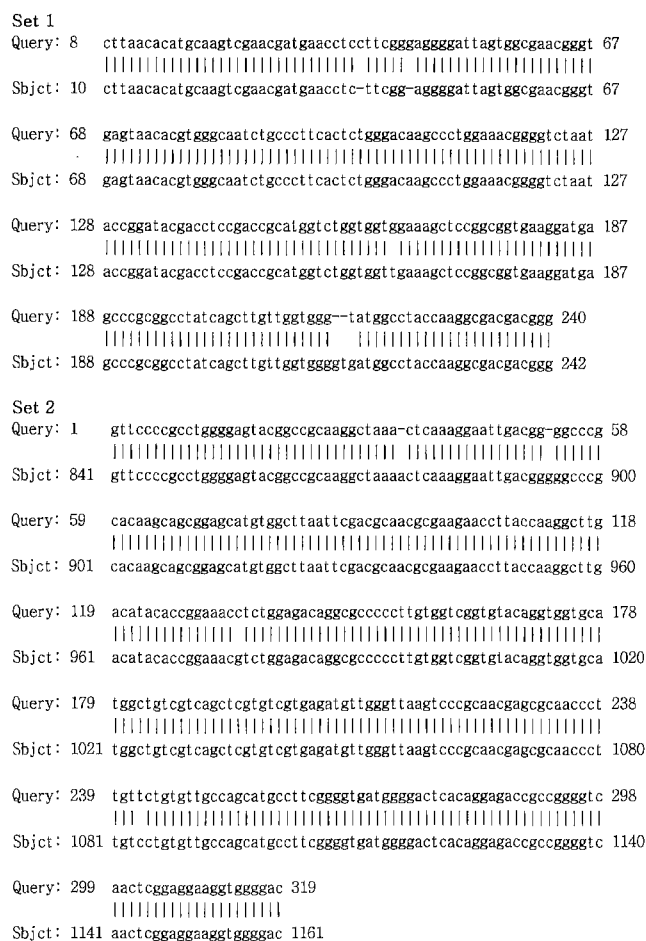


Fig. 3. Multiple sequence alignment of the sequence from primers set 1 and set 2.

Query means *Streptomyces* sp. AMLK-335 and Sbjct means *Streptomyces galbus* (set 1) and *Streptomyces platensis* (set 2).

Identification of Anti-VRE (Vancomycin-Resistant Enterococci) Antibiotics

The structure of anti-VRE antibiotics, which was determined by GC-MS, ¹H- and ¹³C- NMR, UV, and FT-IR data (data not shown), suggested the antibiotic to be cyclo(phe-pro) (Fig. 5). Cyclic dipeptides exist in fermentation broths and cultures of yeast, lichens, and fungi [15]. Among the various types of dipeptide, cyclo(phe-pro) has been reported to be produced by *Rosellinia necatrix* [2]. However, its isolation from *Streptomyces* species has not been reported before.

Antimicrobial Activity of Cyclo(phe-pro)

The activity of the cyclo(phe-pro) produced by *Streptomyces* sp. AMLK-335 against microorganism is shown in Table 2. Cyclo(phe-pro) was effective against microorganism such as *E. faecalis* (K-99-258) and *E. faecium* (K-98-637), and the MIC values were 50 and 25 µg/ml, respectively. This shows that cyclo(phe-pro) was more effective with RP59500 (Quinopristin/Dalfopristin) [22]. Moreover, the MICs

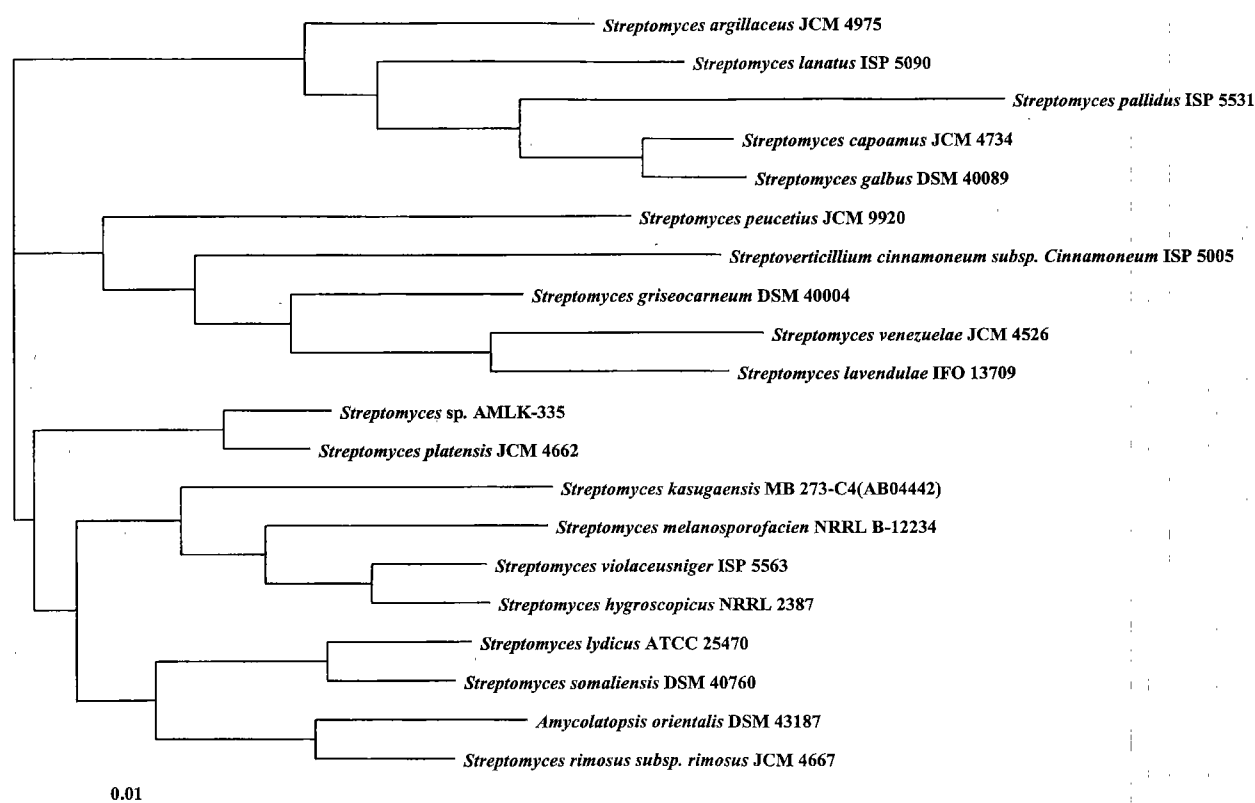


Fig. 4. Dendrogram showing the relationships between *Streptomyces* sp. AMLK-335 and other *Streptomyces* sp. The rooted tree constructed using the neighbor-joining method: the scale bar indicates 0.01 substitution per nucleotide position.

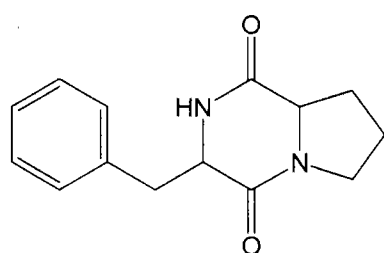


Fig. 5. Chemical structure of the isolated compound, Cyclo(L-phenylalanyl-L-prolyl).

Table 2. Antimicrobial activity of cyclo(phe-pro) produced by *Streptomyces* sp. AMLK-335.

Test organisms	MIC ($\mu\text{g/ml}$)
<i>E. faecalis</i> K-99-258*	50
<i>E. faecium</i> K-98-637*	25
<i>Candida albicans</i> IFO 6258	>100
<i>Bacillus subtilis</i> IAM 1069	50
<i>Micrococcus luteus</i> JCM 1464	12.5
<i>Aspergillus niger</i> ATCC 9642	>100
<i>Streptomyces murinus</i> JCM 4333	>100
<i>Staphylococcus aureus</i> TK 784	0.4
<i>Saccharomyces cerevisiae</i> IFO 1008	50

*Vancomycin-resistant enterococci.

of cyclo(phe-pro) against *Bacillus subtilis* IAM 1069, *Micrococcus luteus* JCM 1464, *Saccharomyces cerevisiae* IFO 1008, and *Staphylococcus aureus* TK 784 strains were 50, 12.5, 50, and 0.4 $\mu\text{g/ml}$, respectively. However, it was ineffective against *Aspergillus niger* ATCC 9642, *Candida albicans* IFO 6258, and *Streptomyces murinus* JCM 4333. These results demonstrate that cyclo(phe-pro) has antimicrobial activity, which may have significant potential as a therapy for a broad range of microbial infections.

Accordingly, further research on the biological activity of the cyclo(phe-pro) is warranted.

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