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Identification of *Streptomyces* sp. KH29, Which Produces an Antibiotic Substance Processing an Inhibitory Activity Against Multidrug-Resistant *Acinetobacter baumannii*

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The Actinomycete strain KH29 is antagonistic to the multidrug-resistant *Acinetobacter baumannii*. Based on the diaminopimelic acid (DAP) type, and the morphological and physiological characteristics observed through the use of scanning electron microscopy (SEM), KH29 was confirmed as belonging to the genus *Streptomyces*. By way of its noted 16S rDNA nucleotide sequences, KH29 was found to have a relationship with *Streptomyces cinnamonensis*. The production of an antibiotic from this strain was found to be most favorable when cultured with glucose, polypeptone, and yeast extract (PY) medium for 6 days at 27°C. The antibiotic produced was identified, through comparisons with reported spectral data including MS and NMR as a cyclo(L-tryptophanyl-L-tryptophanyl). Cyclo(L-Trp-L-Trp), from the PY cultures of KH29, was seen to be highly effective against 41 of 49 multidrug-resistant *Acinetobacter baumannii*. Furthermore, cyclo(L-Trp-L-Trp) had antimicrobial activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Candida albicans*, However, it was ineffective against *Streptomyces murinus*.

Keywords: Cyclo(L-tryptophanyl-L-tryptophanyl), *Acinetobacter baumannii*, S*treptomyces cinnamonensis*

Acinetobacter baumannii is a Gram-negative, nonmotile, encapsulated bacterium that is normally present in soil and water, and is also found as a harmless commensal on the skin of 25% of healthy people [18]. *A. baumannii* has not caused epidemiological problems in the past because of its susceptibility to antibiotic treatment [1, 4, 7, 16]. However, with the widespread usage of antibiotics, many isolates are now resistant to a number of antibiotics, such as aminoglycosides, cephalosporins, quinolones, penicillins, monobactams, and imipenem, leading to nosocomial outbreaks. *A. baumannii* does not require fastidious conditions for growth and is able to prosper under various temperatures and pH conditions [3]. These properties allow *Acinetobacter* species to persist in either moist or dry conditions in hospital environments, thereby contributing to transmission [5]. Recently, *A. baumannii* has emerged as an important nosocomial pathogen in many countries [8]. In 1999 in Korea, an antimicrobial resistance research group reported that 6.2% of *A. baumannii* isolates from certain hospitals were imipenem-resistant. It was then suggested that the use of optical detection methods, such as the disk diffusion assay and Vitek system, be employed in order to screen and characterize those resistant to imipenem [11]. Other outbreaks have been reported in the US military health care system associated with military operations in Iraq and Israel, as well as in many hospitals in the United States and Italy. *Acinetobacter* infections are closely related to the use of ventilators or other invasive devices, and risk factors include neurosurgery, acute respiratory distress syndrome, and head trauma [2, 13]. It is therefore essential to discover new drugs with anti-MDR activity. During the course of our screening program, we isolated a strain from the soil that was seen to possess anti-MDR activity and identified it as *Streptomyces* sp. KH29. The objectives of the present study were to characterize *Streptomyces* sp. KH29 through the use of scanning electron microscopy (SEM) to examine the diaminopimeric acid (DAP) type, morphology, physiological characteristics, and 16S rDNA nucleotide sequences. In order to identify potential antibiotics, the antimicrobial activities of the extracts and compounds from *Streptomyces* sp. KH29 were screened against 41 MDR *A. baumannii* isolates and a number of pathogenic microorganisms.

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Materials and Methods

The Anti-*A. baumannii* Antibiotic-Producing Actinomycete Strain KH29

The Actinomycete strain KH29, which produces an anti-*A. baumannii* antibiotic, was isolated in a medium for Actinomycete isolation from a soil sample collected in Yesan, Korea (S medium: 1% glycerin, 1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% K_2HPO_4, 0.2% NaCl, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% thiamine·HCl, and 0.9% agar, adjusted to pH 7.0–7.2. AV medium: 0.1% glucose, 0.1% glycerol, 0.03% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% NaCl, 0.03% L-asparagine, 0.1% trace salt solution, 1% vitamine solution, 1% antibiotic solution, and 0.9% agar, adjusted to pH 6.4). The organism was grown at 28°C on a modified Bennett's agar slant and stored at 4°C [9].

Identification of the Actinomycete Strain KH29

In order to determine the genus of the Actinomycete strain KH29 and the type of 2,6-diaminopimelic acid, one of the cell wall components of Actinomycete mycelia was analyzed using the methods of the International *Streptomyces* Project (ISP), which were suggested by Shirling and Gottlieb [17], and *Bergey's Manual of Systematic Bacteriology* [22]. The KH29 strain was cultured in 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 H_2O, adjusted to a pH of 7.3 before autoclaving) for 7 days at 28°C by using a rotary-shaking incubator. The cultured broth was filtered with 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 of 6 N HCl, sealed tightly, and hydrolyzed by heating of the tube for 18 h in a boiling water bath. The hydrolysate was filtered with Whatman No.1 filter paper and evaporated to dryness in order 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., NJ, USA). Five μl of 0.01 M DL-DAP (Sigma Chemical Co., St. Louis, USA) containing both the *meso*- and LL-DAP isomers and amino acids (alanine, glycine, and glutamate) were also loaded on plates as a standard [23].

To examine spore chain morphology, the KH29 strain 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 H_2O, adjusted to pH 7.3 before autoclaving). The spore chain morphology of the strain KH29 was examined using light microscopy (×400 magnification) and scanning electron microscopy (SEM) (Model S-800, Hitachi Co., Tokyo, Japan). The SEM specimen was prepared according to the method reported by Williams and Davies [21]. Amongst the morphological categories suggested by Pridham *et al*. [12], the two categories of *Rectiflexibles* and *Spirales* were employed for the evaluation of spore chain morphology.

The Genus *Streptomyces* for 16S rDNA Sequencing

For sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. kit (Promega Co., WI, USA). Amplification of 16S rDNA was performed on the isolated DNA using the primers 5'-GGCGTGCTTAACACATGCA AGTC-3' and 5'-TACCTTGTTACGACTTCGTCCCAA-3'. The amplified products were resolved on 1.5% (w/v) agarose gel, and then excised from the gel and purified. The purified products were cloned into a pGEM-T Easy vector (Promega) and subsequently sequenced using Bi Dye Terminator chemistry (Applied Biosystem Inc., CA, U.S.A.). In addition, dendrograms were constructed utilizing the neighbor-joining method [16] from a distance matrix calculated with ClustalX software [8]. The levels of sequence similarity were calculated and the distances derived were used to infer the phylogenetic relationships.

Extraction and Identification of Anti-*A. baumannii* Antibiotics

The anti-*A. baumannii* 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 100% methanol. The eluate was concentrated *in vacuo* to a small volume and extracted with ethyl acetate (5 l) at a pH of 7.0. The ethyl acetate layer was then concentrated to a small volume and extracted again 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) and eluted with 100% 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=95:5. High-performance liquid chromatography (HPLC, Hewlett-Packard 1100; HP Co., CA, USA) was performed using a diode-array detection system equipped with a μ-Bondapak C18 column (10 μm, 150 mm×3.9 mm; Waters Co., MA, USA) at room temperature. The system was operated at a flow rate of 0.5 ml/min with a methanol:water (70:30) solvent mixture.

Spectroscopic Analysis of Cyclo(Trp-Trp)

The purified compound was identified by GC–MS, ^{1}H and ^{13}CNMR, and FT–IR data. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer. EI–MS and FABMS data were obtained on a JEOL JMS700 mass spectrometer, and GC–MS data on a Hewlett Packard 6890 Series using a Finnigan TSQ 700 operated in the electron ionization mode. IR spectra were recorded on a Bruker IFS- 66/S FT–IR spectrometer.

Medium for Anti-*A. baumannii* Antibiotic Production

The medium used for the seed culture of the KH29 strain (for 2 days) was PC II (10 g dextrose, 2 g polypeptone, 1 g yeast extract, 1 g meat extract, 0.5 g asparagine, 0.1 g thiamine·HCl, and 1 l of H_2O, adjusted to a pH of 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 $CaCO_3$, 0.01 g thiamine·HCl, and 1 l of H_2O, adjusted to pH 7.0 before autoclaving). The 49 clinical *A. baumannii* strains (from *A. baumannii* 1 to *A. baumannii* 49) were isolated from the Korea University Hospital over a 2-year period (2006–2008). Furthermore, *Aspergillus niger* ATCC 9642, *Bacillus subtilis* IAM 1069, *Candida albicans* IFO 6258, *Micrococcus luteus* JCM 1464, *Saccharomyces cerevisiae* IFO 1008, *Strerptomyces murinus* JCM 4333, and *Staphylococcus aureus* TK 784 were also evaluated in this study. The medium used for the anti-*A. baumannii* was a brain heart infusion agar (Difco, USA) and glucose bouillon (GB), and potato dextrose agar (PDA) (Difco, USA) media were used for all other organisms. The assays were performed in triplicate for each experiment. The minimal inhibitory

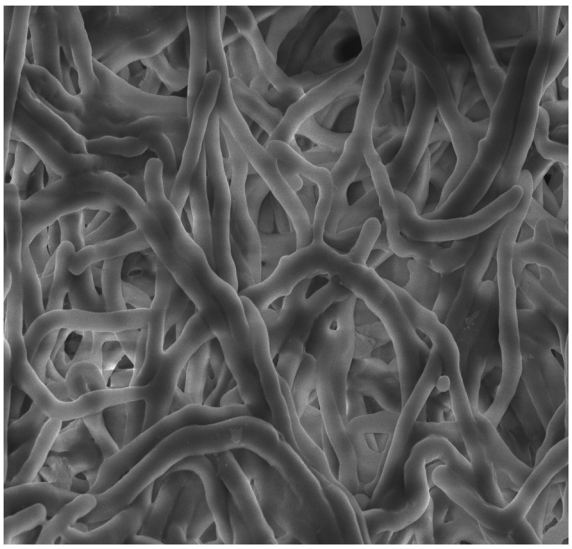

Fig. 1. Scanning electron microphotograph of spore surface of the strain KH29 cultured on YME agar for 14 days at 27°C.

concentrations (MICs) were determined by the conventional agar dilution method according to the National Committee for Clinical Laboratory Standards [19]. In brief, the concentrations of the anti-MDR agent tested ranged from 12.5 to 125 μg/ml. Colony suspensions equal to a 0.5 McFarland standard were prepared and inoculated onto the antibiotic-containing media using a Cathra System replicating device (MCT Medical Inc., MN, USA.) to yield a final inoculum of 10^4 CFU/spot. The plates were incubated in ambient air at 35°C for 24 h. The MICs were defined as the lowest antibiotic concentration showing no growth.

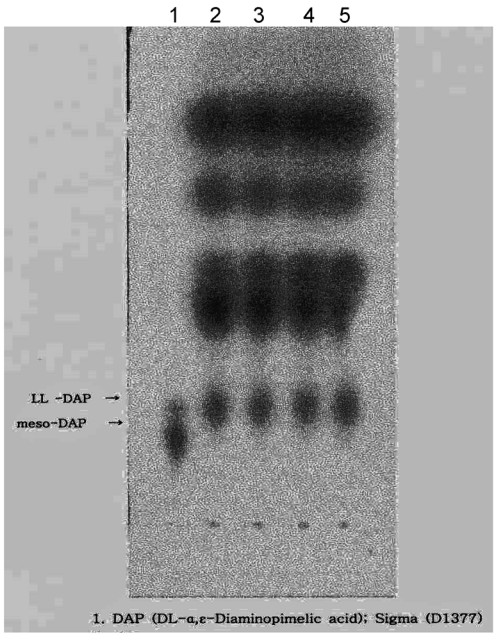

Fig. 2. Thin-layer chromatography (TLC) of cell wall diaminopimelic acid isomers of the stain KH29.
Lane 1, Standard DAP; lanes 2–5, Strain KH29.

RESULTS AND DISCUSSION

Analysis of Diaminopimelic Acid Type and Morphological Characteristics

The cell wall hydrolysates of the strain KH29 were developed on a cellulose TLC plate. The diaminopimelic acid that was present in the cell wall was found to be LL-DAP (Fig. 2). The strain KH29 formed a rectiflexible type of spore chain, as observed by light microscopy. Under SEM, the strain KH29 had cylindrical forms with a smooth surface on aerial mycelia (Fig. 1). A special structure, such as a sporangium, was found in KH29. Based on the DAP type of cell wall and morphological characteristics, KH29 was concluded to belong to the genus *Streptomyces.*

Phylogenetic Analysis of the Strain KH29 Using the 16S rDNA Sequence

The 1,512 bp sequence obtained from the strain KH29 was aligned with all presently available 16S rRNA gene sequences obtained from GenBank databases. From this alignment, a dendrogram was constructed. Phylogenetic analysis of the strain KH29 using the 16S rRNA gene sequence data suggests that the strain is most closely related to *Streptomyces cinnamonensis* (Fig. 3).

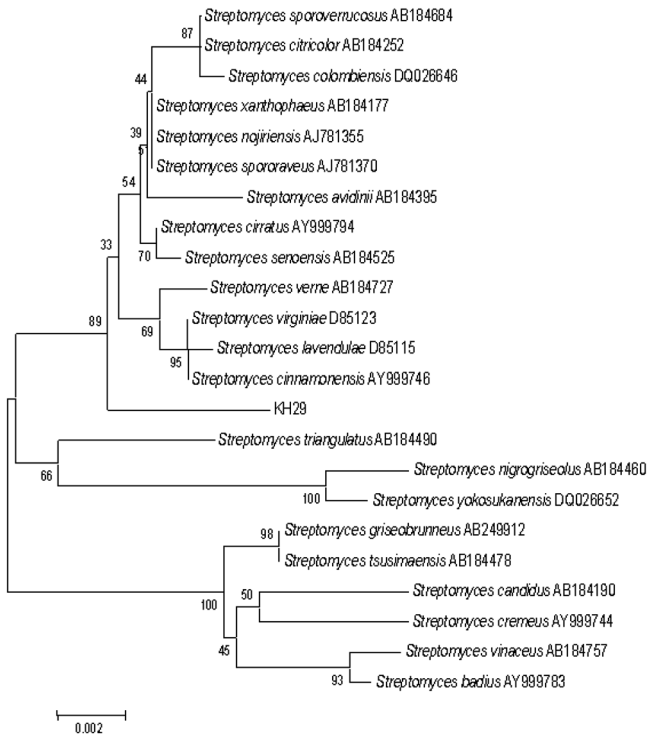

Fig. 3. Dendrogram showing the relationships between *Streptomyces* sp. KH29 and other *Streptomyces* sp.
The rooted tree was constructed using the neighbor-joining method. The scale bar indicates a 0.002 substitution per nucleotide position.

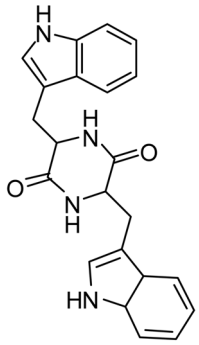

Fig. 4. Chemical structure of the isolated compound, cyclo(L-tryptophanyl-L-tryptophanyl).

Table 2. ^{1}H NMR data of cyclo(Trp-Trp) in DMSO-d_6.

Proton	ppm
Trp-β	2.20
Trp-β	2.70
Trp-α	3.87
Trp-Ar	6.62
Trp-Ar	6.95
Trp-Ar	7.04
Trp-Ar	7.29
Trp-Ar	7.36
NH	7.65
NH(indole)	10.82

Identification of Anti-*A. baumannii* Antibiotics

The active anti-*A. baumannii* compound was isolated from the extracts of the strain KH29 using solvent extraction and chromatography. The resultant compound had a yield of 37 mg per 50-l jar fermenter with a purity of 98% by HPLC. The properties of this compound were identified by GC–MS, ^{1}H and ^{13}C NMR, and FT–IR data, and these results were found to be in agreement with those reported by Graz *et al*. [6], suggesting the antibiotic to be cyclo(Trp-Trp) (Fig. 4). Furthermore, this identification was confirmed using an authentic sample (Lot No. 0555208; Bachem AG, Bubendorf, Swiss). The mass spectra of cyclo(Trp-Trp)(diketopiperazines) showed a parent ion peak at *m/z* 372, the expected cyclic dipeptide form. The characteristic tryptophan side-chain cleavage yielding the fragmentation *m/z* 130 is one of the highest observed fragment ions in the mass spectra. The ion at *m/z* 154 corresponds to the diketopiperazine pyrrolidine fragment. IR spectroscopy of cyclo(Trp-Trp) revealed the specific values of (N–H) 3,214.7 cm^{-1} and (C=O) 1,661.8 cm^{-1}. ^{1}H NMR (Table 2) of cyclo(Trp-Trp) exhibited resonances for 10 protons (δ 2.20, 2.70 from Trp-β, 3.87 from Trp-α, 6.62, 6.95, 7.04, 7.29, 7.36 from Trp-Ar, 7.65 from NH, and 10.82 from NH of indole. ^{13}C NMR data (Table 3) of cyclo(Trp–Trp) also showed resonances for 11 carbons (δ 29.87 from Trp-β, 55.23 from Trp-α, 108.86, 111.30, 118.41, 118.61, 120.86, 124.49, 127.46, 136.17 from Trp-Ar, and 166.90 from C=O).

Table 1. Antimicrobial activity of the cyclo(Trp-Trp) produced by *Streptomyces* sp. KH29.

Test organisms	MIC (μg/ml)
A. baumannii [a] 1–12, 23–29, 38	25
A. baumannii 13–22, 30–35	12.5
A. baumannii 36–41	12.5
A. baumannii 42–49	>100
Candida albicans IFO 6258	50
Bacillus subtilis IAM 1069	50
Micrococcus luteus JCM 1464	12.5
Aspergillus niger ATCC 9642	25
Streptomyces murinus JCM 4333	>100
Staphylococcus aureus TK 784	0.8
Saccharomyces cerevisiae IFO 1008	25

[a]Isolated at the Korea University Hospital over a period of two years (2006–2008).

Antimicrobial Activity of Cyclo(Trp-Trp)

The activity of the cyclo(Trp-Trp) produced by *Streptomyces* sp. KH29 against microorganisms are shown in Table 1. Cyclo(Trp-Trp) was effective against microorganisms such as *A. baumannii* (1–12, 23–29, 38), *A. baumannii* (13–22, 30–50), and *A. baumannii* (36–41), and the MIC values observed were 25, 12.5, and 12.5 μg/ml, respectively. However, cyclo(Trp-Trp) was not effective against 8 of the *A. baumannii* (42–49) strains. This shows that cyclo(Trp-Trp) was more effective in comparison with RP59500 (Quinopristin/Dalfopristin) [20]. Moreover, the MICs of cyclo(Trp-Trp) against *Candida albicans* IFO 6258, *Bacillus subtilis* IAM 1069, *Micrococcus luteus* JCM 1464, *Saccharomyces cerevisiae* IFO 1008, *Aspergillus niger* ATCC 9642, and *Staphylococcus aureus* TK 784

Table 3. ^{13}C NMR data of cyclo(Trp-Trp) in DMSO-d_6.

Carbon	ppm
Trp-β	29.87
Trp-α	55.23
Trp-Ar	108.86
Trp-Ar	111.30
Trp-Ar	118.41
Trp-Ar	118.61
Trp-Ar	120.86
Trp-Ar	124.49
Trp-Ar	127.46
Trp-Ar	136.17
C=O	166.90

were 50, 50, 12.5, 25, 25, and 0.8 μg/ml, respectively. However, it was ineffective against *Streptomyces murinus* JCM 4333. Cyclic dipeptides are known to possess both antibacterial and anti-yeast activities [6]. We have previously reported on the production of cyclic dipeptides, cyclo(Leu-Pro) and cyclo(Phe-Pro), from *Streptomyces* strains, which had both anti-VRE (vancomycin-resistant enterococci) and antibacterial activities [14]. These results demonstrate that cyclic dipeptides, such as cyclo(leu-pro) and cyclo(trp-trp), have antimicrobial activity, which may have significant potential as a therapy for a broad range of microbial infections. Additional investigations of these and other cyclic dipeptides are merited, particularly in relation to aspects such as bioavailability, toxicity, and stability. The latter considerations represent pragmatic considerations and represent the next step towards the creation of novel treatments for bacterial infections.

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