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NOTE

Molecular Taxonomy of a Soil Actinomycete Isolate, KCCM10454 Showing Neuroprotective Activity by 16S rRNA and *rpoB* Gene Analysis

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Epilepsy constitutes a significant public health problem, and even the newest drugs and neurosurgical techniques have proven unable to cure the disease. In order to select a group of isolates which could generate an active compound with neuroprotective or antiepileptic properties, we isolated 517 actinomycete strains from soil samples taken from Jeju Island, in South Korea. We then screened these strains for possible anti-apoptotic effects against serum deprivation-induced hippocampal cell death, using the 3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay as an *in vitro* test. The excitotoxic glutamate analog, kainic acid (KA), was used to induce seizures in experimental mice in our *in vivo* tests. As a result of this testing, we located one strain which exhibited profound neuroprotective activity. This strain was identified as a *Streptomyces* species, and exhibited the rifampin-resistant genotype, Asn(AAC)^{442}, according to the results of 16S rRNA and *rpoB* gene analyses

Key words: epilepsy, antiepileptic activity, hippocampal cell, apoptosis, *Streptomyces*, rifampin resistant genotype

Epilepsy is a neuropathological condition that is characterized by localized bursts of electrical activity (seizures) in the cerebral hemisphere. It appears to be caused by extensive neuronal cell death occurring in diverse regions of the brain. The calcium influx and molecular cascades that appear to contribute to necrotic and apoptotic neuronal death in these areas of the brain, which include the hippocampus, have been implicated as the major molecular mechanism underlying the phenomenology of epilepsy (Mattson, 2000). Epilepsy constitutes a significant public health problem. Despite the widespread use of both classic and newly-developed pharmacological agents that target ion channels, amino acid transmission, or receptors, both mono- and polytherapeutic approaches have almost uniformly proven to be ineffective (Pitkanen and Kubova, 2004). Clearly, there is a current necessity for the development of a new anti-epileptic drug.

In this study, we have conducted a screening program designed to locate a compound that exerts antiepileptic effects. We opted to use microbial metabolites from soil actinomycete strains, since bioactive chemicals and proteins have previously been isolated from such strains. Recently, Kim *et al.* (2004) reported that, among *Streptomyces* spp., approximately 10 % of tested strains proved to harbor a natural rifampin resistance genotype, designated as Asn (AAC)^{442}. This genotype is characterized by an AAC codon, which encodes for asparagine, at the site corresponding to codon 442 of the *S. coelicolor* RNA polymerase β subunit. In rifampin-susceptible strains, a TCG or TCC codon, both of which would encode for serine, are found at this site. Therefore, in this study, in order to increase our chances of locating an appropriate compound, we targeted actinomycete strains that exhibited the rifampin-resistant genotype as our source of microbial metabolites.

Twenty soil samples were collected from several sites near Halla Mountain, on Jeju Island, in Korea. Isolates were prepared using three different types of reduced arginine starch salts agar, all of which contained rifampin at

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a concentration of 20 ug/ml. 50 ug/ml of cycloheximide was autoclaved and added to the isolation media, to inhibit fungal growth, and heat-labile nystatin was sterilized by filtration, and also added at a concentration of 50 ug/ml. We isolated the *Streptomyces* strains from the soil samples according to the previously-described method (Kim *et al.*, 2002; Kim *et al.*, 2001). In brief, approximately 1 g of soil was placed in a Petri dish, ground, and then heated at 60°C for 90 min in a dry oven. The dried soil samples were transferred to sterilized bottles, and sterile distilled water was added. The soil suspensions were then mixed vigorously and allowed to stand for 30 min. Portions of the supernatant (0.1 ml) were then inoculated onto plates, which were subsequently incubated at 30°C for 10 days. Colonies that exhibited the typical characteristics of actinomycetes, but exhibited diverse morphologies, were then extracted from the plates and transferred to Bennett agar for strain maintenance. Finally, we collected the 517 actinomycete isolates that clearly evidenced rifampin resistance. Samples of each selected colony were extracted from the agar, and inoculated into 30 ml of Bennet broth in a 100 ml capped tube. The inoculated broths were then cultured in a shaking incubator at 28°C for 14 days. The fermentation broths were concentrated under reduced pressure, and filtered.

In order to preliminarily select out the antiepileptic actinomycete strains, we screened the 517 isolates based on their anti-apoptotic effects against serum deprivation-induced apoptosis in the HT-22 line, a type of immortalized mouse hippocampal cell that phenotypically resembles neuronal precursor cells. We utilized 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay in this *in vitro* test (Lobner, 2000). The HT-22 cell line has been used in the past, primarily as a model system for the study of oxidative glutamate toxicity (Maher and Schubert, 2000). The HT-22 cells were grown to confluency in Dulbecco's modified essential medium (DMEM), supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS) and 1 mg/ml streptomycin at 37°C, in an atmosphere containing 5% CO_2. The HT-22 cells were plated in 96-well plates, at a density of 10,000 cells/ml. After 24 h of normal cell culturing, we initiated our analysis of the anti-apoptotic effects of these actinomycetes culture broths. 160 μl of serum-free DMEM solution and 40 μl culture broth were added to each well, then cultured for 24 h. For negative and positive controls, we used serum free DMEM solution only (negative) and DMEM solution supplemented with serum with no added culture broth (positive). Neuronal injury was quantified by measuring of the reduction of MTT, which could be visualized by the formation of a dark blue formazan product. MTT was added to each well 24 h after beginning serum deprivation. After 4 h of incubation, the medium was removed, and the cells were dissolved with DMSO. We then conducted a spectrophotometric determination of the formazan products

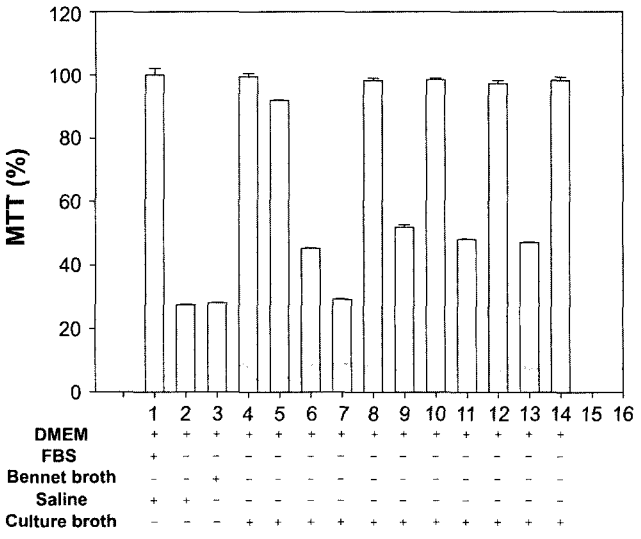

Fig. 1. Protective effects against serum deprivation-induced apoptotic cell death in the hippocampal cell line, HT-22, as the result of incubation with culture broth from actinomycete isolates. Mean data are shown, representative of at least three individual experiments ($P< 0.05$). Compared to the controls, the culture broths from six isolates (CB14, CB67, CB 79, CB104, CB124, and CB214) exhibited protective effects more than 50% more potent than those exhibited by the controls. The other isolates showed no, or minor, protective qualities. 1, positive control; 2, negative control; 3, negative control with added bennet broth; 4, CB14 ; 5, CB67; 6, CB68 ; 7, CB70; 8, CB79; 9, CB100; 10, CB104; 11, CB112; 12, CB124; 13, CB197; 14, CB214.

generated, at 540 nm using a multiplate reader. We then selected the isolates evidencing anti-apoptotic properties, by comparing the MTT results of the tested samples with those of the negative and positive controls. Out of the 517 original actinomycete culture broth samples, 6 isolates (CB14, CB67, CB79, CB104, CB124, and CB214) exhibited anti-apoptotic activities in the *in vitro* test, generating at least 150% of the formazan product seen in the control samples (Fig. 1).

We then initiated the final phase of selection for the actinomycete strains with antiepileptic activity. The 6 isolates selected in the previous phase were tested for their neuroprotective effects in the hippocampus, using KA-induced seizures in experimental mice as an *in vivo* test. In order to evaluate the isolates' neuroprotective effects against KA-induced hippocampal cell death, we used ICR mice (Samtaco Inc., Korea) as experimental animals. In brief, 100 μl of filtered culture broth from each isolate was administered intraperitoneally to three ICR mice. Saline-injected animals were used as negative controls. After 30 min, the mice were injected intraperitoneally (i.p) with a single dose of KA (25 mg/kg) (Sigma, USA), dissolved in 0.9% NaCl. 48 h after the administration of the KA, all of the test animals (n=3) were anesthetized with ketamine (30 mg/kg, i.p) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The animals' brains were extracted, post-

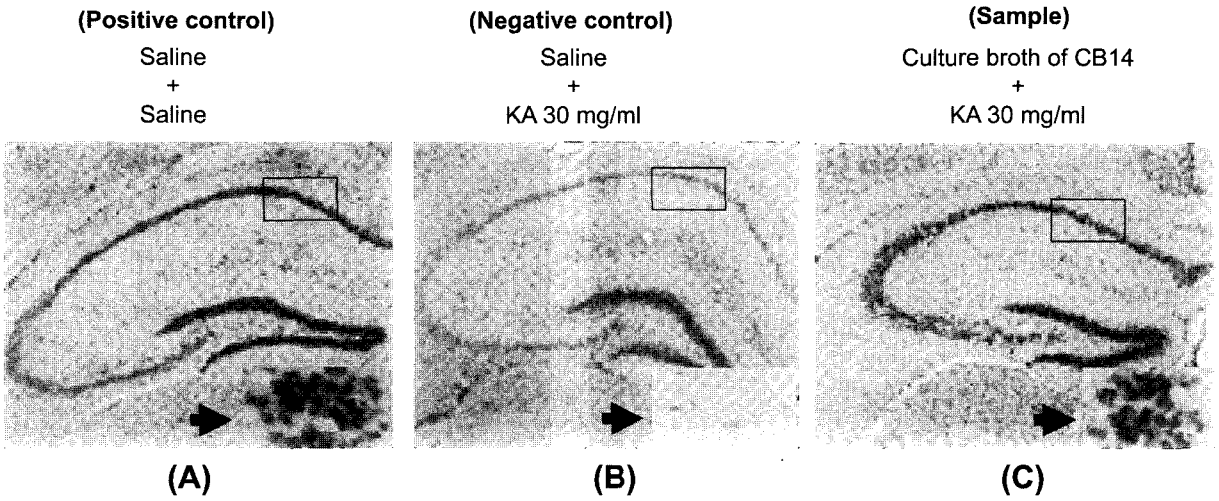

Fig. 2. Anti-epileptic effects against KA-induced hippocampal cell damage in mice by CB14 culture broth. Regions stained with crezyl violet indicated viable hippocampal cells. The boxed regions were magnified (× 40). In the mice treated only with KA, most of the pyramidal cell layer was disrupted and replaced by a dark material. However, 48 h after KA injection, some of the extract-injected mice exhibited less severe apoptotic neuronal damage.

fixed for 4 h, then maintained in 20% sucrose solution overnight at 4°C Serial sections were cut to a thickness of 30 μM using a freezing microtome, and collected in 6-well plates. One of these wells in each plate was stained with crezyl violet (Sigma, USA) in order to determine the extent of neuronal cell loss in each animal. The administration of KA induced neurodegeneration in the CA1 and CA3 hippocampal subfields. The crezyl violet indicated cellular pyknosis associated with neuronal degeneration (Fig. 2). The analyses of the KA-induced hippocampal neuronal losses occurring in each animal are shown in Table 1. Forty eight h after the injection of KA, we detected significant differences in hippocampal morphology between the PBS-injected mice and the extract-injected mice. In the PBS injection mice, the majority of the pyramidal cell layer in the CA1 and CA3 regions had been disrupted, and had been replaced by a dark substance. Some of the extract-injected mice, however, exhibited apoptotic neuronal damage to a lesser degree. In particular, the mice injected with the CB14 isolate displayed substantially lower neuronal loss scores in the CA1 and CA3 hippocampal regions. In order to evaluate the degree to which KA-induced hippocampal cell damage had occurred in the mice, the stained sections from each set of 3 mice were assigned the following scores, according to the criteria developed by Morrison *et al.* (1996): "little damage", characterized by occasional single-cell degeneration in the CA3 region; "mild damage", characterized by small regions of degenerated CA3 pyramidal cells; and "severe damage", characterized by an extended neuronal degeneration zone, neuronal cell loss, and tissue sclerosis, which frequently included both dorsal and ventral hippocampal CA3 regions. Among the six selected isolates that displayed anti-apoptotic activities in the HT-22 cells, only one (CB14) exhibited a significantly potent neuroprotective effect in the KA-induced seizure mouse model. The other isolates exhibited no neuroprotective effects, or only mild neuroprotective effects (Table 1 and Fig. 2). Thus, we ultimately concluded that CB14 produces an active compound that exerts some anti-epileptic effects.

Table 1. Neuroprotective effects against KA induced hippocampal cell death of culture broth from six isolates

Culture broth	Degree of neuronal damage (No. of animals)			
	None	Little	Mild	Severe
Saline (n=3)	0	0	1	2
CB14 (n=3)	2	1	0	0
CB67 (n=3)	0	0	0	3
CB79 (n=3)	0	0	1	2
CB104 (n=3)	0	0	2	1
CB124 (n=3)	0	0	0	3
CB214 (n=3)	0	0	0	3

We then attempted to classify and identify CB14 via 16S rRNA and *rpoB* gene analysis. The chromosomal DNA of CB14 was extracted according to the bead beater-phenol extraction method (Kim *et al.*, 2003; Kim *et al.*, 2004). In order to disrupt the streptomycete cell wall, bacterial mixtures containing phenol and glass beads were subjected to vibration on a Mini-Bead beater, as described previously. In brief, the aqueous phase was then transferred to a clean tube and the DNA pellet was precipitated with isopropyl alcohol. The pellet was then solubilized with 60 ul TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Two microliters of purified DNA were used for a PCR template. The 16S rRNA gene fragment was then

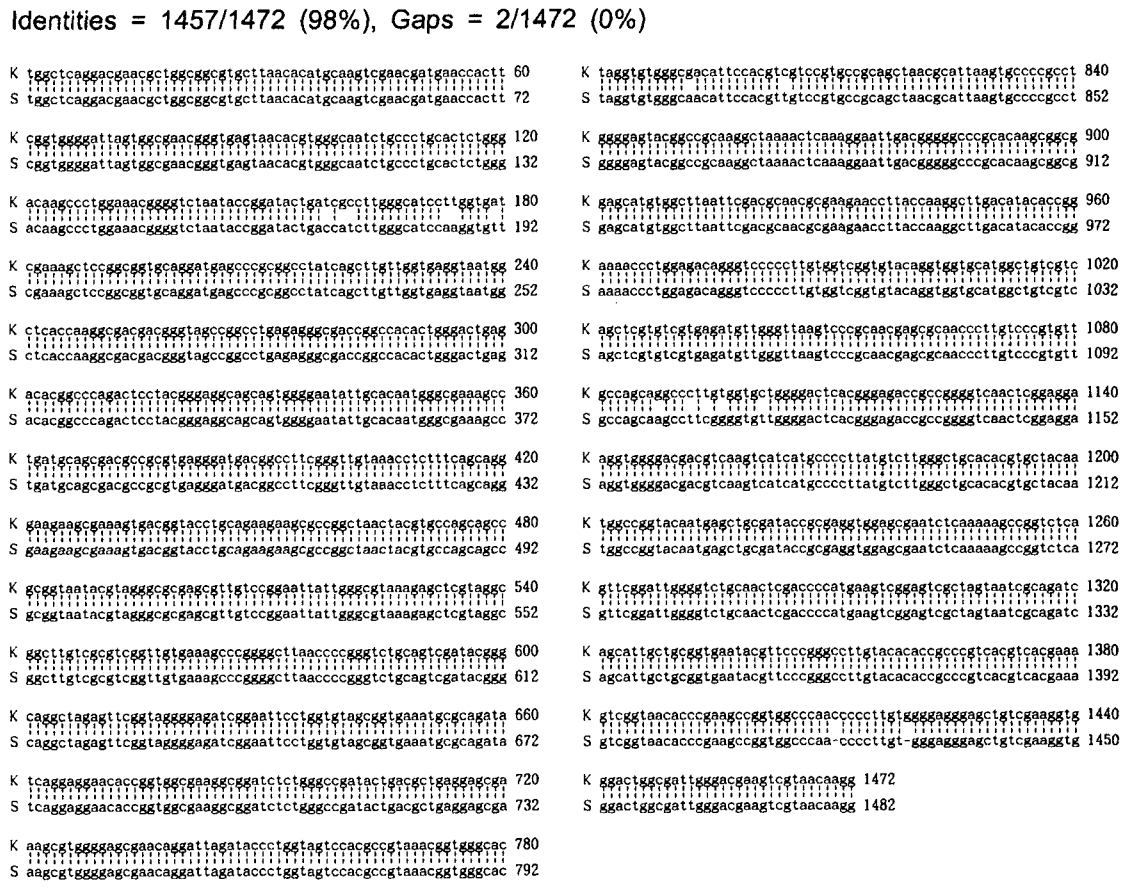

Fig. 3. The results obtained by BLASTN (K: KCCM10454, S: *Streptomyces caelestis*). The 16S rDNA sequence of KCCM10454 was analyzed with BLAST (National Center for Biotechnology Information). The results indicate that the strain shares 98% homology (1457/1472) with *Streptomyces caelestis* (Genbank No. X80824).

amplified by using primers 285 (forward: 5'-GAG AGT TTG ATC CTG GCT ACA-3'; *Escherichia coli* bp 9-29) and 261 (reverse: 5-AAG GAG GTG ATC CAG CCG CA; *E. coli* bp 1542-1523), as previously described (Kirschner *et al.*, 1992). The purified PCR products (1472-bp) were then ligated to PCR®II-TOPO® using the TOPO TA Cloning® Kit (Invitrogen, USA). The ligation products were subsequently transformed into chemically competent *E. coli* (TOP10), according to the manufacturer's instructions. The PCR product sequences were determined using the four internal primers (p510r 5'-TAT TAC CGC GGC TGC TG-3', p364f 5'-GGC AGC AGT GGG GAA TAT TG-3', p783f 5'-TAG ATA CCC TGG TAG TCC AC-3', and p1037f 5'-TCG TCA GCT CGT GTC GTG AG-3'), with an Applied Biosystems model 373A automatic sequencer and a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, United Kingdom). The 16S rDNA sequence of CB14 is shown in Fig. 3. This sequence was then subjected to BLAST analysis (National Center for Biotechnology Information). Our results indicated that the strain exhibits a 98% degree of homology (1457/1472) with *Streptomyces caelestis* (Genbank No. X80824) (Fig. 3).

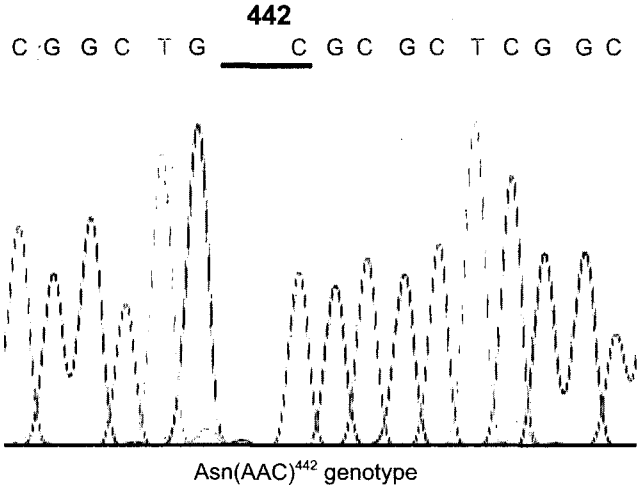

Fig. 4. *rpoB* gene sequences of the CB14 strain corresponding to the 442th codon of the *S. coelicolor* RNA polymerase β subunit. This strain has an AAC sequence, which encodes for asparagine, at this codon, which is associated with natural rifampin resistance in some bacteria.

We also performed *rpoB* gene analysis in order to identify and determine the genotype responsible for the nat-

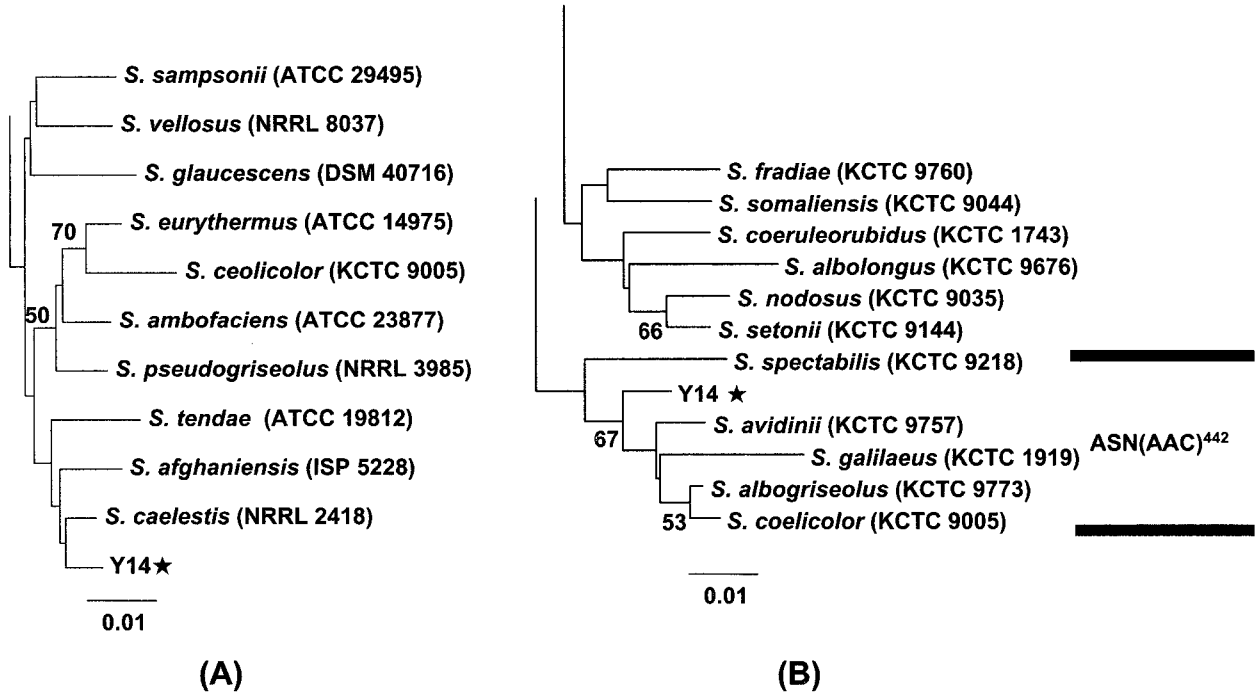

Fig. 5. Phylogenetic analyses based on partial (1465-1480 bp) 16S rDNA sequences (A) and partial (306-bp) *rpoB* sequences (B) of *Streptomyces* reference strains, for the determination of the phylogenetic locations of the CB14 strain. Both trees were constructed via the Neighbor-Joining method. The percentage numbers at the nodes indicate the the bootstrap levels supported from 1000 resampled data sets. Bootstrap values of less than 50% are not shown.

ural rifampin resistance exhibited by the CB14 strain. The 306 bp sequences of the *rpoB* gene were determined according to the previously described method (Kim *et al.*, 2003; Kim *et al.*, 2004). In brief, a set of primers [SRPOF1, 5'-TC GAC CAC TTC GGC AAC CGC-3' and SRPOR1 5'-TC GAT CGG GCA CAT GCG GCC-3'] was used to amplify a 352-bp *rpoB* gene from selected *Streptomyces* strains. The nucleotide sequences (306-bp) of the purified PCR products (352-bp) were then determined directly with forward and reverse primers, for PCR amplification. CB14 exhibited an AAC nucleotide sequence, which encodes for asparagines, at the region corresponding to the 442th codon of the *S. coelicolor* RNA polymerase β subunit. This sequence, in the rifampin-susceptible strains of *Streptomyces,* is either TCG or TCC, which encodes for serine (Fig. 4). Therefore, the CB14 strain was determined to exhibit the natural rifampin resistance genotype, Asn (AAC)^{442}.

According to the 1472 bp 16S rDNA sequences of the CB14 strain, we conducted a phylogenetic analysis, in order to delineate the precise phylogenetic relatedness of this strain to 40 reference strains in the Genbank database (Fig. 5A). We also performed a phylogenetic analysis based on the 306 bp *rpoB* sequences of the CB14 strain, compared to those of the same 67 reference strains used in the 16S rDNA sequence analysis (Fig. 5B). The nucleotide sequences of the two genes were aligned, and their similarities were calculated with the multiple-alignment algorithm provided in the Megalign software package (DNASTAR, Window version 3.12e). Phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei, 1987). The neighbor-joining was carried out with the help of the MEGA version 2.1 program (Kumar *et al.*, 2001). The resultant neighbor-joining tree, and its topology, were then evaluated via bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Our phylogenetic results confirmed that CB14 belongs within the *Streptomyces* genus, and belongs to a rifampin-resistant genotypic group (Fig. 5A and B). The strain was submitted to the Korean Culture Center of Microorganisms (KCCM) at Seoul, Korea, under registration number, KCCM10454.

In conclusion, even though the compound CB14 remains to be completely classified and characterized, we are able to report our results with regard to the isolation and identification of a naturally rifampin-resistant *Streptomyces* sp., which exhibits profound anti-epileptic properties. This strain, henceforth referred to as KCCM10454, could be used effectively in the development of anti-epileptic drugs in the future. To our knowledge, this constitutes the first report regarding the selection and isolation of a naturally rifampin-resistant *Streptomyces* strain that producing bioactive compounds such as the one characterized in this study.

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