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Metabolomics-Based Chemotaxonomic Classification of *Streptomyces* spp. and Its Correlation with Antibacterial Activity

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Secondary metabolite-based chemotaxonomic classification of *Streptomyces* (8 species, 14 strains) was performed using ultraperformance liquid chromatography-quadrupole-time-of-flight-mass spectrometry with multivariate statistical analysis. Most strains were generally well separated by grouping under each species. In particular, *S. rimosus* was discriminated from the remaining seven species (*S. coelicolor*, *S. griseus*, *S. indigoferus*, *S. peucetius*, *S. rubrolavendulae*, *S. scabiei*, and *S. virginiae*) in partial least squares discriminant analysis, and oxytetracycline and rimocidin were identified as *S. rimosus*-specific metabolites. *S. rimosus* also showed high antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*, the pathogen responsible for rice bacterial blight. This study demonstrated that metabolite-based chemotaxonomic classification is an effective tool for distinguishing *Streptomyces* spp. and for determining their species-specific metabolites.

Keywords: *Streptomyces*, chemotaxonomy, antibacterial activity

Introduction

Streptomyces is a group of gram-positive filamentous bacteria that grow in soil and decaying vegetation. It is the largest genus of Actinobacteria and includes over 500 species [61]. Streptomycetes have historically been examined for their ability to biologically control plant disease and animal pathogens [22, 23, 41]. Among various species of Streptomycetes, *S. somaliensis* and *S. sudanensis* cause actinomycosis in animals [40, 49], and *S. scabiei* causes common scab in potatoes [13, 35]. *Streptomyces* is a particularly well-known agent against leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. The genus *Xanthomonas*, comprising gram-negative plant pathogenic bacteria, causes a variety of diseases in plants such as citrus canker, walnut blight, rice leaf blight, and bacterial spot [20, 33, 38, 55]. Secondary metabolites of *Streptomyces* are associated with the production of antibiotics and toxic substances in animals and plants. For example, antibiotics such as alnumycin, fistupyrone, and vinylamycin have been obtained from *Streptomyces* spp. [6, 30, 31]. *S. coelicolor* and *S. hygroscopicus* are known to produce antibiotic metabolites such as perimycin and herbimycin [8, 43]. In addition, particular species of *Streptomyces* such as *S. scabiei*, *S. ipomoeae*, and *S. somaliensis* are harmful to humans and animals. Therefore, these species are crucial to clearly classify and differentiate the various species. Traditional taxonomic procedures based on phenotypic traits and DNA relatedness involve classification of new microorganisms mostly using DNA sequencing [27, 37]. Bacterial identification has been generally based on their morphological, physiological, and chemical characteristics [59]. However, solely using one taxonomic method is not sufficient for bacterial classification. Classification of bacterial species in the genus *Streptomyces* has typically been supported by other types of investigation, such as chemical, enzymatic, serological, and hybridization studies, and RNA/DNA sequence analysis [12, 57]. Despite the wide variety of investigations, taxonomic approaches to identifying *Streptomyces* species remain difficult because of the various morphological, cultural, physiological, and

biochemical characteristics that are observed at the inter- and intraspecies levels [1]. Recently, several studies using a chromatographic approach to classify bacteria have been reported [21, 56, 25]. The classification of microorganisms based on metabolite profiling has emerged as a useful chemotaxonomic tool that provides detailed information about the differences and similarities between species [7, 32, 51, 58]. In addition, liquid chromatography mass spectrometry (LC-MS) has been used to identify active antimicrobial substances in target microorganisms [10, 17].

In this study, the secondary metabolites of *Streptomyces* were profiled to classify *Streptomyces* strains based on cluster-specific properties by using ultraperformance liquid chromatography-quadrupole-time-of-flight-mass spectrometry (UPLC-Q-TOF-MS). We also evaluated the growth inhibition of *Xanthomonas oryzae* pv. *oryzae* by *Streptomyces* species extracts and tentatively identified antibacterial metabolites.

Materials and Methods

Chemicals and Reagents

Methanol, water, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Yeast extract, malt extract, and nutrient broth (NB) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Agar powder, calcium carbonate, and glucose were purchased from Junsei Chemical (Tokyo, Japan). Glucose/soybean meal/sodium chloride (GSS) broth was purchased from MB Cell (Seoul, Korea). 2,4-Diacetylphloroglucinol (DAPG) was purchased from Toronto Research Chemicals (Ontario, Canada) as a positive control. Formic acid and oxytetracycline hydrochloride and standard compounds were obtained from Sigma-Aldrich. (St. Louis, MO, USA)

Strains

The *Streptomyces* strains used in this study consisted of 8 species and 14 strains, which are *S. coelicolor* (KACC 20100), *S. griseus* (KACC 20084 and KACC 20731), *S. indigoferus* (MJM 8645), *S. peucetius* (KCTC 9038 and KCTC 9199), *S. rimosus* (KACC 20082 and KACC 21078), *S. rubrolavendulae* (MJM 4426), *S. scabiei* (KACC 20135, KACC 20200 and KACC 20227), and *S. virginiae* (KCTC 1747 and KACC 14680).These strains were obtained from the Korean Agricultural Culture Collection (KACC, Korea) and the Korean Collection for Type Cultures (KCTC, Korea). *S. indigoferus* MJM8645 and *S. rubrolavendulae* MJM4426, which were isolated from soil and identified by 16S rRNA sequence alignment, were obtained from Bioscience and Bioinformatics, Myongji University, Korea.

Culture Condition

All *Streptomyces* strains were cultured on yeast malt agar (YMA; 4 g yeast extract, 10 g malt extract, 4 g glucose, and 20 g agar per liter) plates for 3 days at 28°C. One piece of agar (6 mm) containing *Streptomyces* was transferred into 50 ml of yeast malt broth (YMB) in a 250 ml Erlenmeyer flask and maintained at 28°C for 3 days with shaking. A 4 ml aliquot of this culture was added to 80 ml of GSS broth in a 250 ml baffled flask and incubated for 10 days at 28°C for high production of metabolites.

For the antibacterial assay, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was used. Optimal growth of *Xoo* is reportedly on yeast extract glucose carbonate (YGC) agar containing 5 g yeast extract, 10 g glucose, 30 g calcium carbonate, and 15 g agar per liter. *Xoo* was grown on YGC agar at 28°C for 2 days and identified on the basis of its morphology. One bacterial colony was transferred into 5 ml of NB in a 13 ml tube and further incubated at 28°C with shaking at 200 rpm for 24 h.

Extraction Conditions

The culture broth of *Streptomyces* was centrifuged at 4°C and 5,000 rpm for 10 min, and the supernatant removed. The remaining pellets were washed 3 times with 20 ml of phosphate-buffered saline. Twenty milliliters of methanol was added to the washed pellets, which were then sonicated for 20 min and extracted for 9 h at 28°C and 200 rpm in a shaker incubator. The extract solution was evaporated using a speed-vacuum machine (Biotron, Seoul, Korea)

UPLC-Q-TOF-MS Analysis

A UPLC Acquity System (Waters, Milford, MA, USA) equipped with a binary solvent delivery system, a UV detector, and an autosampler was combined with a Waters Q-TOF Premier MS system (Micromass MS Technologies, Manchester, UK). An Acquity UPLC BEH C18 column (100 × 2.1 mm i.d. × 1.7 µm particle size; Waters) was used and the column oven temperature was set at 37°C. The mobile phase consisted of water (A) and acetonitrile (B) with 0.1% formic acid (v/v) at a flow rate of 0.3 ml/min. The initial condition was 5% solvent B for 1 min followed by a linear gradient over 10 min, ending at 100% solvent B. The full-scan mass spectral range was 100–1,000 *m/z*. MS was performed with the following conditions: ion source temperature at 100°C; desolvation gas flow at 300 l/h; cone gas flow at 0 l/h; cone voltage at 40 V; and capillary voltage at 2.5 V.

LC-ESI-MS/MS Analysis

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed using a Varian 500-MS ion trap mass spectrometer (Varian, Palo Alto, CA, USA), which consisted of an LC binary pump (Varian 212), a photodiode array detector (Prostar 335), and an autosampler (Prostar 410). The LC system was equipped with a Varian PurSuit XRs C18 column (100 × 2.1 mm i.d. × 3 µm particle size). Mobile phases consisted of water (A) and acetonitrile (B) with 0.1% formic acid (v/v). The initial mobile phase consisted of 90% A and 10% B, which was maintained for 2 min, followed by an increase to 90% B over 25 min, which was maintained at 90% B for 5 min, and then

rapidly decreased to 10% B, which was maintained for 5 min. The flow rate was set to 0.2 ml/min and 10 µl of sample was injected. The full-scan mass spectral range was 100–1,000 *m/z*. The running parameters for analyzing the samples were as follows: spray needle voltage, 5 kV; capillary voltage, 80 V; drying temperature, 300°C; drying gas (nitrogen) pressure, 20 psi; nebulizer gas (air) pressure, 40 psi. Tandem mass spectrometry analysis was carried out using scan-type turbo data-dependent scanning (DDS) under the same conditions. LC-ESI-MS/MS data were analyzed using the MS workstation software (ver. 6.9; Varian, USA).

Data Processing and Multivariate Statistical Analysis

The UPLC-Q-TOF-MS data were acquired with MassLynx software (ver. 4.1; Waters), and raw files were converted to a NetCDF file (*.cdf). After conversion, peak extraction, retention-time correction, and alignment were performed using the metAlign software package (http://www.metalign.nl). After analysis, the resulting data file (*.csv) was then transferred to a Microsoft Excel data sheet for sequential multivariate analysis. Multivariate statistical analysis was performed using the SIMCA-P+ ver. 12.0 (Umetrics, Umea, Sweden). Principal component analysis, partial least squares discriminant analysis (PLS-DA), and hierarchical clustering analysis (HCA) were employed to determine the distribution of *Streptomyces* strains. Numerical value of R2X and Q2X showed statistical significance and explained variation in the PLS model. R2X was a fraction of the Sum of Squares (SS) of the entire X explained by the current component. Variables grouped by PLS-DA and HCA, which are significantly different metabolites separating *Streptomyces* strains, was determined on the basis of the variable importance of the projection (VIP) value (VIP > 0.7) and *p*-value ($p < 0.05$). The *p*-values of different metabolite-based cluster groups were determined using STATISTICA (ver. 7.0; StatSoft, Tulsa, OK, USA).

Phylogenetic Analysis

The 16S ribosomal RNA (rRNA) sequences of 14 strains of *Streptomyces* spp. were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The 16S rRNA sequences were aligned and analyzed by MEGA ver. 4.0 (The Biodesign Institute, Tempe, AZ, USA), and a phylogenetic tree for the datasets was inferred from the neighbor-joining method.

Antibacterial Activity and Isolation of Active Compound

The modified broth microdilution method [5, 26] was used to screen *Streptomyces* for antibacterial activity. The optical density (OD) value of *Xoo* culture medium was adjusted to 0.5 at UV 600 nm by a GENESYS 6 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted 1:20 (v/v) in NB medium, and then the diluted inoculum (190 µl) was seeded in each well of a 96-well plate. For screening of antibacterial activity, each well was treated with 10 µl of crude *Streptomyces* extract (1,000 µg/ml). The plates were incubated at 28°C for 24 h, and the OD of the bacteria was measured at 570 nm using an EL808 Microplate Reader (BioTek, Winooski, VT, USA).

The MIC (minimum inhibitory concentration) values of *S. rimosus* (KACC 20082 and KACC 21078), which showed high antibacterial activity, were measured to confirm the antibacterial activity compared with the positive control, DAPG [19, 44]. *S. rimosus* extract was serially diluted 2-fold to obtain a final sample concentration ranging from 2 to 500 µg/ml, and incubated for 24 h at 28°C; the OD was measured before and after incubation. The MIC of oxytetracycline, which was tentatively considered an active compound, was also determined. The MIC was defined as the lowest concentration of test sample resulting in 90% growth inhibition. All experiments were carried out in triplicate. The MIC value was calculated using the following formula:

$$\text{Increase in bacterial growth (\%)} = \left[1 - \frac{(C_{24} - C_0) - (T_{24} - T_0)}{(C_{24} - C_0)}\right] \times 100$$

where C_{24} = control (not treated), incubated for 24 h; C_0 = control (not treated) at 0 h; T_{24} = treated, incubated for 24 h; and T_0 = treated at 0 h.

We performed preparative high-performance liquid chromatography (prep-HPLC) with a YMC-Pack Pro C18 reversed-phase column (250 × 4.6 mm i.d. × 5 µm particle size) to identify active compounds. All fractions were assayed for antibacterial activity by MIC and active fractions were analyzed by UPLC-Q-TOF-MS and LC-ESI-MS/MS. Putative active compounds were identified on the basis of comparative analysis of the mass spectrum, molecular weight, molecular formula, and UV absorbance data, relative to those of standard compounds.

Results

Comparison of Metabolite-Based Taxonomy and Phylogenetic Taxonomy Studies of *Streptomyces*

16S rRNA sequences of the 8 *Streptomyces* species (14 strains) were determined and compared by MEGA4.0 software (Fig. 1A). The phylogenetic tree obtained from the 14 *Streptomyces* strains was mainly divided into two characteristic branches. The first branch consisted of *S. rimosus*, *S. coelicolor*, and *S. rubrolavendulae* (bootstrap value 97%), and they were divided into two groups: *S. rimosus* group, bootstrap value 100%; and *S. coelicolor* and *S. rubrolavendulae* group, bootstrap value 87%. The second branch included *S. indigoferus*, *S. scabiei*, *S. griseus*, *S. peucetius*, and *S. virginiae*. They were divided in two parts, *S. indigoferus* and *S. scabiei* (bootstrap value 84%), and *S. griseus*, *S. peucetius*, and *S. virginiae* (bootstrap value 90%). Each part was again further subdivided. In general, most strains were well separated by grouping under each species.

The HCA dendrogram constructed by secondary metabolites analyzed by UPLC-Q-TOF-MS is shown in Fig. 1B. The

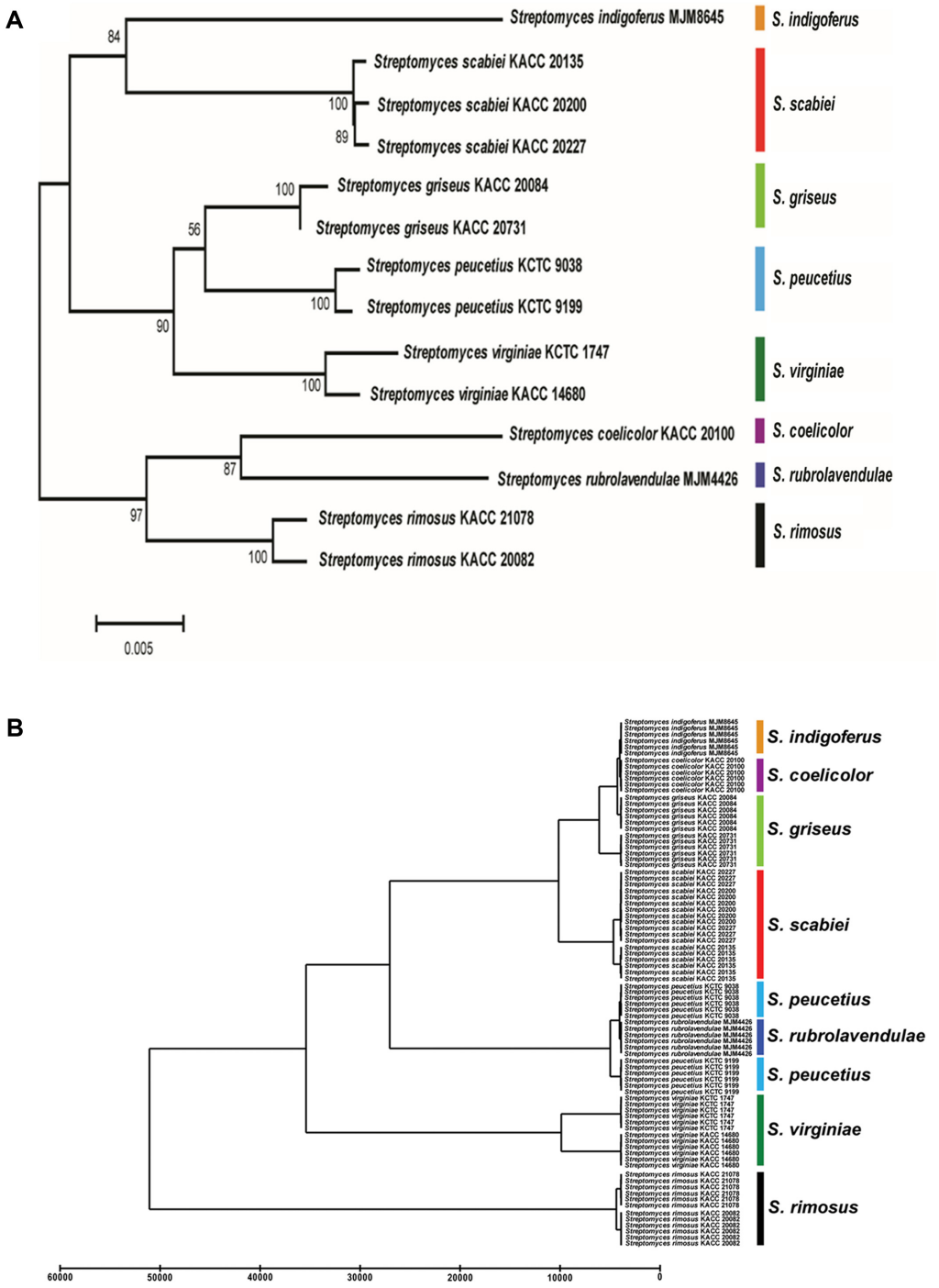

Fig. 1. Taxonomy studies of the 14 *Streptomyces* strains.

(**A**) Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences. The numbers at the branching points are the percentages of occurrence in 1,000 bootstrapped trees. The bar indicates a distance of 0.005 substitutions per site. (**B**) Metabolite-based hierarchical clustering analysis (HCA) of *Streptomyces* strains analyzed by UPLC-Q-TOF-MS.

dendrogram was divided into two branches: S. *rimosus* and other seven species. Other species were divided into two characteristic branches: *S. virginiae* and other species (*S. indigoferus*, *S. coelicolor*, *S. griseus*, *S. scabiei*, *S. peucetius*, and *S. rubrolavendulae*). These six species were again divided into two sub-branches. The first sub-branch includes

S. indigoferus, *S. coelicolor*, *S. griseus*, and *S. scabiei*. The second sub-branch includes *S. peucetius* and *S. rubrolavendulae*. Both dendrograms were typically well separated for each species.

Secondary Metabolite-Based Separation of *Streptomyces* and Identification of Species-Specific Metabolites

The metabolites of the eight *Streptomyces* species (14 strains) were analyzed by UPLC-Q-TOF-MS, and each species was separated by PLS-DA (Fig. 2). The PLS1 and PLS3 scores were found to be 11.0% and 7.2% of the total variation, respectively, as observed in the PLS-DA model (R2X = 0.567, R2Y = 0.985, Q2Y = 0.972). In particular, *S. rimosus* and other strains were significantly classified as PLS1. Metabolites selected by VIP > 0.7 and p-value < 0.05 were determined as the potential variables that separated each species in the PLS-DA model. Selected metabolites were tentatively identified by molecular weight, elemental composition, and mDa using UPLC-Q-TOF-MS software, and MS^n fragmentation and UV λ_{max}(nm) analyzed by LC–ESI-MS/MS were used to support the identification. Twenty-one metabolites such as oxytetracycline (**3**), altamycin A (**4**), 2-hydroxystaurosporinone (**5**), USF-19A (**7**), staurosporine (**9**), rimocidin (**13**), strevertene A (**14**), AN-201 III (**20**), and 13 unidentified metabolites were detected (Table 1). The

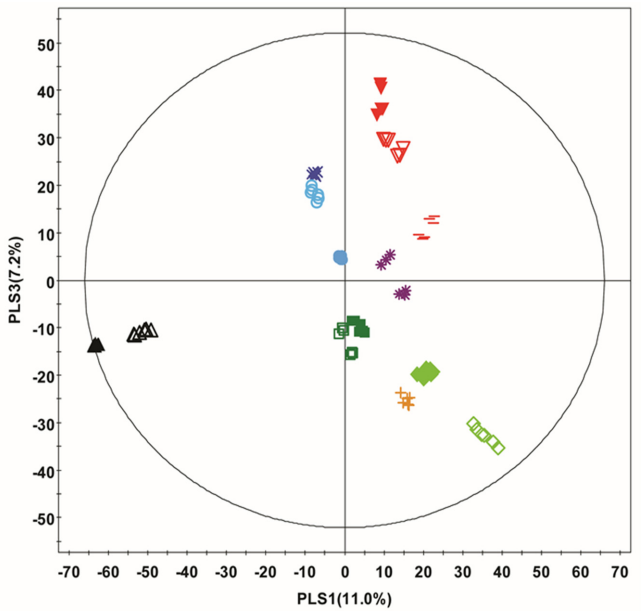

Fig. 2. PLS-DA score plot derived from the UPLC-Q-TOF-MS dataset of *Streptomyces* spp.

Identical species are represented by the same color. *S. coelicolor*: ✳ 20100; *S. griseus*: ■ 1747, □ 14680; *S. indigoferus*: ✚ 8645; *S. peucetius*: ● 9038, ○ 9199; *S. rimosus*: ▲ 20082, △ 21078; *S. rubrolavendulae*: X 4426; *S. scabiei*: ▼ 20200, ▽ 20227, – 20135; *S. virginiae*: ◆ 20731 ◇ 20084.

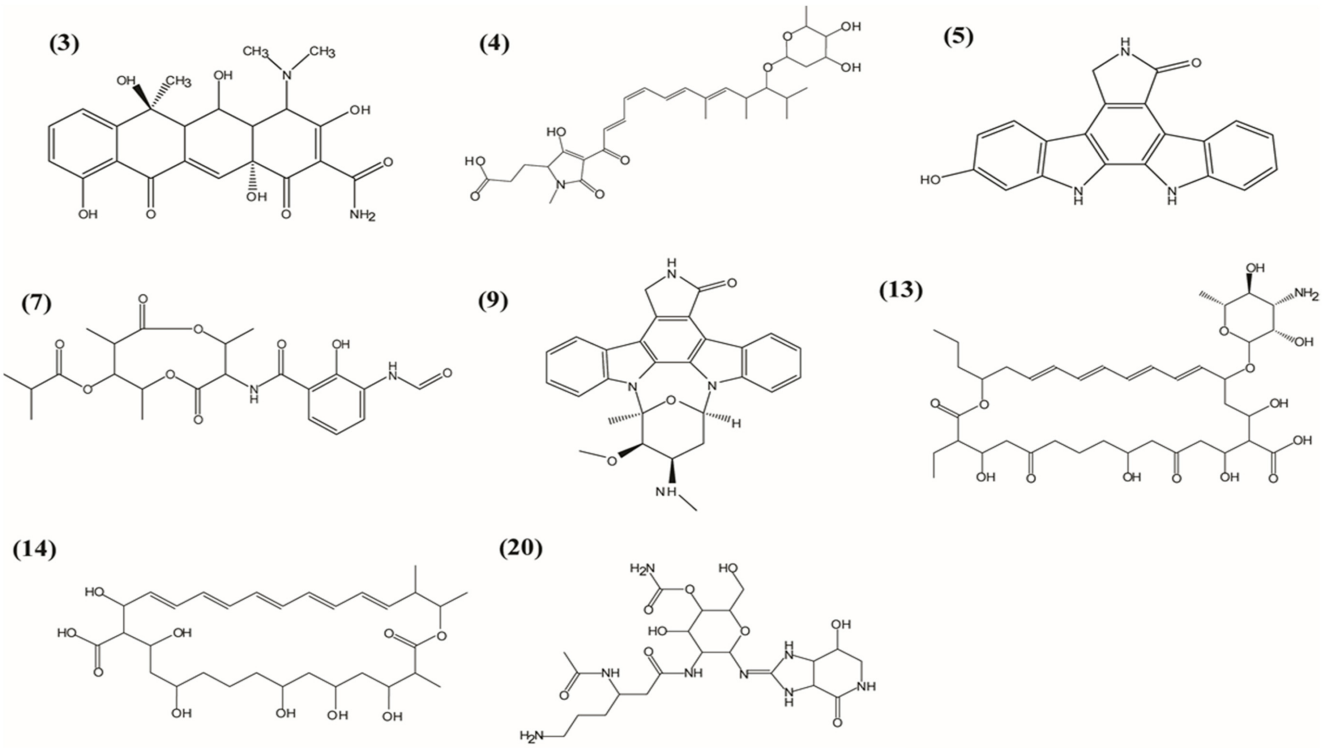

Fig. 3. Structures of significantly different metabolites among *Streptomyces* spp.

The numbers of the compounds in parentheses are the same as those referred to in Tables 1 and 2.

structures of the tentatively identified metabolites are shown in Fig. 3. As shown in Table 2, 2-hydroxystaurosporinone (**5**), staurosporine (**9**), and three unidentified metabolites (**1**, **8**, **17**) were found in *S. rubrolavendulae*, whereas oxytetracycline (**3**), altamycin (**4**), rimocidin (**13**), and four unidentified metabolites (**2**, **15**, **16**, **19**) were detected in *S. rimosus*. Furthermore, three unidentified metabolites (**10**, **11**, **12**) were *S. scabiei*-specific metabolites, and strevertene A (**14**) and unidentified metabolites (**21**) were from *S. virginiae*.

Antibacterial Activity of *Streptomyces* spp. and Their Active Compounds

Crude extracts of the 14 *Streptomyces* strains were tested for antibacterial activity against *Xoo*. As shown in Table 3, *S. rimosus* (KACC 20082 and KACC 21078) and *S. scabiei* (KACC 20200) displayed the highest relative antibacterial activity, whereas the other strains showed less activity or no activity. To confirm these antibacterial activities, we measured the MIC and IC_{50} values for *S. rimosus* KACC 20082 and KACC 21078 (Table 4). The IC_{50} value was calculated as the concentration that caused 50% growth inhibition of *Xoo*. The IC_{50} values of *S. rimosus* strains KACC 20082 and KACC 21078 were 4.09 μg/ml and 3.00 μg/ml, respectively, whereas that of the positive control DAPG was 6.37 μg/ml.

To isolate the active compounds from *S. rimosus*, we performed prep-HPLC analysis. The 12–14 (min) fractions by prep-HPLC exhibited the greatest suppression of *Xoo* growth (Fig. S1) at more than 100% inhibition. These active fractions were further analyzed by UPLC-Q-TOF-MS and LC-ESI-MS/MS to identify the active compounds. As a result, the active compound was identified as an oxytetracycline by its molecular weight, elemental composition, MS^n fragmentation, and UV λ_{max} (nm) as compared with a standard compound. The MIC and IC_{50} of oxytetracycline were 0.08 μg/ml and 0.04 μg/ml, respectively (Table 4).

Table 1. Tentative identification of significantly different metabolites of *Streptomyces* spp.

No.	Tentative metabolite[a]	UPLC-Q-TOF-MS						LC-ESI-MS/MS		Reference
		RT[b] (min)	Experimental mass (*m/z*)		MW[c]	MF[d]	mDa[e]	MS^n fragment ion (*m/z*)	UV λmax (nm)	
			$[M-H]^-$	$[M+H]^+$						
1	N. I.[f]	3.25	447.2078	449.2237	488	-	-	449>413,389>353	211	-
2	N. I.	3.65	444.1740	446.1902	445	-	-	-	-	-
3	Oxytetracycline	3.83	459.1410	461.1569	460	$C_{22}H_{24}N_2O_9$	0.9	461>426>337	202, 225, 268, 356	[28]
4	Altamycin A	4.13	560.2764	562.2972	561	$C_{30}H_{43}NO_9$	-1.7	-	-	[4]
5	2-Hydroxystaurosporinone	4.57	326.0954	328.1107	327	$C_{20}H_{13}N_3O_2$	-0.9	-	-	[14]
6	N. I.	4.41	-	265.1002	-	-	-	-	-	-
7	USF-19A	4.74	463.1647	465.1896	464	$C_{22}H_{28}N_2O_9$	0.6	-	-	[36]
8	N. I.	4.97	570.3332	572.3502	571	-	-	-	-	-
9	Staurosporine	5.13	511.1997[g]	467.2180	466	$C_{28}H_{26}N_4O_3$	0.3	467>338>295	245, 291, 333, 353, 370	[39]
10	N. I.	5.50	-	210.0589	-	-	-	-	-	-
11	N. I.	5.65	-	823.4517	-	-	-	823>476>391	209, 221	-
12	N. I.	5.89	-	190.0345	-	-	-	-	-	-
13	Rimocidin	6.03	766.3915	768.4174	767	$C_{39}H_{61}NO_{14}$	-0.1	766>704>523[h]	281, 304	[16]
14	Strevertene A	6.26	579.3109	581.3324	580	$C_{31}H_{48}O_{10}$	-0.2	-	-	[34]
15	N. I.	6.55	581.2494	583.2501	582	-	-	583>297>269	207, 217, 225	-
16	N. I.	6.71	621.3182	645.3199[i]	622	-	-	-	-	-
17	N. I.	6.75	-	399.0647	-	-	-	-	-	-
18	N. I.	6.89	-	285.0724	-	-	-	-	-	-
19	N. I.	6.91	-	551.2975	-		-	-	-	-
20	AN-201III	7.19	543.2526	567.2515[i]	544	$C_{21}H_{36}N_8O_9$	0.8	-	-	[2]
21	N. I.	8.99	535.3471	559.3349[i]	536	-	-	-	-	-

[a]Identified metabolites based on variable importance projection (VIP) analysis with a cut-off value of 0.7 and a *p*-value < 0.05; [b]Retention time; [c]MW, Molecular weight; [d]MF, Molecular formula; [e]mDa stands for error in milliDaltons; [f]N.I., Not identified; [g]$[M+FA-H]^-$; [h]Negative mode; [i]$[M+Na]^+$

Table 2. Production of species-specific secondary metabolites among *Streptomyces* spp. analyzed by UPLC-Q-TOF-MS in positive mode.

No.	Tentative metabolite	*S. indigoferus*	*S. coelicolor*	*S. griseus*		*S. scabiei*			*S. peucetius*		*S. rubrolavendulae*	*S. virginiae*		*S. rimosus*	
		MJM 8645	KACC 20100	KACC 20084	KACC 20731	KACC 20135	KACC 20200	KACC 20227	KCTC 9038	KCTC 9199	MJM 4426	KCTC 1747	KACC 14680	KACC 20082	KACC 21078
1	N. I.	-	-	-	-	-	-	-	-	-	++	-	-	-	-
2	N. I.	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++
3	Oxytetracycline	-	-	-	-	-	-	-	-	-	-	-	-	+++	++
4	Altamycin A	-	-	-	-	-	-	-	-	-	-	-	-	++	-
5	2-Hydroxystaurosporinone	-	-	-	-	-	-	-	-	-	++	-	-	-	-
6	N. I.	+++	+++	+++	++	+++	++	+++	+++	+++	+++	+	+++	+	+
7	USF-19A	++	-	+	-	+	-	-	-	+	-	+	++	++	+
8	N. I.	-	-	-	-	-	-	-	-	-	+	-	-	-	-
9	Staurosporine	-	-	-	-	-	-	-	-	-	+++	-	-	-	-
10	N. I.	-	-	-	-	-	+++	++	-	-	-	-	-	-	-
11	N. I.	-	-	-	-	-	+++	++	-	-	-	-	-	-	-
12	N. I.	-	-	-	-	-	++	++	-	-	-	-	-	-	-
13	Rimocidin	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++
14	Strevertene A	-	-	-	-	-	-	-	-	-	-	-	++	-	-
15	N. I.	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++
16	N. I.	-	-	-	-	-	-	-	-	-	-	-	-	++	+++
17	N. I.	-	-	-	-	-	-	-	-	-	+++	-	-	-	-
18	N. I.	-	-	-	-	-	+	-	-	+++	-	-	-	-	-
19	N. I.	-	-	-	-	-	-	-	-	-	-	-	-	-	++
20	AN-201III	-	+	-	++	-	++	++	-	++	++	+	-	-	-
21	N. I.	-	-	-	-	-	-	-	-	-	-	+	++	-	-

The production of each metabolite was calculated by the peak area from an UPLC-Q-TOF-MS chromatogram.
+: >0.5 count, ++: >10 count, +++: >100 count, -: <0.5 count or not detected.

Discussion

In this study, we sought to classify *Streptomyces* species based on their secondary metabolites. The metabolite-based and 16S rRNA-based dendrograms exhibited the same patterns of species discrimination. In particular, *S. rimosus* was separated from other species by PLS1 and PLS3 (Fig. 2). To distinguish each species on the basis of species-specific metabolites, the PLS-DA model was used and 21 metabolites such as oxytetracycline (**3**), altamycin A (**4**) 2-hydroxystaurosporinone (**5**), staurosporine (**9**), rimocidin (**13**), strevertene A (**14**), AN-201III (**20**), and 14 unidentified metabolites were tentatively identified. 2-Hydroxystaurosporinone (**5**), staurosporine (**9**), and two unidentified metabolites (**1**, **17**) were detected as *S. rubrolavendulae*-specific metabolites. 2-Hydroxystaurosporinone and staurosporine are alkaloid metabolites [39, 42], and 2-hydroxystaurosporinone (**5**) is derived from staurosporine [14, 29]. Alkaloid groups are produced by many kinds of organisms, including fungi, bacteria, animals, and plants [24]. Several *Streptomyces* species are known to produce antibiotics and related alkaloid compounds. Staurosporine is a *bis*-indole alkaloid originally isolated in 1977 from *S. staurosporeus*. This member of the indolocarbazole alkaloid family has several well-known biological activities, due to its potential as an anticancer drug [48]. Staurosporine has been shown to inhibit protein kinases to control ATP binding to the kinases [60], and it affects cell viability by participating in apoptosis [9]. The *S. virginiae*-specific metabolites were strevertene A (**14**) and unidentified compound (**21**) (Table 2). Strevertene A is a polyketide compound that was isolated from fermentation products of *Streptomyces* spp. [52], and was detected in one strain of *S. virginiae* (KACC 14680) in the present study (Table 2). Oxytetracycline (**3**), altamycin

Table 3. *Streptomyces* strains and their antibacterial activity against *Xoo*.

Streptomyces species	Strains No.[a]	Antibacterial activity of *Xoo*[b]
S. indigoferus	MJM 8645	-
S. coelicolor	KACC 20100	-
S. griseus	KACC 20084	-
	KACC 20731	-
S. scabiei	KACC 20135	-
	KACC 20200	+
	KACC 20227	-
S. peucetius	KCTC 9038	-
	KCTC 9199	-
S. rubrolavendulae	MJM 4426	-
S. virginiae	KCTC 1747	-
	KACC 14680	-
S. rimosus	KACC 20082	++
	KACC 21078	++

[a]KACC, Korean Agricultural Culture Collection.
KCTC, Korean Collection for Type Culture.
MJM, Collection of Myoungji University.
[b]Inhibition % of *Xoo* growth: -, <20; +, >50; ++, >90.
Xoo: *Xanthomonas oryzae* pv. *oryzae*.

(**4**), and rimocidin (**13**) were identified as *S. rimosus*-specific metabolites. These are tetraene-related compounds that contain a polyketide group and four carbon-carbon double bonds with a large macrolactone ring [46, 54]. Oxytetracycline and rimocidin were isolated from *S. rimosus* species in a previous study [18, 50]. Polyketides are produced by almost all living organisms and are a diverse array of natural products. They are particularly highly produced in *Streptomyces* and have various activities such as antibacterial, antifungal, immunosuppressant, antiparasitic, antitumor, and pharmacological properties [3, 53].

The antimicrobial activities of *Streptomyces* spp. against various microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Aspergillus niger*, *Candida albicans*, *Aspergillus fumigates*, and *Aspergillus flavus* have been previously reported [43, 59]. In the present study, evaluation of the antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* of eight *Streptomyces* species (14 strains) showed that *S. rimosus* had relatively high antibacterial activity (Table 3). To identify the active antibacterial compounds in *S. rimosus*, we performed prep-HPLC analysis and active fractions were analyzed by UPLC-Q-TOF-MS. These results confirmed oxytetracycline as an active compound. Oxytetracycline is a broad-spectrum tetracycline group of antibiotics that

Table 4. MIC and IC_{50} values for antibacterial activity against *Xoo*.

Tested sample	Concentrations (μg/ml)	
	MIC[a]	IC_{50}[b]
S. rimosus KACC 20082	4.48 ± 0.12	3.00 ± 0.28
S. rimosus KACC 21078	7.50 ± 0.16	4.09 ± 0.17
Oxytetracycline	0.08 ± 0.01	0.04 ± 0.15
DAPG	11.01 ± 0.17	6.37 ± 0.29

[a]Minimum Inhibitory Concentration.
[b]Inhibitory concentration at 50%.
MIC and IC_{50} are expressed as average values from three independent experiments.
All values are expressed as the mean ± SD.

interferes with the ability of bacteria to produce essential proteins [47]. Various biological activities of oxytetracycline have previously been reported, including antibacterial [45], anti-acne, and anti-inflammatory effects [15]. In particular, oxytetracycline was mainly used for fire blight management of fruit in America [11]. In this study, eight *Streptomyces* species (14 strains) were classified based on secondary metabolite profiling analyzed by UPLC-Q-TOF-MS. Among them, *S. rimosus* showed the highest antibacterial activity against *Xoo*. As a result of activity-guided prep-HPLC, oxytetracycline was identified as an *S. rimosus*-specific antibacterial metabolite. This research indicates that chemotaxonomic studies are an effective tool to supplement conventional bacterial classification methods as well as for the evaluation of species-specific bioactivity.

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