Screening the Antibacterial Activities of *Streptomyces* Extracts against Phytopathogens *Xanthomonas oryzae* pathovar *oryzae*, *Xanthomonas campestris* pathovar *vesicatoria*, and *Pectobacterium carotovorum* pathovar *carotovorum*

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**Abstract** *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), *X. campestris* pv. *vesicatoria* (*Xcv*), and *Pectobacterium carotovorum* pv. *carotovorum* (*Pcc*) are the causative agents of bacterial blight in rice, bacterial spot in pepper, and bacterial soft rot in carrot and cabbage, respectively. To isolate novel microbial extracts with antimicrobial activities against these bacteria, approximately 5,300 different *Streptomyces* extracts were prepared and tested. Microbial cultures from various *Streptomyces* strains isolated from the Jeju Island, Baekam, Mankyoung river, Jiri mountain etc. in Korea were extracted into three different factions -secreted hydrophobic, secreted hydrophilic, and mycelia- using ethyl acetate, water, and methanol. Initially, 34, 29, and 10 extracts were selected as having antibacterial activities against *Xoo*, *Xcv*, and *Pcc*, respectively. Extracts 1169G4, 1172E9, and 1172E10 had the highest growth inhibition activities against both *Xoo* and *Xcv*; and extracts 1151H7 and 1152H7 showed the highest growth inhibition activities against *Pcc*.

**Keywords** antimicrobial activity · *Pectobacterium carotovorum* pathovar *carotovorum* · *Streptomyces* · *Xanthomonas campestris* pathovar *vesicatoria* · *Xanthomonas oryzae* pathovar *oryzae*

**Introduction**

Filamentous bacteria belonging to the genus *Streptomyces* produce over 50% of the antibiotics discovered to date (Taddei et al., 2006; Jayapal et al., 2007). *Streptomyces* bacteria and other Actinomycetes produce a diverse array of antibiotics, including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrodides, nucleosides, peptides, polyenes, polyethers, and tetracyclines (Mellouli et al., 2003).

Rice, pepper, and cabbage are the first, second, and third most cultivated crops, respectively, in Korea. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. campestris* pv. *vesicatoria* (*Xcv*) are the causal agents of bacterial blight in rice and bacterial spot in pepper, respectively (Yang et al., 2005; Adhikari et al., 2010). *Pectobacterium carotovorum* pv. *carotovorum* (*Pcc*) causes soft rot in a wide range of plants, including cabbage (Mole et al., 2010). Because these bacteria cause severe crop damage and reduce yield, prevention and treatment of these diseases have been the focus of intense research in both the academy and industry (Khush, 1997). In 2005, the whole genomes of *Xoo* and *Xcv* were sequenced (Frank Thieme, 2005; Lee et al., 2005). The emergence of resistance against *Pcc* was reported in 2007 (T. Luzzatto, 2007).

Chemical pesticides are routinely used to control plant pathogens in agricultural fields. However, recently, indiscriminate usage of...
chemical pesticides has raised many concerns, including environmental toxicity (Vivek K. Bajpai, 2010) and the possibility of generating antibiotic-resistant microbes. Therefore, biopesticides made from secondary metabolites from microbes or plants are being preferred over chemical pesticides.

The main objective of this study was to isolate novel antimicrobial Streptomyces extracts against the phytopathogens mentioned. Streptomyces species are ubiquitous in the soil, and play an important ecological role in the turnover of organic material. Furthermore, the diverse secondary metabolites produced by Streptomyces species are good sources of novel bioactive compounds. To date, more than half of the world’s antibiotics (over 50 different antibiotics) have been isolated from Streptomyces. Furthermore, the diverse secondary metabolites produced by Streptomyces species are good sources of novel bioactive compounds. To date, more than half of the world’s antibiotics (over 50 different antibiotics) have been isolated from Streptomyces. In this study, we prepared and screened 5,328 Streptomyces extracts, and tested their antimicrobial activities against Xoo, Xcv, and Pcc.

Materials and Methods

Strains and Culture Conditions. X. oryzae pv. oryzae KACC10331 (Xoo), X. campestris pv. vescicatoria KACC11127 (Xcv) and P. carotovorum pv. carotovorum KACC10227 (Pcc) were provided by the Korea Agriculture Culture Collection (KACC). Xoo, Xcv, and Pcc were grown in YPD (1.0% yeast extract, 2.0% peptone, and 0.1% yeast extract, 0.2% bacto-peptone, and 0.1% beef extract), or DYC medium (2.5% dextrin, 1.2% dry yeast, 2.0% corn starch liqueur, 0.1% NaBr, and 0.1% CoCl2 at pH 7.0). The dried extract was dissolved in 0.85% NaCl solution and serially diluted. The diluted soil suspension were spread on Humic acid- vitamin (HV) agar medium (0.1% humic acid, 0.05% Na3HPO4, 0.071% KCl, 0.005% MgSO4·7H2O, 0.001% FeSO4·7H2O, 0.002% CaCO3, 0.2% B-Vitamin solution, 1.8% agar at pH 7.2) and incubated at 28°C for 14 days. The colony with representative actinomycetes were selected and identified by 16S rDNA sequence. Then Streptomyces strains were cultured in three different media: GSS medium for nutrient-rich condition, Bennett’s medium for moderate-nutrition condition, and DYC medium for nutrient-poor condition. Three extract fractions were prepared from each Streptomyces culture broth. 30 mL of the culture broth were centrifuged at 5,000×g for 10 min. Mycelia were collected as a pellet, and extracted with 3 mL of methanol. This fraction was called “mycelia isolated from supernatant (MIS)”. After pelleting the mycelia, the supernatant was further extracted with 30 mL of ethyl acetate and dried. The resulting dried extract was dissolved in 3 mL of methanol. This was called the “secreted hydrophobic (SHB) fraction”. The remaining ethyl acetate-insoluble fraction was called the “secreted hydrophilic (SHL) fraction”. Thirty microliter of each fraction was distributed into 96-well plate and dried completely before further use. The dry weights were measured to enable accurate calculation of GI50 (dose required to cause 50% growth inhibition).

Preparation of the Streptomyces extracts. For preparation of Streptomyces extracts library, one gram of soil was suspended in 10 milliliter of 0.85% NaCl solution and serially diluted.

| Table 1 | Antibacterial extracts screened in the first and second round against Xoo, Xcv, and Pcc |
|---------|-----------------------------------------------|
| SHL |
| 1st |
| 11764A, 1176D7, 1175A4, 1173E4, 1172E10, 1169H4, 1169G4, 1159B10, 1149F4, 1139G10, 1136G7, 1124A7, 1120F10, 1119F10, 1119C10, 1117E4 | 1175A4, 1169G4, 1169E10, 1144H7, 1144A7, 1141H7, 1141A10, 1133H10, 1132H7, 1131H4, 1128H4, 1128C4, 1128B4, 1121F10, 1120F10, 1117E10 | 1153E7, 1152H7, 1151H7, 1158G7 |
| 2nd |
| 1175A3, 1173E3, 1172E9, 1169G3, 1164E6, 1161F3, 1161E3, 1160D3, 1159B9, 1158A6, 1149F3, 1144B6, 1135H3, 1120F9, 1119C6, 1117E3 | 1172E9, 1152H3, 1144A6, 1141H6, 1132H6, 1131H3, 1128H6, 1128D3, 1128C6, 1128A3 | | 1158H6, 1158G6, 1157A6, 1155E9 |
| MIS |
| 1172E10, 1169G4 | 1172E10, 1169G4 | 1152H7, 1151H7 |
| SHB |
| 1st |
| 11764A, 1176D7, 1175A4, 1173E4, 1172E10, 1169H4, 1169G4, 1159B10, 1149F4, 1139G10, 1136G7, 1124A7, 1120F10, 1119F10, 1119C10, 1117E4 | 1175A4, 1169G4, 1169E10, 1144H7, 1144A7, 1141H7, 1141A10, 1133H10, 1132H7, 1131H4, 1128H4, 1128C4, 1128B4, 1121F10, 1120F10, 1117E10 | 1153E7, 1152H7, 1151H7, 1158G7 |
| 2nd |
| 1175A3, 1173E3, 1172E9, 1169G3, 1164E6, 1161F3, 1161E3, 1160D3, 1159B9, 1158A6, 1149F3, 1144B6, 1135H3, 1120F9, 1119C6, 1117E3 | 1172E9, 1152H3, 1144A6, 1141H6, 1132H6, 1131H3, 1128H6, 1128D3, 1128C6, 1128A3 | | 1158H6, 1158G6, 1157A6, 1155E9 |
| MIS |
| 1172E9 | 1172E9 | | 1152H7, 1151H7 |
bacterial culture with 6% dimethylsulfoxide (DMSO), respectively. The *Streptomyces* extracts were dissolved in 40 µL of 50% DMSO, and further diluted in 100 µL of YPD medium. *Xoo*, *Xcv*, and *Pcc* were grown to the early exponential growth stage (absorbance at 600 nm \(A_{600}\) = 0.2), and 160 µL of each bacterial culture was dispensed into separate 96-well micro-plates (Nunc\®', US). Then, 40 µL of previously diluted *Streptomyces* extract was added to each well. The cultures were grown at 28°C with continuous shaking at 700 rpm. The \(A_{600}\) values of the wells in the culture plates were measured every 4 h for 20 h using a multimode micro-plate reader (Infinite® M200 PRO, TECAN, Switzerland).

To measure the antibacterial activity of each extract, we calculated the growth inhibition rate (GIR) at 20 h for *Xoo* and *Xcv* and at 12 h for *Pcc* using the following formula:

\[
GIR = \frac{1 - CB}{AB} \times 100
\]

where \(A\) was the \(A_{600}\) value of the bacterial culture with 6% DMSO; \(B = 0.2\), which was the \(A_{600}\) value of the bacterial culture when the extract was added; and \(C\) was the \(A_{600}\) value of the bacterial culture after treatment with the extract. For the second growth inhibition screen, the extracts selected from the first assay were tested three times using the same procedure.

The amount of growth inhibition induced by each extract was measured at five different dosages \((1, 10^{-1}, 10^{-2}, 10^{-3}, \text{ and } 10^{-4}\) dilutions) and compared with the controls. From this, the GI50 was calculated. All the tests were carried out in triplicate.
Results

Preparation of extracts from Streptomyces. Streptomyces strains produce diverse physiological secondary metabolites depending on the culture conditions. To generate various secondary metabolites from each Streptomyces strain, three culture media with different nutritional levels were used to grow the Streptomyces cells. In addition, three extract fractions were prepared for each Streptomyces culture. The MIS was separated from the supernatant; SHBs were extracted from the supernatant fraction using ethyl acetate; and SHLs were obtained from the residual water layer. In total, 5,328 extracts were prepared and screened for their antimicrobial activities against the three plant pathogens, *Xoo*, *Xcv*, and *Pcc*.

First round of screening for antimicrobial activity. Extracts showing more than 50% cell growth inhibition activity were considered antimicrobial extracts; extracts 34, 29, and 10 were selected because they exhibited antimicrobial activity against *Xoo*, *Xcv*, and *Pcc*, respectively. These three extracts constitute 0.64, 0.53, and 0.19% of all the extracts tested in the study (Fig. 1 and Table 1).

Extract 1172H9, the most effective antimicrobial extract against *Xoo*, inhibited 90% of *Xoo* cell growth. Of the top 34 extracts against *Xoo*, two were SHLs, 16 were MISs, and 16 were SHBs (Fig. 2A). Extract 1131H3 exhibited approximately 100% growth-inhibition effect against *Xoo*, *Xcv*, and *Pcc*, respectively. These three extracts constitute 0.64, 0.53, and 0.19% of all the extracts tested in the study (Fig. 1 and Table 1).

Second round of screening for antimicrobial activity. Extracts showing high growth-inhibition activity in the first round of screening were tested three times to confirm their antimicrobial activities. 1169G4 (SHB), 1172E9 (MIS), and 1172E10 (SHB) had the highest growth-inhibition activities against both *Xoo* and *Xcv* (Fig. 3A and 3B). 1172E9 (MIS) and 1172E10 (SHB) are from the same *Streptomyces* strain from Jeju island. 1169G4 (SHB) is also from Jeju island (S-Table 1). 1152H7 (SHB) and 1151H7 (SHB) showed the highest growth-inhibition activity against *Pcc* (Fig. 3C). 1152H7 (SHB) and 1151H7 (SHB) are from Baekam, Gyoungsangbukdo (S-Table 1). 1152H7 (SHB) showed slightly higher antibacterial activity than 1151H7 (SHB). In total, five different extracts -1169G4 (SHB), 1172E9 (MIS), 1172E10 (SHB), 1152H7 (SHB), and 1151H7 (SHB)- were found to possess antibacterial activities against *Xoo*, *Xcv*, and *Pcc*. Interestingly, all the extracts, except 1172E9 (MIS), were SHBs.

The GI50s of the five antimicrobial extracts were measured by serial dilution of the extracts (Table 2). 1172E9 (MIS) and 1172E10 (SHB) showed the highest antibacterial activities against *Xoo* and *Xcv* (Fig. 4 and 5). In particular, 1172E10 (SHB) showed a GI50 value of less than µg·mL\(^{-1}\). 1152H7 (SHB) had a slightly lower GI50 value of less than µg·mL\(^{-1}\).
higher antibacterial activity than 1151H7 (SHB) against *Pcc* (Fig. 6). The antimicrobial activities of all five extracts were less than µg·mL$^{-1}$. 1172E10 (SHB: MJM strain No. 10146GE) had the highest antimicrobial activity against *Xoo* and *Xcv* with GI50s of 0.3 and 0.4 µg·mL$^{-1}$, respectively. 1152H7 (SHB: MJM strain No. 9973DE) had the highest antimicrobial activity against *Pcc*, with a GI50 of 0.5 µg·mL$^{-1}$.

**Discussion**

Antibiotics are secondary metabolites that inhibit the growth of other microbial organisms. Growing incidences antibiotic-resistance among deadly pathogens has increased the need to discover novel antibiotics. Over the last few decades, new technologies for drug discovery have been developed, such as high throughput screening.
and combinatorial chemistry. Moreover, sequencing of pathogen genomes has led to the identification of new drug targets, and many chemical compounds have been synthesized by combinatorial chemistry and screened using cell-free assay systems and high-throughput screening systems. However, most screened compounds do not penetrate the pathogen to reach the target, or are easily degraded in vivo. Despite these technological advances, only five new classes of antibiotics have been introduced for human use (Talbot et al., 2006). Therefore, many studies are now focusing on screening secondary metabolites from microbes, especially Streptomyces, to identify novel antimicrobials. Streptomyces bacteria produce numerous secondary metabolites, and approximately 55% of currently used antibiotics are produced by the genus Streptomyces.

Five Streptomyces extracts showing high antimicrobial activity against Xoo, Xcv, and Pcc were screened. Of the five extracts, four (1152H7, 1151H7, 1169G4, and 1172E10) were secreted and hydrophobic, and only one extract (1172E9) was non-secreted. The secretory and hydrophobic properties of the extracts aid in the penetration of bacterial cell walls (Nikaido H, 1976; Savage et al., 2002). In particular, 1169G4, 1172E9, and 1172E10 were effective against both Xcv and Xoo. The genus Xanthomonas belongs to Proteobacteria, and it causes a variety of diseases in numerous crops. The extracts identified in this study may have applications as a novel antimicrobial drug against Xanthomonas, including Xoo and Xcv.

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References

Adhikari SR, Supakankunti S, and Khan MM (2010) Kala azar in Nepal: estimating the effects of socioeconomic factors on disease incidence. Kathmandu Univ Med J (KUMJ) 8, 73–9.
Frank Thieme RK, Thomas Bekel, Carolin Berger, Jens Boch, Daniela Böttner, Camila Caldana et al. (2005) Insights into Genome Plasticity and Pathogenicity of the Plant Pathogenic Bacterium Xanthomonas campestris pv. vesicatoria Revealed by the Complete Genome Sequence. Journal of Bacteriology 187, 7254–66.
Jayapal KP, Lian W, Glod F, Sherman DH, and Hu WS (2007) Comparative genomic hybridizations reveal absence of large Streptomyces coelicolor genomic islands in Streptomyces lividans. Bmc Genomics 8, 229
N. Huang, E. R. Angeles, J. Domingo, G. Magniant, S. Singh, G. Zhang et al. (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. TAG Theoretical and Applied Genetics 95, 313–20.
Lee BM, Park YJ, Park DS, Kang HW, Kim JG, Song ES et al. (2005) The genome sequence of Xanthomonas oryzae pathovar oryzae KACC10331, the bacterial blight pathogen of rice. Nucleic Acids Res 33, 577–86.
Melloul E, Ben Amen-Mehdi R, Stoud S, Salem M, and Bejar S (2003) Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated Streptomyces sp US24 strain. Research in Microbiology 154, 345–52.
Mole B, Habibi S, Dangl JI, and Grant SR (2010) Glucanote metabolism is required for virulence of the soft-rot pathogen Pectobacterium carotovorum. Mol Plant Microbe Interact 23, 1335–44.
Nikaido H (1976) Outer membrane of Salmonella typhimurium. Transmembrane diffusion of some hydrophobic substances. Biochimica et Biophysica Acta 433, 118–32.
Savage PB, Li C, Taotafa U, Ding B, and Guan Q (2002) Antimicrobial properties of cationic steroid antibiotics. FEBS Microbiol Lett 217, 1–7.
T. Luzzatto MY Yishay, A. Lipsky, A. Ion, E. Belauov, and I. Yedidia (2007) Efficient, long-lasting resistance against the soft rot bacterium Pectobacterium carotovorum in calla lily provided by the plant activator methyl jasmonate. Plant Pathology 56, 692–701.
Taddie A, Rodriguez MJ, Marquez-Vilchez E, and Castelli C (2006) Isolation and identification of Streptomyces spp. from Venezuelan soils: Morphological and biochemical studies. I. Microbiological Research 161, 222–31.
Talbot GH, Bradley J, Edwards JE, Jr., Gilbert D, Scheld M, and Bartlett JG (2006) Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin Infect Dis 42, 657–68.
Vivek K. Bajpai NTD, Hwa-Jin Suh, and Sun Chul Kang (2010) Antibacterial Activity of Essential Oil and Extracts of Cleistocalyx operculatus Buds Against the Bacteria of Xanthomonas spp. Journal of the American Oil Chemists’ Society 87, 1341–9.
Yang W, Sacks EJ, Lewis Ivey ML, Miller SA, and Francis DM (2005) Resistance in Lycopersicon esculentum Intraspecific Crosses to Race T1 Strains of Xanthomonas campestris pv. vesicatoria Causing Bacterial Spot of Tomato. Phytopathology 95, 519–27.