

Occurrence of Antibiotic-resistant Bacteria to Streptomycin and Oxytetracycline in Agricultural Soils of Gangwon State

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Abstract: Soil can be a natural reservoir of antibiotic-resistant bacteria (ARB) and is capable of transferring them to the environment. The spreading of ARB can be affected by geographical factors such as land type and altitude. This study investigated the occurrence of antibiotic-resistant bacteria from agricultural soils in five regions of Gangwon State, South Korea, focusing on two commonly used agricultural antibiotics, streptomycin (ST) and oxytetracycline (OTC). Soil samples were collected from two plane land fields (Cheorwon, Chuncheon) and three high land fields (Gangneung, Jeongseon, Taebaek) for isolation of ARB against ST and OTC. Thirty-four bacterial strains were isolated, showing resistance to ST and OTC. Examination of cross-resistance between the two antibiotics found that some strains resistant to ST also exhibited reduced susceptibility to OTC, and vice versa. Remarkably, the bacterial strains, CW1 and TB7, showed the highest cross-resistance between the two antibiotics and they were from either plane land fields or high land fields. The strains were identified as diverse genera such as *Pseudomonas* sp., *Lysobacter* sp., *Telluria* sp., *Pseudarthrobacter* sp., *Stenotrophomonas* sp., *Microbacterium* sp., and *Flavobacterium* sp. The results in this study infer the potential for the spread of ARB or related genes from high land in sloped fields. Taken together, it should be emphasized that a need for careful monitoring of the occurrence of ARB in different types of fields to protect the environment as well as sustainable agriculture by preventing the further spread of these bacteria and related antibiotic-resistant genes in the environment.

Keywords: Agricultural antibiotics, Antibiotics resistance, High land field, Oxytetracycline, Streptomycin

<https://doi.org/10.5338/KJEA.2025.44.03>

Agric. Environ. Sci. 2025, 44, 21-28

Received: December 9, 2024
Revised: December 17, 2024
Accepted: January 2, 2025
Published: January 15, 2025

Online ISSN: 2233-4173
Print ISSN: 1225-3537



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Introduction

The extensive use of antibiotics in agricultural industries has contributed to protecting crops and livestock from severe pathogens. Simultaneously, it has given rise to the development of antibiotic-resistant bacteria (ARB) and their prevalence in the natural environment. It can lead to the transfer of their antibiotic-resistant genes (ARGs) between bacteria via horizontal gene transfer, which have been considered as emerging contaminants [1]. Over the decades, the detection of ARB and ARGs has been reported in various environments, including wastewater, ground-

water, aquaculture, and agricultural soils with the potential to threaten public health and crop production [2-5].

Soil can be a natural reservoir of ARB and is capable of transferring them to the environment through environmental factors such as runoff and soil erosion. The degradability of residual antibiotics and the dissemination of ARB can be affected by climatic factors such as annual temperature and precipitation [6]. In addition, geographical traits, such as land type and altitude, are important influencing factors in the spread of ARB and ARGs. In other words, the presence of ARB in high land in sloped fields can be a source of nonpoint contamination that is difficult to pinpoint diffuse source and typically spread over large areas. Therefore, basic studies on the monitoring of ARB in diverse types of fields with different geographical and climatic properties are needed for a comprehensive investigation of antibiotic resistance.

In this study, the occurrence of bacterial strains resistant to streptomycin (ST) and oxytetracycline (OTC), two of the most widely used agricultural antibiotics, was investigated from agricultural soils of Gangwon State, South Korea, including plane land fields (Cheorwon, Chuncheon) and high land in sloped fields (Gangneung, Jeongseon, Taebaek). The study highlights the need for close monitoring of ARB in different types of fields to protect the environment as well as sustainable agriculture.

Materials and Methods

Antibiotics

Streptomycin (ST) and oxytetracycline (OTC) were purchased from Sigma-Aldrich (St. Louis, MO), and were of analytical grade. Based on the properties of the chemicals, stock solutions (20,000 mg/L) of the antibiotics were prepared using sterile double-distilled water (SDDW) for ST and dimethyl sulfoxide (DMSO) for OTC (Table 1).

Isolation of antibiotic-resistant bacteria from agricultural soils

To isolate antibiotic-resistant bacteria, agricultural soils were collected from five cities in Gangwon State, including Cheorwon, Chuncheon, Gangneung, Jeongseon, Taebaek (Figure 1). Gangwon State is located in the northern part of South Korea and covered with rocky mountains, therefore, its annual temperature is rather lower than other regions. These unique geographical

Table 1. Selective chemical properties of antibiotics used in this study (<https://pubchem.ncbi.nlm.nih.gov/>)

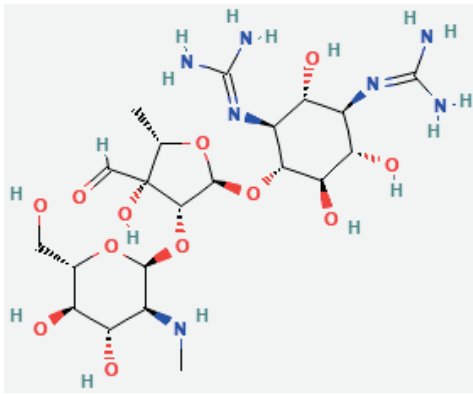
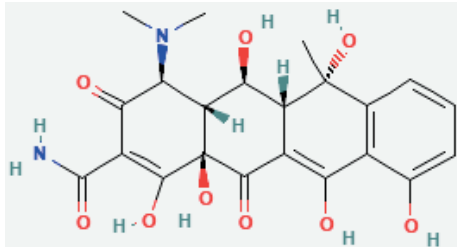
Chemical properties	Streptomycin	Oxytetracycline
Chemical structure		
Chemical formula	C ₂₁ H ₃₉ N ₇ O ₁₂	C ₂₂ H ₂₄ N ₂ O ₉
Molecular weight (g·mol ⁻¹)	581.6	460.4
Water solubility (mg·mL ⁻¹)	very soluble >30	slightly soluble 3.13 × 10 ⁻¹
Melting point (°C)	230	184.5
Vapor pressure (mm Hg) at 25°C	5.8 × 10 ⁻²⁸	9.7 × 10 ⁻²⁵



Figure 1. Soil sampling sites in Gangwon State of South Korea.

and climatic traits have developed crop cultivation in high land in sloped fields. Therefore, Gangneung, Jeongseon, and Taebaek were chosen as representatives for high land fields in Gangwon State. Additionally, Cheorwon and Chuncheon were selected as representatives for plane land fields, which had been used to cultivate paprika and corn, common crops.

Topsoil at a depth of 10 cm was collected and mixed well to homogenized soil sample before enriching cultivation. A 0.2 g (1%, w/v) of each soil sample was resuspended in mineral salt medium (MSM) containing 20 ppm of either ST or OTC, and incubated at 30°C and 150 rpm for 1 hour. Subsequently, the soil suspension was serially diluted to 10^{-2} and 10^{-3} using MSM. The MSM composition was as follows: $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (5.2 g/L), KH_2PO_4 (2.7 g/L), KOH (0.14 g/L), nitrilotriacetic acid (0.2 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.06 g/L), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.18 mg/L), $(\text{NH}_4)_2\text{SO}_4$ (0.1 g/L), and trace metals [4]. A 100 μL aliquot of each diluted sample was spread onto R2A agar medium containing 20 ppm of either ST or OTC, and the plates were incubated at 30°C for 2 days. The colonies that grew in the presence of the antibiotics were repeatedly streaked onto a fresh medium to obtain pure isolates. All isolated strains were stored at -80°C in a freezer for further experiments.

Antibiotic susceptibility testing using the paper disc method

The isolates were incubated in NB liquid medium at 30°C and 150 rpm overnight, and the culture was used as inoculum for the antibiotic susceptibility test at various concentrations ranging from 20 ppm to 100 ppm. The bacterial culture was evenly spread onto R2A agar plates using a sterile swab and left for 5 minutes to allow absorption of the culture into the agar medium. Subsequently, 50 μL of each antibiotic stock solution was dropped onto sterile paper discs ($\varnothing$ 6 mm, Advantec, Tokyo, Japan) placed on the agar surface. The plates were then incubated at 30°C for 2 days. SDDW and DMSO were used as controls for the chemicals, and the experiment was performed in triplicates. Antibiotic resistance was determined by observing the inhibition zones around the paper discs.

Cross-resistance test to antibiotics

Selective isolates showing high resistance to ST were tested cross-resistance to OTC. Parallely, selective isolates showing high resistance to OTC were tested for resistance to ST. The testing was performed as described above. The isolates were categorized based on the maximum concentration at which they grew; Group 1 (100 ppm), Group 2 (80 ppm), Group 3 (40 ppm),

Group 4 (20 ppm), Group 5 (no growth at any concentration).

16S rRNA sequencing for bacterial identification

To identify the selected isolates, genomic DNA was extracted using the Genomic DNA Extraction Kit (Bioneer Co., Daejeon, South Korea). The 16S rRNA genes of the genomic DNA were amplified by polymerase chain reaction (PCR) using universal primers: 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. The PCR reaction was performed using AccuPower® Taq PCR Premix (Bioneer Co.) under the following conditions: denaturation at 95°C for 20 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 1 minute, for a total of 30 cycles. The PCR product of the bacterial 16S rRNA gene was purified using AccuPrep® PCR Purification Kit (Bioneer Co.), and the presence of a 1.4 kb fragment was confirmed through 1% agarose gel electrophoresis. The purified bacterial 16S rRNA gene was sequenced by Macrogen (Daejeon, South Korea). The sequencing data covered up to 700 bp, and microbial identification was performed using BLASTN (blast.ncbi.nlm.nih.gov) on the NCBI database, with more than 98.5% sequence similarity. To ensure the accuracy of the identification, only genus-level classification information was considered, and the 16S rRNA gene sequence was compared with the sequences of type strains using EzBioCloud (<https://www.ezbiocloud.net/>).

Results and Discussion

Antibiotic-resistant bacteria isolated from agricultural soils of Gangwon State

Among the hundreds of colonies, a total of 34 colonies grew faster on MSB medium in the presence of the respective antibiotic. Specifically, 13 and 21 strains were isolated from MSB containing 20 ppm of either ST or OTC (Table 2). The number of isolates varied depending on the types of fields and the maximal resistance concentrations. Eleven strains were isolated from plane land fields, and 23 strains were from high land in sloped fields. Specifically, among the 13 strains resistant to ST, 4 strains were from the soil in plane land fields (CW and CC), and 9 strains were from the soil of high land in sloped fields (GN, JS, TB). Similarly, among the 21 strains resistant to OTC, 7 strains were from plane land fields, and 14 strains were from high land in sloped fields. Although the total number of isolates to resist ST was fewer than those resistant to OTC, the maximal resistance concentration, grouped as G1, was higher for ST. Among ST-resistant isolates, CW1, CC1, CC3, JS4, and TB2 showed growth at all tested concentrations, including 100 ppm, indicating that these strains were able to withstand higher concentrations of the antibiotic. Meanwhile, CC6, TB7, and TB9 showed the highest resistance among OTC-resistant isolates.

There are various influencing factors on the dissemination of ARGs in the environment, including mean annual temperature, precipitation, anthropogenic activities, and geographical properties [6]. Specifically, altitude was strongly and positively related to the abundance of ARGs, showing a higher accumulation of ARGs in cropland of Earth's highest elevated plateau, Qinghai-Tibet Plateau, than in low-longitude regions [7]. Therefore, it should not be ignored that geographical properties of high land fields could distribute ARB itself or their ARGs into diverse environments like fresh water.

The high land in sloped fields collected soil samples in this study, Gangneung, Jeongseon, and Taebaek, are located in mountainous regions with an altitude of 1100 m. The fields had been used for cultivating kimchi cabbage during the summer since the 1960's with repeated use of pesticides, antibiotics, and fertilizer for a long time. Unfortunately, it recently faced climate changes such as longer summer periods with repeated extreme heating and rain, which brought about serious diverse problems in the environment such as soil erosion and runoff. These geographical and climatic conditions can remarkably affect ARGs transfer risks [8]. This suggests that the occurrence of ARB and ARGs in high land fields could be carefully considered to sustain crop cultivation in high land fields and to conserve a sustainable agricultural environment.

Table 2. Bacterial strains with a high resistance against streptomycin and oxytetracycline

Site site	Streptomycin			Oxytetracycline		
	Strain name	Maximal resistance concentration	Group ^a	Strain name	Maximal resistance concentration	Group ^a
Cheorwon	CW1	100	G1	CW2	20	G4
				CW3	40	G3
				CW4	20	G4
Chuncheon	CC1	100	G1	CC4	40	G3
	CC2	20	G4	CC5	40	G3
	CC3	100	G1	CC6	100	G1
				CC7	40	G3
Gangneung	GN1	100	G1	GN3	20	G4
	GN2	80	G2	GN4	20	G4
				GN5	40	G3
				GN6	40	G3
Jeongseon	JS1	20	G4	JS5	20	G4
	JS2	0	G5	JS6	40	G3
	JS3	40	G3	JS7	80	G2
	JS4	100	G1	JS8	20	G4
Taebaek	TB1	20	G4	TB5	40	G3
	TB2	100	G1	TB6	40	G3
	TB3	20	G4	TB7	100	G1
				TB8	40	G3
				TB9	100	G1
				TB10	20	G4

^a Bacteria were divided in to five groups according to their growth in the presence of antibiotic: G1 (100 µg/mL), G2 (80 µg/mL), G3 (40 µg/mL), G4 (20 µg/mL), G5 (no growth at any concentrations).

Determination of cross-resistance against streptomycin and oxytetracycline

The cross-resistance test of the 34 isolates was conducted using varying concentrations of ST and OTC, ranging from 20, 40, 80, and to 100 ppm. The strains were exposed to these concentrations on R2A agar plates, and the inhibition zones were measured to assess the cross-resistance level. Some ST-resistant strains exhibited cross-resistance to OTC, as indicated by smaller or absent inhibition zones at higher concentrations of OTC (Table 3). These strains showed reduced susceptibility to OTC, despite being originally isolated for their resistance to ST. Among the ST-resistant strains, CW1 showed the highest cross-resistance, surviving up to 40 ppm of OTC. However, other strains exhibited minimal (~20 ppm) or no cross-resistance, suggesting that these ST-resistant strains had reduced susceptibility to OTC, which indicates the potential for simultaneous resistance to both antibiotics.

This strong resistance profile in certain strains reflects their ability to survive and proliferate in environments with elevated levels of ST, confirming the persistence of antibiotic resistance. It suggests that these strains can thrive in environments with elevated levels of OTC as well. These results highlight the prevalence of ARB in agricultural environments and underscore the risks associated with the overuse of antibiotics like ST in agricultural practices. Meanwhile, CC6 and TB7 exhibited the highest cross-resistance to 40 ppm of ST (Table 3). The results showed that some OTC-resistant strains also exhibited cross-resistance to ST, as evidenced by smaller inhibition zones at higher concentrations of ST. These strains demonstrated reduced susceptibility to ST, even though they were originally isolated for their resistance to OTC. This suggests that OTC resistance in these strains may be linked to increased resistance to ST, highlighting the potential for antibiotic cross-resistance in agricultural environments.

Table 3. Bacterial strains with a cross resistance between streptomycin and oxytetracycline

No.	Land type	Strain name	Maximal resistance concentration (µg/mL) ^a		Identification	Reference strain ^b
			Streptomycin	Oxytetracycline		
1	Plane land field	CW1	100	40	<i>Pseudomonas</i> sp.	<i>Pseudomonas knackmussii</i>
2		CC1	100	20	<i>Pseudomonas</i> sp.	<i>Pseudomonas ceruminis</i> <i>Pseudomonas tructae</i> <i>Pseudomonas huaxiensis</i> <i>Pseudomonas putida</i> <i>Pseudomonas monteilii</i>
3		CC3	100	20	<i>Pseudomonas</i> sp.	<i>Pseudomonas knackmussii</i>
4		CC6	40	100	<i>Lysobacter</i> sp.	<i>Lysobacter enzymogenes</i> (99.63%)
5	High land in sloped field	GN1	100	0	<i>Pseudomonas</i> sp.	<i>Pseudomonas koreensis</i>
6		GN2	80	0	<i>Pseudomonas</i> sp.	<i>Pseudomonas koreensis</i>
7		GN4	0	20	<i>Telluria</i> sp.	<i>Telluria cellulolytica</i> (99.20%)
8		JS2	0	40	<i>Pseudarthrobacter</i> sp.	<i>Pseudarthrobacter oxydans</i> (99.85%)
9		JS7	0	80	<i>Stenotrophomonas</i> sp.	<i>Stenotrophomonas</i> sp. (99.93%)
10		TB2	100	0	<i>Microbacterium</i> sp.	<i>Microbacterium phyllosphaerae</i>
11		TB7	40	100	<i>Flavobacterium</i> sp.	<i>Flavobacterium chungangensis</i> (98.04%)
12		TB9	20	100	<i>Stenotrophomonas</i> sp.	<i>Stenotrophomonas lacuserhaili</i> (99.57%)

^a Bacteria were divided in to five groups according to their growth in the presence of antibiotic: G1 (100 µg/mL), G2 (80 µg/mL), G3 (40 µg/mL), G4 (20 µg/mL), G5 (no growth at any concentrations).

^b Bacterial strains were identified based on NCBI-Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) results. Percentage (%) of identification and query cover are 100% unless not mentioned.

The findings point to the possibility of cross-resistance between ST and OTC, emphasizing the challenges posed by multidrug-resistant bacteria in agricultural environments. The presence of cross-resistant bacteria against ST and OTC highlights the importance of careful antibiotic application and monitoring of ARB in agricultural environment.

Identification of antibiotic-resistant bacteria

The isolates were identified as *Pseudomonas* sp., *Lysobacter* sp., *Telluria* sp., *Pseudarthrobacter* sp., *Stenotrophomonas* sp., *Microbacterium* sp., and *Flavobacterium* sp. Notably, the strains from high land in sloped fields exhibited different genera compared to those from plain land fields. Generally, *Pseudomonas* sp. and *Stenotrophomonas* sp. are well-known as multidrug-resistant bacteria, and their major antibiotic resistance mechanisms can be classified as three: intrinsic, acquired, and adaptive resistance [9]. Firstly, intrinsic resistance includes having low outer membrane permeability, expressing efflux pumps that expel the drugs out of bacterial cells, and producing antibiotic-inactivating enzymes. Secondly, acquired resistance can be achieved by either horizontal transfer of resistance genes or mutational changes in the bacterial genes. Lastly, adaptive resistance involves the formation of biofilms in the surrounding environment, which can serve as a diffusion barrier to limit antibiotic access to the bacterial cells. These strategies to gain antibiotic resistance would be complicated and depend on bacterial species and their surviving environments. In addition, it is noteworthy that *Telluria* sp. is less commonly associated with antibiotic resistance. Therefore, it can be assumed that this bacterial species may acquire ARGs from the environment by horizontal gene transfer. However, further investigation on antibiotic resistance mechanism and the core genome of *Telluria* sp. would be needed to support this hypothesis.

Conclusion

In this study, antibiotic-resistant bacteria to agricultural antibiotics, streptomycin and oxytetracycline, were isolated from two types of lands: plain land fields and high land in sloped fields of Gangwon State. The isolates, which were capable of resisting and growing at various concentrations of antibiotics ranging from 20 to 100 ppm, were tested to examine cross-resistance between streptomycin and oxytetracycline. The strains identified included diverse genera such as *Pseudomonas* sp., *Lysobacter* sp., *Telluria* sp., *Pseudarthrobacter* sp., *Stenotrophomonas* sp., *Microbacterium* sp., and *Flavobacterium* sp.. The occurrence of antibiotic-resistant bacteria from the high land in sloped fields could lead to the spread of ARB as well as ARGs by geographical and environmental factors such as soil erosion and runoff. To mitigate the spread of them in the environment, the comprehensive management in arable land in the sloped fields should be considered for sustainable agriculture, including terracing, contour farming, water management, crop rotation, soil stabilization, slope-specific infrastructure, etc. Taken together, the results of this study suggest that careful monitoring of antibiotic-resistant bacteria is needed in high land in sloped fields to prevent their spread into open natural environments.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: C.S. performed the experiments and interpreted data, and revised the manuscript. J.L. performed the experiments and interpreted data. Y.Y. designed the study and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Notes: The authors declare no conflict of interest.

Acknowledgments: This study was supported by 2023 Research Grant from Kangwon National University and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2024-00348474).

Additional Information:

Supplementary information The online version contains supplementary material available at <https://doi.org/10.5338/KJEA.2025.44.03>

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Peer review information Korean Journal of Environmental Agriculture thanks the anonymous reviewers for their contribution to the peer review of this work.

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