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Comparison of Resistance Acquisition and Mechanisms in *Erwinia amylovora* against Agrochemicals Used for Fire Blight Control

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Agrochemicals containing antibiotics are authorized to manage fire blight that has been occurring in Korea since 2015. The minimum inhibitory concentration (MIC) of each antibiotic against *Erwinia amylovora*, the causal pathogen of fire blight, has increased over the years due to the pathogen's frequent exposure to antibiotics, indicating the necessity to prepare for the emergence of antibiotic resistance. In this study, *E. amylovora* was exposed to stepwise increasing concentrations of eight different agrochemicals, each containing single or mixed antibiotics, and gene mutation and changes in MIC were assessed. Streptomycin and oxolinic acid induced an amino acid substitution in RpsL and GyrA, respectively, resulting in a rapid increase in MIC. Oxytetracycline initially induced amino acid substitutions or frameshifts in AcrR, followed by substitutions of 30S small ribosomal protein subunit S10 or AcrB, further increasing MIC. *E. amylovora* acquired resistance in the order of oxolinic acid, streptomycin, and oxytetracycline at varying exposure frequencies. Resistance acquisition was slower against agrochemicals containing mixed antibiotics than those with single antibiotics. However, gene mutations conferring antibiotic resistance emerged sequentially to both antibiotics in the mixed formulations. Results suggested that frequent application of mixed antibiotics could lead to the emergence of multidrug-resistant *E. amylovora* isolates. This study provided essential insights into preventing the emergence of antibiotic-resistant *E. amylovora* and understanding the underlying mechanisms of resistance acquisition.

Keywords **:** antibiotic resistance, fire blight, GyrA, multidrug efflux transporter, RpsL

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Erwinia amylovora is a casual bacterium of fire blight that causes severe threats to apple and pear production worldwide. Agrochemicals composed of antibiotics, such as streptomycin, oxytetracycline, and oxolinic acid, are used for fire blight control. However, frequent application of agrochemicals can induce the emergence of antibiotic-resistant strains, consequently reducing control efficacy (McManus et al., 2002; Stockwell and Duffy, 2012; Sundin and Wang, 2018).

E. amylovora isolates collected from 2015 to 2022 in Korea did not exhibit resistance to streptomycin, oxytetracycline, and oxolinic acid, which are registered for fire blight control in Korea (Ham et al., 2023, 2024; Lee et al., 2018). However, the minimum inhibitory concentration (MIC) of antibiotics to *E. amylovora* isolates increased year by year, except for oxolinic acid, raising concerns about the occurrence of resistant isolates. The occurrence of streptomycin-resistant *E. amylovora* isolates has been reported in several

countries, including the United States and Israel, and genetic mutations and mechanisms in the resistance have been revealed (Forster et al., 2015; McGhee and Sundin, 2011; McManus et al., 2002). Although oxytetracycline is used for a short duration compared to streptomycin for fire blight control, a few occurrences of resistant *E. amylovora* against oxytetracycline have been reported recently in the United States (Sundin et al., 2023). Mutations in 16S ribosomal RNA, ribosome protection proteins, AraC transcriptional activator, two-component system, and genes associated with intrinsic efflux in tetracycline resistance in human pathogenic bacteria have been reported (Grossman, 2016; Sundin and Wang, 2018). However, there are no reports of oxytetracycline resistance in *E. amylovora* due to chromosome variation occurring naturally. Oxolinic acid is also widely used in Korea to control bacterial diseases, such as leaf blight and grain rot in rice plants and soft rot in cabbage. Oxolinic resistance in *Burkholderia glumae*, a causal pathogen of bacterial grain rot, was induced due to a point mutation in DNA gyrase A (Maeda et al., 2007). Although oxolinic acid is not widely used for fire blight control, oxolinic acid-resistant bacterial outbreak occurred in Israel (Kleitman et al., 2005; Manulis et al., 2003), and repeated exposure of *E. amylovora* to oxolinic acid induced point mutations in *gyrA*, which conferred resistance to oxolinic acid (Ham et al., 2022).

Agrochemicals containing antibiotics as active ingredients are registered to control bacterial diseases in Korea. They are composed of single antibiotics, such as streptomycin, oxytetracycline dihydrate, oxytetracycline calcium alkyltrimethylammonium, and oxolinic acid, or mixed with two antibiotics, such as streptomycin + oxytetracycline calcium alkyltrimethylammonium, streptomycin + oxytetracycline hydrochloride, oxolinic acid + streptomycin, and validamycin A + streptomycin. Because the experimental use of *E. amylovora* is strictly controlled by the law in Korea, it is impossible to conduct field experiments in commercial orchards. Therefore, agrochemicals approved for use in Korea are mandatorily registered by a governmental agency based on the official use in other countries (Lee et al., 2018). There is little understanding of how the frequent use of agrochemicals containing single or mixed antibiotics affects the emergence of antibiotic resistance in *E. amylovora*.

In this study, *E. amylovora* was exposed to stepwise increasing concentrations of various agrochemicals containing single or combined antibiotics to assess the development of antibiotic-resistant isolates *in vitro*. This study identified the underlying genetic mutations responsible for resistance acquisition and evaluated the corresponding increase in MICs. The findings offered insights for developing strategies to prevent and minimize the emergence of antibiotic resistance in *E. amylovora* against commercially used agrochemicals.

Materials and Methods

Agrochemicals used for this study and preparation of media. Eight different agrochemicals (Table 1) containing single antibiotics (streptomycin, oxolinic acid, and oxytetracycline) and mixed antibiotics (streptomycin + oxolinic acid and streptomycin + oxytetracycline) were prepared at 100 times higher concentration than the recommended application dosage. The 100-fold stocks of each agrochemical

Table 1. List of agrochemicals used in this study

Common name (formulation)	Active ingredient (%)	Dilution times to use (antibiotic concentration)	Abbreviation used in this study
Streptomycin (WP)	Streptomycin (20%)	2,000 (100 μg/ml)	S100
Oxolinic acid (WP)	Oxolinic acid (20%)	1,000 (200 μg/ml)	OA200
Oxytetracycline (WP)	Oxytetracycline calcium alkyltrimethylammonium (17%)	2,000 (85 μg/ml)	OT85_1
Oxytetracycline (WG)	Oxytetracycline dihydrate (34%)	4,000 (85 μg/ml)	OT85_2
Oxolinic acid + streptomycin (WP)	Oxolinic acid (10%), streptomycin (15%)	1,000 (oxolinic acid 100 μg/ml, streptomycin 150 μg/ml)	OA100 + S150
	Oxolinic acid (17%), streptomycin (3%)	2,000 (oxolinic acid 85 μg/ml, streptomycin 15 μg/ml)	OA85 + S15
Streptomycin + oxytetracycline (WP)	Streptomycin (15%), oxytetracycline calcium alkyltrimethylammonium (1.5%)	2,000 (streptomycin 75 μg/ml, oxytetracycline 7.5 μg/ml)	S75 + OT7.5_3
Streptomycin + oxytetracycline (SG)	Streptomycin (15%), oxytetracycline hydrochloride (1.5%)	2,000 (streptomycin 75 μg/ml, oxytetracycline 7.5 μg/ml)	S75 + OT7.5_4

WP, wettable powder; WG, wettable granule; SG, soluble granule.

were serially diluted twofold (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512 times), and a 30 µl aliquot of each dilution was added to 3 ml tryptic soy broth (TSB; Difco, Detroit, MI, USA) in a 14 ml tube to assess *E. amylovora* growth.

Stepwise exposure of *E. amylovora* to each agrochemical. *E. amylovora* isolates were exposed to progressive antibiotic concentrations following the methods described by Entenza et al. (2010) with slight modifications. Briefly, *E. amylovora* TS3128 was cultured in TSB overnight, and a 100 µl culture was inoculated into a series of prepared tubes containing a range of agrochemical concentrations from 1/512-fold dilutions with twofold increments (Supplementary Fig. 1). The tubes were incubated at 27°C with 200 rpm for 48 h, and 100 µl from the tube with the highest agrochemical concentration where the optical density at 600 nm exceeded 1 were transferred to a new series of tubes with increasing agrochemical concentrations and cultured again. Each series of cultures refers to the cycle. *E. amylovora* cells in each cycle were used for DNA extraction, and their MICs were recorded. The experiments were repeated twice, with two replicates.

Gene sequencing and analysis. The genomic DNA of oxytetracycline-resistant *E. amylovora* isolates (1_B and 1_S) was extracted by the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Whole-genome sequencing was conducted by Macrogen Corp. (Seoul, Korea). Briefly, the library was constructed using the TruSeq Nano DNA High Throughput Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The constructed library was quantified by the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA) and qualified by an Agilent Technologies 4200 TapeStation D1000 Screen Tape (Agilent Technologies, Santa Clara, CA, USA). NovaSeq (Illumina) paired-end (2 × 150 bp) sequencing was conducted, and the quality was confirmed using FastQC (version 0.11.7). Subsequently, adapters were removed using Trimmomatic (version 0.38). *De novo* assembly was performed using SPAdes (version 3.15.0), followed by annotation using Prokka (version 1.14.6), InterProScan (version 5.34-73.0), and PSI-Blast (version 2.12.0) with EggNOG DB (version 4.3).

Variant calling analysis. Variant calling between wild-type (WT) and oxytetracycline-resistant strain was performed by Macrogen. The filtered reads of mutant sequences were mapped to the genome of *E. amylovora* TS3128 (GenBank accession no. GCA_013375015.1) using the Burrows-Wheeler Aligner (version 0.7.17) and mem algorithm. The duplicated reads were removed by Sambaba (version 0.6.7), and variant calling was performed by SAMtools (version 1.9) and BCFtools (version 1.6). Single nucleotide polymorphisms with a Phred score of >30 and short indels were captured, classified, and used to determine the genome location. Gene information from the captured data was confirmed, and changes in amino acids were predicted using SnpEff (version 4.3t).

Analysis of mutated regions in resistant *E. amylovora* isolates. At the end of each cycle of progressive antibiotic exposure, bacterial cells were pelleted, and genomic DNA was extracted by the Wizard Genomic DNA Purification Kit according to the manufacturer's instructions. *rpsL* (30S small ribosomal protein subunit S12) was investigated for streptomycin-resistant *E. amylovora* using primers AJ42 (5′-GAAGCAAAAAGCTAAAACCAGGAGCT-3′) and AJ44 (5′-CGTGGCATGGAAATACTCCG-3′) (Chiou and Jones, 1995). EagyrA primers (F: 5′-CACCGGTCAATATCGAAGAAGAGT-3′ and R: 5′-TACCCACGGCGATCCCAGAAGAAC-3′) were used to determine oxolinic acid resistance in *gyrA* (DNA gyrase subunit A) (Ham et al., 2022). The mutated regions in oxytetracycline-resistant *E. amylovora* confirmed by variant calling were amplified using each primer set: S10_473 (F: 5′-CGC TGC CGA CCC GTA AAG AG-3′ and R: 5′-TGC GGA ATT CCC ACA GAC CAC-3′), AcrB_590 (F: 5′-CGA CGA TGG CGC CAA AAC TAC GAC-3′ and R: 5′-CCC CAC GCC GGA GAC ACG ACT-3′), and AcrR_534 (F: 5′-TCG CAA ATG TAT GTA AGT CTG AAG-3′ and R: 5′-GCG GCG CGT CGG GTA TC-3′).

The reaction mixture for polymerase chain reaction (PCR) was 50 ng DNA template, 0.2 µM forward and reverse primers, 1× buffer, 0.2 mM deoxynucleotide triphosphate, 4 mM $MgCl_2$, and 1.25 U GoTaq Flexi DNA polymerase (Promega) at a final volume of 25 µl. PCR was conducted by a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA), with the following conditions: 5 min predenaturation at 95°C, 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C (AJ42/44) or 58°C (EagyrA), 30 s extension at 72°C, and 10 min final extension at 72°C. The size of the amplicons was 550 and 507 bp, which was confirmed by electrophoresis with 1% agarose for streptomycin and oxolinic acid, respectively. PCR for oxytetracycline-resistant isolates was performed as described above at an annealing temperature of 64°C, which produced 473, 590, and 534 bp amplicons for S10 (30S small ribosomal protein subunit S10), AcrB (multidrug efflux RND trans-

porter permease subunit AcrB), and AcrR (multidrug efflux transporter transcriptional repressor AcrR), respectively. PCR amplicons were sequenced by Macrogen using primers used for PCR amplification and aligned using SeqMan of DNASTAR (version 15.1.0).

Results

Mutation patterns of *E. amylovora* isolates against oxolinic acid and streptomycin.

The MIC of *E. amylovora* against oxolinic acid WP (OA200) increased from 0.4 to 3.1 μg/ml (for the 1-1 and 1-2 sets) and from 0.8 to 3.1 μg/ml (for the 2-1 and 2-2 sets) after one cycle of exposure to the diluted OA200 (Table 2, Fig. 1A), and a point mutation in *gyrA* was induced with the amino acid substitutions on G69T (substitution from G to T at amino acid position 69), G81C, and S83R in GyrA of *E. amylovora* (Table 3). The lines of the 1-1 and 2-1 sets in Fig. 1A and B did not ap-

Table 2. Minimum inhibitory concentration (MIC) range (μg/ml) at the initial occurrence of amino acid changes by the stepwise treatment of agrochemicals to *Erwinia amylovora*

Agrochemicals		Treatment	Control[a]	GyrA	RpsL	AcrR	S10[b]	AcrB
Single	OA200	1-1	0.4	3.1	-	-	-	-
		1-2	0.4	3.1	-	-	-	-
		2-1	0.8	3.1	-	-	-	-
		2-2	0.8	3.1	-	-	-	-
	S100	1-1	1.6	-	33.3	-	-	-
		1-2	1.6	-	33.3	-	-	-
		2-1	3.1	-	100	-	-	-
		2-2	3.1	-	100	-	-	-
	OT85_1	1-1	2.7	-	-	14.2	21.3	-
		1-2	2.7	-	-	14.2	42.5	-
		2-1	1.3	-	-	2.7	-	-
		2-2	1.3	-	-	2.7	15.5	-
	OT85_2	1-1	0.7	-	-	10.6	28.3	28.3
		1-2	0.7	-	-	17	17	28.3
		2-1	1.3	-	-	3.5	85	-
		2-2	1.3	-	-	85	85	-
Mixed	OA100 + S150	1-1	OA: 0.1, S: 0.1	1.6	4.7	-	-	-
		1-2	OA: 0.1, S: 0.1	1.6	4.7	-	-	-
		2-1	OA: 0.4, S: 0.6	0.8	75	-	-	-
		2-2	OA: 0.4, S: 0.6	1.0	12.5	-	-	-
	OA85 + S15	1-1	OA: 0.2, S: 0.03	5.3	30	-	-	-
		1-2	OA: 0.2, S: 0.03	5.3	30	-	-	-
		2-1	OA: 0.7, S: 0.1	5.3	45	-	-	-
		2-2	OA: 0.7, S: 0.1	1.3	60	-	-	-
	S75 + OT7.5_3	1-1	S: 2.3, OT: 0.2	-	12.5	7.5	60	-
		1-2	S: 2.3, OT: 0.2	-	12.5	7.5	-	-
		2-1	S: 2.3, OT: 0.2	-	75	1.3	37.5	-
		2-2	S: 2.3, OT: 0.2	-	37.5	3.8	37.5	-
	S75 + OT7.5_4	1-1	S: 2.3, OT: 0.2	-	37.5	1.5	30	-
		1-2	S: 2.3, OT: 0.2	-	37.5	1.5	45	-
		2-1	S: 2.3, OT: 0.2	-	75	0.9	22.5	-
		2-2	S: 2.3, OT: 0.2	-	37.5	3.8	22.5	-

[a]Untreated WT.
[b]S10: 30S ribosomal protein S10.

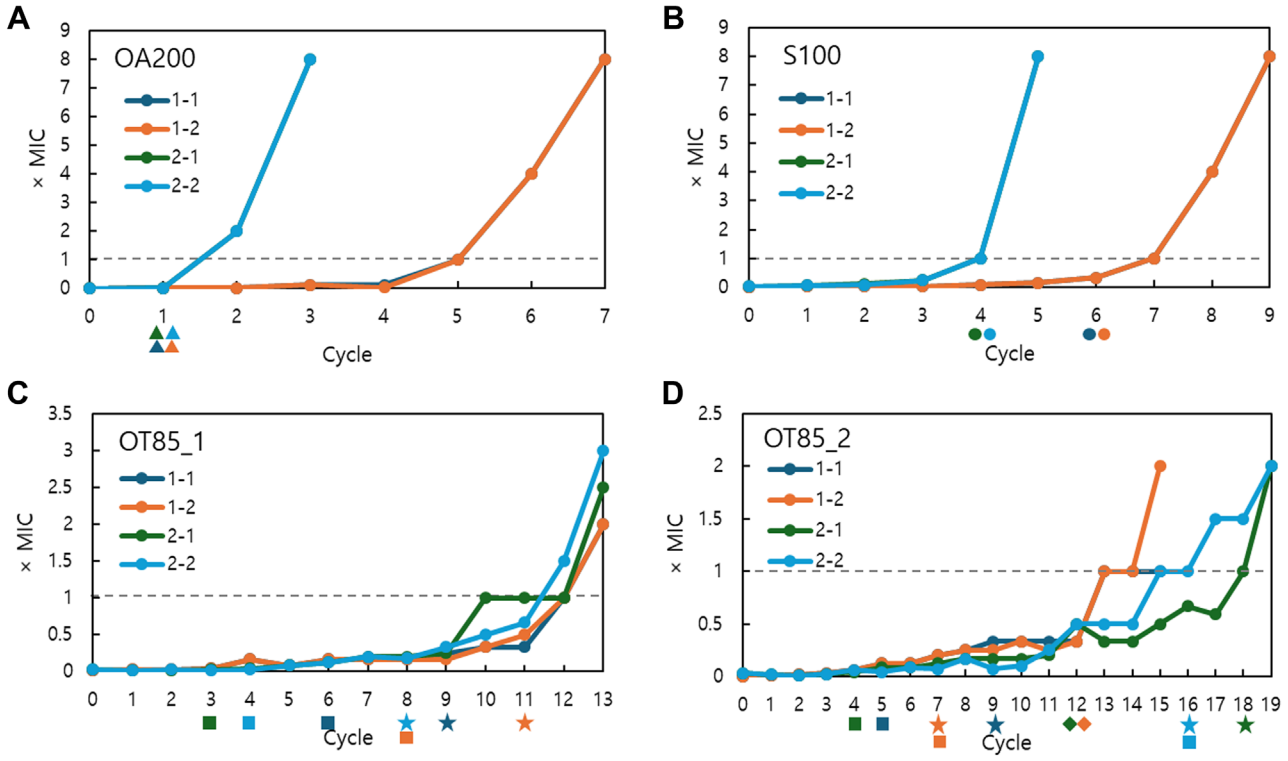

Fig. 1. Increase of minimum inhibitory concentration (MIC) in *Erwinia amylovora* by the stepwise increasing treatment of agrochemicals containing a single antibiotic. (A) OA200, oxolinic acid at concentration of 200 μg/ml. (B) S100, streptomycin (100 μg/ml). (C) OT85_1, oxytetracycline calcium alkyltrimethylammonium (85 μg/ml). (D) OT85_2, oxytetracycline dihydrate (85 μg/ml). Symbols under the x-axis represent the starting point of gene mutations of *gyrA* (▲), *rpsL* (●), *acrR* (■), S10 (★), and *acrB* (◆) in *E. amylovora*. The dashed line represents the antibiotic concentration in the recommended dosage of each agrochemical. The Y-axis shows the fold increase of MIC values relative to the recommended dosage of agrochemicals, with the recommended rate set as 1-fold. 1-1, 1-2, 2-1, and 2-2 refer to the individual replicates, with each set performed in duplicate including two technical replications.

pear in the figures because the increase of MICs in the 1-1 and 1-2 and 2-1 and 2-2 sets were identical. These mutants survived in 1,200 μg/ml oxolinic acid solution, indicating resistance acquisition to a high oxolinic acid concentration. All these amino acid substitutions in mutants occurred in the quinolone resistance-determining region from A67 to Q106 in GyrA (Maeda et al., 2004; Yoshida et al., 1990). In summary, *E. amylovora* can obtain resistance to agrochemicals containing oxolinic acid by a single exposure with low antibiotic concentrations. The MIC of *E. amylovora* to S100 increased in the third cycle and drastically reached the recommended dosage (100 μg/ml) in the fourth or seventh cycle (Fig. 1B). MIC increased from 1.6 to 33.3 μg/ml in the sixth cycle in the 1-1 and 1-2 sets and from 3.1 to 100 μg/ml in the fourth cycle for the 2-1 and 2-2 sets, respectively (Table 2, Fig. 1B), with an amino acid substitution in RpsL: K43N, K88R, P91S, P91T, and G92C (Table 4). Results indicated that repetitive exposure of *E. amylovora* to streptomycin-containing agrochemicals (S100) can induce resistance in short repetitive exposures.

Genetic mutations associated with oxytetracycline resistance in *E. amylovora* isolates. Variant calling identified 130 variations from oxytetracycline-resistant mutant isolates (1_B and 1_S) compared to WT (TS3128). Among these variations, 73 and 57 variations were identified in the chromosome and plasmid, respectively. Twenty-seven variations in coding DNA sequences were identical in both oxytetracycline-resistant strains (Table 5). Among these variations, S10, AcrB, and AcrR were involved in tetracycline resistance in previous studies (Grossman, 2016). Therefore, they were further analyzed to identify mutations from oxytetracycline-resistant *E. amylovora* isolates of this study.

Repeated treatment of oxytetracycline calcium alkyltrimethylammonium WP (OT85_1) and oxytetracycline dihydrate WG (OT85_2) induced a gradual increase of MIC. MIC reached the recommended dosage after 10

cycles in OT85_1 and 13 cycles in OT85_2 (Fig. 1C and D). In OT85_1, MIC increased from 2.7 to 14.2 μg/ml in the sixth and eighth cycles in the 1-1 and 1-2 sets and from 1.3 to 2.7 μg/ml in the third and fourth cycles in the 2-1 and 2-2 sets, respectively (Table 2), with frameshift at various positions in *acrR* (Table 6). AcrR mutations by OT85_1 were in positions 40-41, 54-55, 87-90, and 178, resulting in a frameshift of amino acid sequences (Table 6). When the amino acid substitution at 57 positions (V57L) of the S10 gene appeared, MIC increased to 15.5, 21.3, and 42.5 μg/ml in the 8th, 9th, and 11th cycles in the 2-2, 1-1, and 1-2 sets.

When OT85_2 was repeatedly treated, MIC increased from 0.7 to 10.6 μg/ml in the 5th cycle in the 1-1 set, from 0.7 to 17 μg/ml in the 7th cycle in the 1-2 set, from 1.3 to 3.5 μg/ml in the 4th cycle in the 2-1 set, and from 1.3 to 85 μg/ml in the 16th cycle in the 2-2 set. The amino acids in positions 13-19 and 48-64 of AcrR changed, resulting in a frameshift of AcrR. Subsequently, an amino acid substitution of V57L in the S10 gene emerged at an MIC of 28.3 μg/ml in the 9th cycle in the 1-1 set, 17 μg/ml in the 7th cycle in the 1-2 set, and 85 μg/ml in 18th and 16th cycles in the 2-1 and 2-2 sets, respectively. V139F in the AcrB in the 12th cycle sequentially emerged after the emergence of AcrR and S10 mutations with 28.3 μg/ml MIC value in the 1-1 and 1-2 sets, respectively (Fig. 1D). In summary, oxytetracycline resistance initially appeared predominantly due to frameshift mutations in AcrR, followed by amino acid substitution in S10 or AcrB, which further increased the MIC of *E. amylovora* against oxytetracycline.

Mutation patterns of *E. amylovora* isolates against antibiotic mixtures. Treatment of oxolinic acid and streptomycin mixture WP (OA100 + S150) induced *gyrA* mutation in *E. amylovora* with an MIC increase from 0.1 to 1.6 μg/ml

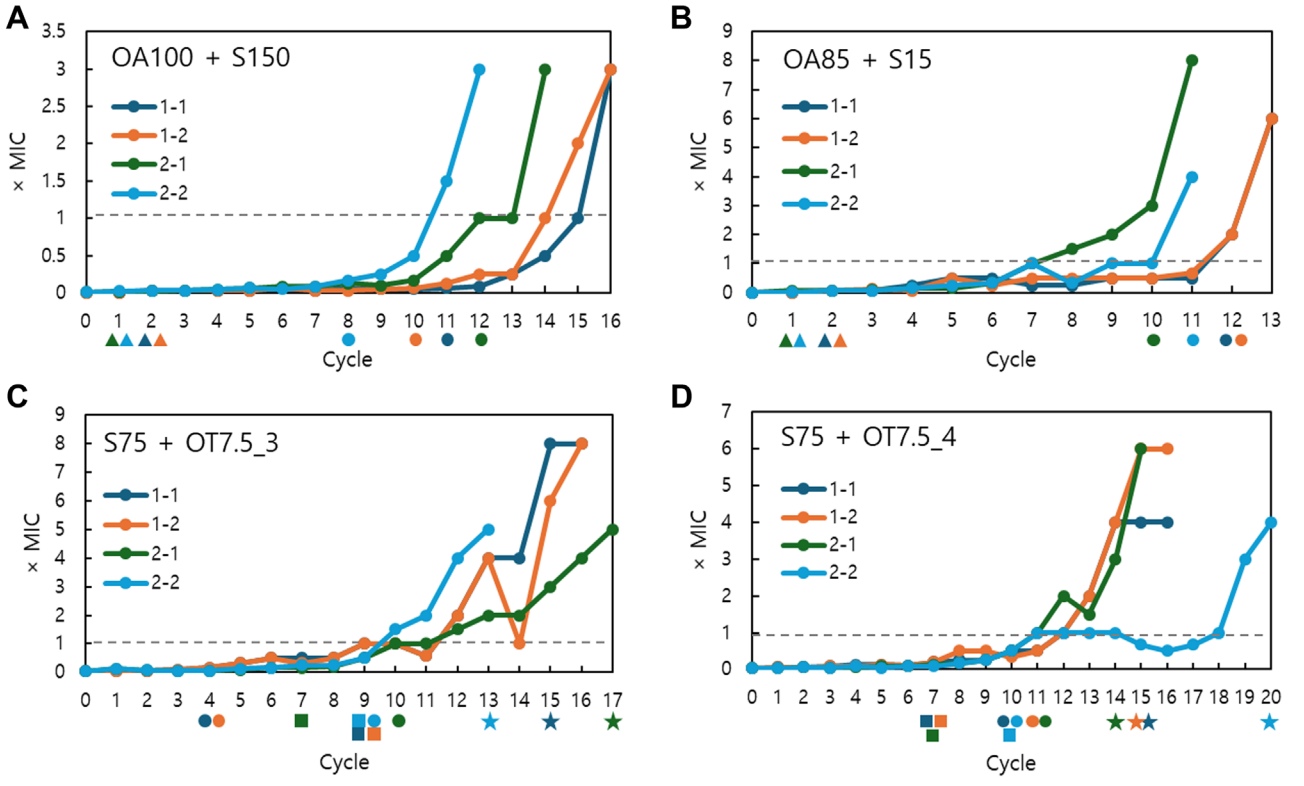

Fig. 2. Increase in minimum inhibitory concentration (MIC) in *Erwinia amylovora* by the stepwise increasing treatment of agrochemicals containing mixed antibiotics. Combination of oxolinic acid (OA) and streptomycin (S). (A) OA100 + S150, a mixture of oxolinic acid and streptomycin at 100 μg/ml + 150 μg/ml, respectively. (B) OA85 + S15, oxolinic acid and streptomycin (85 μg/ml + 15 μg/ml), combination of streptomycin (S) and oxytetracycline (OT). (C) S75 + OT7.5_3, streptomycin and oxytetracycline calcium alkyl trimethylammonium (75 μg/ml + 7.5 μg/ml). (D) S75 + OT7.5_4, streptomycin and oxytetracycline hydrochloride (75 μg/ml + 7.5 μg/ml). Symbols under the x-axis represent the starting point of gene mutations of *gyrA* (▲), *rpsL* (●), *acrR* (■), and S10 (★) in *E. amylovora*. The dashed line represents the recommended dosage of each agrochemical. The Y-axis shows the fold increase of MIC values relative to the recommended dosage of agrochemicals, with the recommended rate set as 1-fold. 1-1, 1-2, 2-1, and 2-2 refer to the individual replicates, with each set performed in duplicate including two technical replications.

in the second cycle in the 1-1 and 1-2 sets and from 0.4 to 0.8 or 1.0 μg/ml in the first cycle in the 2-1 and 2-2 sets, respectively. Subsequently, OA100 + S150 induced *rpsL* mutation in the 8th, 10th, 11th, and 12th cycles with an MIC of 12.5, 4.7, 4.7, and 75 μg/ml in the 2-2, 1-2, 1-1, and 2-1 sets, respectively (Table 2, Fig. 2A). The MIC of *E. amylovora* against OA100 + S150 increased slowly until the *rpsL* mutation emerged, indicating that *gyrA* and *rpsL* mutations can be induced by the mixture of antibiotics. Amino acid substitutions in GyrA frequently appeared as G69T and G81C, whereas D97Y was detected in one case in *E. amylovora* (Table 3). The D97Y mutant emerged at oxolinic acid concentrations of 0.8 and 1.6 μg/ml in the 1-2 set and disappeared as antibiotic concentration increased, suggesting that the mutant cannot withstand high oxolinic acid concentrations. In RpsL, amino acid substitutions of K43R, R86S, R86C, K88T, P91S, and G92C emerged (Table 4). The G residue was inserted at the 92nd amino acid position in some mutants that can survive against 300 μg/ml streptomycin. Insertion of the G residue at the 92nd position of RpsL was previously reported in *Streptomyces coelicolor*, which induced high resistance of the bacterium against paromomycin (Wang et al., 2009). Treatment of another oxolinic acid and streptomycin mixture WP (OA85 + S15) showed similar increasing patterns of MIC, with *gyrA* mutations after one or two cycles of mixture treatments with an MIC of 5.3, 1.3, 5.3, and 5.3 μg/ml in the 2-1, 2-2, 1-1, and 1-2 sets, followed by *rpsL* mutations after the 10th, 11th, and 12th cycles with an MIC of 45, 60, 30, and 30 μg/ml in the 2-1, 2-2, 1-1, and 1-2 sets, respectively (Table 2, Fig. 2B). However, MIC increased to the recommended dosage before the emergence of *rpsL* mutations due to 10 times lower streptomycin concentration (15 μg/ml) in the mixture compared to OA100 + S150. Amino acid substitutions emerged at G69T and G81C in GyrA and R86S, K88T, and P91S in RpsL (Tables 3 and 4). Results indicated that *E. amylovora* isolates acquired antibiotic resistance sequentially and eventually resistant to oxolinic acid and streptomycin contained in agrochemicals with gene mutations.

Table 3. Amino acid substitutions in GyrA of oxolinic acid-resistant *Erwinia amylovora*

Strain/treatment	Location			
	69	81	83	97
TS3128[a]	G	G	S	D
TS3128/OA200	T	C	R	-
TS3128/OA100+S150	T	C	-	Y
TS3128/OA85+S15	T	C	-	-

[a]WT control.

Table 4. Amino acid changes in RpsL in streptomycin-resistant *Erwinia amylovora*

Strain/treatment	Location				
	43	86	88	91	92
TS3128[a]	K	R	K	P	G
TS3128/S100	N	-	R	S, T	C
TS3128/OA100+S150	R	S, C	-	-	C, +G[b]
TS3128/OA85+S15	-	S	T	S	-
TS3128/S75+OT7.5_3	R	-	R	-	-
TS3128/S75+OT7.5_4	R	-	-	-	-

[a]WT control.
[b]Insertion.

Streptomycin + oxytetracycline calcium alkyltrimethylammonium WP (S75 + OT7.5_3) induced streptomycin resistance by point mutation in RpsL after the 4th, 4th, 9th, and 10th cycles with an MIC of 12.5, 12.5, 37.5, and 75 μg/ml in each replication of 1-1, 1-2, 2-2, and 2-1, respectively. Streptomycin + oxytetracycline hydrochloride SG (S75 + OT7.5_4) induced point mutation in RpsL after the 10th, 10th, 11th, and 11th cycles with an MIC of 37.5, 37.5, 37.5, and 75 μg/ml in each replication of 1-1, 2-2, 1-2, and 2-1, respectively (Table 2, Fig. 2C and D). Furthermore, the MIC of *E. amylovora* in the mixture increased when the S10 mutation emerged. S75 + OT7.5_3 treatment induced the amino acid substitution in AcrR after the 7th, 9th, 9th, and 9th cycles with an MIC of 1.3, 3.8, 7.5, and 7.5 μg/ml in each replication of 2-1, 2-2, 1-1, and 1-2, respectively, and induced an amino acid substitution in the S10 gene with an MIC of 37.5, 37.5, and 60 μg/ml in the 13th, 17th, and 15th cycles in each replication of 2-2, 2-1, and 1-1, respectively. S75 + OT7.5_4 treatment induced amino acid substitution in AcrR after the 7th, 7th, 7th, and 10th cycles with an MIC of 0.9, 1.5, 1.5, and 3.8 μg/ml in each replication of 2-1, 1-1, 1-2, and 2-2, respectively, and induced an amino acid substitution in the S10 gene with an MIC of 22.5, 22.5, 30, and 45 μg/ml in the 14th, 20th, 15th, and 15th cycles in each replication of 2-1, 2-2, 1-1, and 1-2, respectively. Amino acid substitution mutants K43R and K88R emerged in RpsL (Table 4), and A9V, T12A, A20V, and the 130th Q changed to stop codon in the AcrR gene (Table 6). In the S75 + OT7.5_3 treatment, the amino acid was inserted at the 112th position, causing a frameshift of the amino acid sequences in AcrR. S10 gene mutations, such as V57L, emerged after AcrR mutation, similar to oxytetracycline dihydrate treatment. Results indicated that *E. amylovora* isolates acquired antibiotic resistance sequen-

Table 5. Nucleotide sequence difference between oxytetracycline-resistant and wild-type *Erwinia amylovora*

NCBI gene ID	Position	Annotated function	TS3128 (WT)	1_B (mutant)	1_S (mutant)
HU055_RS00080	18,039	Tyrosine protein phosphatase	TCCCCCCC	TCCCCCCCC	TCCCCCCCC
HU055_RS00355	85,806	Insulinase family protein	AA	AAACA|ACCA	AAACA|ACCA
HU055_RS01250	287,841	30S ribosomal protein S10	G	C	C
HU055_RS02325	502,318	DUF1190 family protein	GCCCCC	GCCCCCC	GCCCCCC
HU055_RS02365	510,480	DUF1062 domain-containing protein	TGGGGGG	TGGGGGGG	TGGGGGGG
HU055_RS02405	525,661	SDR family NAD(P)-dependent oxidoreductase	CGGGGG	CGGGGGG	CGGGGGG
HU055_RS02410	530,185	SDR family NAD(P)-dependent oxidoreductase	TGGGGGGGG	TGGGGGGGGG	TGGGGGGGGG
HU055_RS05025	1,089,007	Multidrug efflux RND transporter permease subunit (AcrB)	C	A	A
HU055_RS05035	1,090,799	Multidrug efflux transporter transcriptional repressor AcrR	CGCGCAATCACATTATAGATGCGGC	CGC	CGC
HU055_RS17600	1,643,549	Invasion protein	GAAAAA	GAAAAAA	GAAAAAA
HU055_RS08630	1,859,689	Rrf2 family transcriptional regulator	TGGTACTTTAA	T	T
HU055_RS08795	1,899,880	Acyltransferase	CAAAAA	CAAAAAA	CAAAAAA
HU055_RS10025	2,157,080	Cation:proton antiporter	A	C	C
HU055_RS10610	2,282,120	UDP-glucose 4-epimerase GalE	TCCCCC	TCCCCCC	TCCCCCC
HU055_RS11875	2,569,107	N-acetylmuramoyl-L-alanine amidase AmiA	TCCCCCCCC	TCCCCCCCCC	TCCCCCCCCC
HU055_RS12820	2,793,999	Class 1b ribonucleoside-diphosphate reductase subunit alpha	ACCCCC	ACCCCCC	ACCCCCC
HU055_RS13090	2,852,889	Type I methionyl aminopeptidase	A	G	G
HU055_RS13090	2,852,890	Type I methionyl aminopeptidase	G	T	T
HU055_RS13090	2,852,891	Type I methionyl aminopeptidase	T	G	G
HU055_RS13090	2,852,891	Type I methionyl aminopeptidase	TG	TGG	TGG
HU055_RS14215	3,124,282	DUF1311 domain-containing protein	CTTTTTT	CTTTTTTT	CTTTTTTT
HU055_RS15220	3,328,365	Type IV secretion protein Rhs	TGGGGGG	TGGGGGGG	TGGGGGGG
HU055_RS17735	3,364,345	Acyltransferase	GCCCCCCCC	GCCCCCCCCC	GCCCCCCCCC
HU055_RS17760	3,745,995	Glucosyltransferase	CGGGGGG	CGGGGGGG	CGGGGGGG
HU055_RS17290	14,525	AAA family ATPase	AGG	AGGG	AGGG
HU055_RS17790	22,677	MCP four-helix bundle domain-containing protein	GAAAAA	GAAAAAA	GAAAAAA
HU055_RS17795	23,775	Chemotaxis protein	GAA	GAAA	GAAA

Table 6. AcrR, S10, and AcrB mutations in oxytetracycline-resistant *Erwinia amylovora*

Strain/treatment	Location												
	AcrR											S10[a]	AcrB
	9[b]	12	13-19	20	40-41	48-64	54-55	87-90	112-116	130	178	57	139
TS3128[c]	A	T	RNHIIDA	A	DIAAGAGV	-	NK	SVM	IIYHK	Q	W	V	V
TS3128/OT85_1	-	-	-	-	DIAAGV	-	K.	LRD	-	-	(.)	L	-
TS3128/OT85_2	-	-	-LSSVFLN	-	-	ITSLADIAA-GAGVTRGA	-	-	-	-	-	L	F
TS3128/S75 + OT7.5_3	-	A	-	V	-	-	-	-	NHLP.	(.)	-	L	-
TS3128/S75 + OT7.5_4	V	A	-	-	-	-	-	-		(.)	-	L	-

[a]S10: 30S ribosomal protein S10.
[b]Location of the amino acid sequence in the gene.
[c]WT control.

tially and eventually to streptomycin and oxytetracycline contained in agrochemicals with gene mutations. These results suggested that the frequent application of mixed antibiotics could lead to the emergence of multidrug-resistant *E. amylovora* isolates.

Discussion

In this study, eight different agrochemicals registered for fire blight control in Korea were progressively treated to *E. amylovora* to understand the underlying mechanisms and potential synergisms of the resistance. Oxolinic acid, which belongs to quinolone antibiotics and inhibits DNA replication by binding to DNA gyrase and topoisomerase IV, inhibits bacterial growth and is used to control various diseases in Japan and Israel (Stockwell and Duffy, 2012; Sundin and Wang, 2018). In this study, oxolinic acid-resistant *E. amylovora* was constructed by repeated contact with agrochemicals containing oxolinic acid *in vitro*, and amino acid substitutions were identified at positions 69, 81, 83, and 97 of GyrA in *E. amylovora*. Amino acid substitutions of S83R and G81C were previously reported from the constructed oxolinic acid-resistant *E. amylovora* (Ham et al., 2022), whereas the other mutations were identified in this study. In *B. glumae*, the S83I mutant in GyrA showed higher oxolinic resistance than the S83R mutant. The amino acid substitution at different locations of GyrA affected the fitness and pathogenicity of the mutants (Maeda et al., 2007). Only the S83R mutant showed pathogenicity in the rice plant, although it had a low growth rate compared to WT. Resistance to oxolinic acid in *E. amylovora* emerged after one to two treatments with a low concentration (<5.3 μg/ml). In Israel, oxolinic acid, used as a streptomycin alternative to control the fire blight in 1997, induced resistant isolates 2 years after its use (Manulis et al., 2000, 2003). Considering that the streptomycin-resistant *E. amylovora* was found in 1971, after its initial use in the 1950s (McManus et al., 2002; Miller and Schroth, 1972), results indicated that oxolinic acid should be used cautiously, as oxolinic acid-resistant *E. amylovora* can emerge readily under any circumstances. It is recommended to avoid applying oxolinic acid on rainy days, which could dilute its active concentration, and apply it at the recommended concentration of 200 μg/ml in the field to prevent resistance acquisition to oxolinic acid.

Streptomycin resistance occurs mainly by a point mutation in the *rpsL* gene on chromosome or insertion of *strA-strB* genes, which encodes for aminoglycoside phosphotransferases, in the plasmid (Chiou and Jones, 1995; McManus et al., 2002). In this study, amino acid substitu-

tions were identified at various positions (locations 43, 86, 88, 91, and 92) in RpsL by repeated treatment of streptomycin formulations. In the previous study, the most frequent mutation was K43R, followed by P91L and K88R. K43N, K43T, and G92D mutations were also reported in *E. amylovora* (Escursell et al., 2021). The K43R mutant showed a similar growth rate to WT without antibiotic pressure, but P91L and G92D can only survive under streptomycin supplication. K43N and K43T mutants in RpsL showed reduced populations under repeated cultivation without streptomycin supplementation, indicating that substitution with different amino acids induces varying levels of environmental fitness.

Oxytetracycline is used for fire blight control in the United States and other countries as an alternative to streptomycin, but there are few reports on the emergence of resistant *E. amylovora* in the field (McManus and Jones, 1994; Schnabel and Jones, 1999; Sundin et al., 2023). The resistance acquired by horizontal gene transfer with a plasmid possessing the *tet* genes has been reported in other bacteria (Herbert et al., 2022; Schnabel and Jones, 1999). Resistance arising from chromosomal mutations is associated with efflux genes of OmpF, OmpC, and AcrAB (Mortimer and Piddock, 1993), RND family (Hirata et al., 2004), genes related to ribosomal protection, 30S ribosomal subunit, and 16S rRNA (Brodersen et al., 2000), and AraC family genes (MarA, RamA, SoxS, RobA, and RarA) (Martin and Rosner, 2001). In this study, mutations of AcrR, S10, and AcrB from the variant calling analysis of oxytetracycline-resistant isolates were confirmed. AcrAB was encoded in an operon controlled by the transcriptional repressor AcrR. AcrB is a component of the RND family efflux pump, contributing to the extrusion of antibiotic molecules (Anes et al., 2015; Deng et al., 2013). However, AcrR mutation inhibits its binding to the AcrAB promoter, relieving the transcriptional repression (Deng et al., 2013; Gu et al., 2008). Therefore, mutations of these genes affect the extrusion of tetracycline molecules from the cells, causing tetracycline resistance. S10 is the component of the 30S small subunit of the ribosome, interacting with each other for accurate protein synthesis. As tetracycline blocks protein synthesis by binding to the 30S ribosomal subunit and inhibiting the access of aminoacyl-transfer RNA, the mutation of this gene can induce tetracycline resistance (Brodersen et al., 2000; Grossman, 2016). This study confirmed that oxytetracycline induces the mutations initially in AcrR of *E. amylovora* at low concentrations, and mutations in S10 and AcrB genes also occur as MIC gradually increases. When exposed to high oxytetracycline concentrations (OT85 treatment), frameshift mutations emerged in the gene, whereas low oxytetracycline concentrations (S75 + OT7.5 treatment) usually resulted in amino acid substitutions. Various mutation positions were identified in the AcrR gene, whereas only one substitution (V57L) emerged in the S10 gene. Several studies have demonstrated that changes or deletions in residues from 53 to 60 of 30S ribosomal subunit protein S10 are related to tetracycline resistance (Grossman, 2016). Furthermore, tigecycline, the derivative of tetracycline, conferred resistance to *Escherichia coli* by V57 mutation (Beabout et al., 2015; Izghirean et al., 2021). However, the increase of MIC caused by the S10 mutation alone was <0.5 μg/ml, which was not enough to classify as a resistant mutant by the European Committee on Antimicrobial Susceptibility Testing criteria (Izghirean et al., 2021). Results indicated that AcrR and S10 gene mutations are essential for *E. amylovora* to survive in high oxytetracycline concentrations. Resistance acquisition patterns differed from streptomycin or oxolinic acid resistance, where MIC increased rapidly due to the single amino acid substitution in the responsible genes.

Producing reproducible MIC values *in vitro* is challenging because even minor variations of culturing conditions, such as pH and incubation time, can drastically affect the MIC. A twofold scale for MIC level assessment results in more than twofold difference in MIC values when variation occurs, as already recognized in many reports (Krajewska et al., 2023; Mouton et al., 2017). Nevertheless, the twofold dilution system for MIC determination is still the most commonly used method (Wiegand et al., 2008). Therefore, this study performed assessments twice with two technical replications. The MIC level and starting point of gene mutations were different between the first (1-1, 1-2) and second (2-1, 2-2) replicates (Figs. 1 and 2). Hence, an individual set of results was described to recognize the variation and understand the occurrence of resistance beyond the variations.

The average time required for gene mutation through stepwise exposure to agrochemicals was 1.3 cycles in GyrA, 8.8 cycles in RpsL, and 13.4 cycles for AcrR and S10 in *E. amylovora*, suggesting that antibiotic resistance emerged sequentially in the order of oxolinic acid, streptomycin, and oxytetracycline in *E. amylovora*. Comparing the emergence of the resistance between single and mixed agrochemicals, MIC increased, and target gene mutations occurred more slowly with mixed agrochemicals than with the single antibiotic composition. However, gene mutations appeared sequentially in the order of GyrA, RpsL, AcrR, and S10 by the stepwise exposure of mixed agrochemicals, raising concerns about the emergence of multiantibiotic resistance. This study analyzed chromosomal mutations over

repetitive oxolinic acid, streptomycin, and oxytetracycline treatment in *E. amylovora*. However, various chemical pressures and transposon-mediated gene insertions causing antibiotic resistance should also be considered to broaden our understanding of complex natural circumstances. This study provided fundamental information on genetic mutations in the chromosome and the dynamics of antibiotic resistance emergence with agrochemicals containing single or mixed antibiotics and contributed significantly to the establishment of measures to prevent the emergence of antibiotic-resistant *E. amylovora*. This study strongly recommended the cautious use of agrochemicals composed of mixed antibiotics, as there is a risk of simultaneous mutations and the acquisitions of resistance to all antibiotics included.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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