

Survey of Oxolinic Acid-Resistant *Erwinia amylovora* in Korean Apple and Pear Orchards, and the Fitness Impact of Constructed Mutants

Hyeonheui Ham ^{1,2*}, Ga-Ram Oh¹, Dong Suk Park¹, and Yong Hoon Lee ^{2*}

¹Crop Protection Division, National Institute of Agricultural Sciences, Rural Development Administration, Wanju 55365, Korea

²Division of Biotechnology, Jeonbuk National University, Iksan 54596, Korea

(Received on April 26, 2022; Revised on July 27, 2022; Accepted on August 9, 2022)

Fire blight caused by *Erwinia amylovora* (Ea) is a devastating disease in apple and pear trees. Oxolinic acid (OA), a quinolone family antibiotic that inhibits DNA gyrase, has been employed to control fire blight in South Korea since 2015. The continuous use of this bactericide has resulted in the emergence of OA-resistant strains in bacterial pathogens in other countries. To investigate the occurrence of OA-resistant Ea strains in South Korea, we collected a total of 516 Ea isolates from diseased apple and pear trees in 2020-2021 and assessed their sensitivities to OA. We found that all isolates were susceptible to OA. To explore the possibility of emerging OA-resistant Ea by continuous application of OA, we exposed Ea strains to a range of OA concentrations and constructed OA-resistant mutant strains. Resistance was associated with mutations in the GyrA at codons 81 and 83, which result in glycine to cysteine and serine to arginine amino acid substitutions, respectively. The *in vitro* growth of the mutants in nutrient media and

their virulence in immature apple fruits were lower than those of wild-type. Our results suggest that OA-resistance decreases the fitness of Ea. Future work should clarify the mechanisms by which OA-resistance decreases virulence of this plant pathogen. Continuous monitoring of OA-resistance in Ea is required to maintain the efficacy of this potent bactericide.

Keywords : antibiotic resistance, *Erwinia amylovora*, *gyrA*, oxolinic acid

Erwinia amylovora (Ea) is a Gram-negative bacterium and a major cause of fire blight in rosaceous plants such as apple and pear trees. Ea can infect the host by invading flowers, shoot tips, wounds, and natural openings. Following invasion, Ea causes systemic infection, which ultimately leads to the death of the tree (Momol et al., 1998; Norelli et al., 2003). Huge numbers of apple and pear trees are destroyed by fire blight every year in countries including the USA, Europe, and South Korea (Bonn and van der Zwet, 2000; Calzolari et al., 1999; Park et al., 2017).

Once Ea disseminates systemically within the tree, it is exceptionally difficult to control the disease (Aćimović et al., 2015). It is therefore recommended to eradicate potential pathogens before they invade the host. Chemicals, biological products, and physical practices including pruning and removing cankers from trees have been employed to control fire blight. In particular, chemical control with antibiotics is common except for Europe, and streptomycin has been the most effective bactericide in control of fire blight (McManus et al., 2002). Overuse of antibiotics, however, can lead to the emergence of resistant bacteria. In United States, streptomycin has been used in the control of fire blight since the 1950s. Following the emergence of

*Co-corresponding authors.

H. Ham

Phone) +82-63-238-3276, FAX) +82-63-238-3838

E-mail) hhham@korea.kr

https://orcid.org/0000-0002-4795-1773

Y. H. Lee

Phone) +82-63-850-0841, FAX) +82-63-850-0834

E-mail) yonghoonlee@jbnu.ac.kr

https://orcid.org/0000-0001-9921-3871

Handling Editor : Hee Jin Park

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Articles can be freely viewed online at www.ppjonline.org.

streptomycin-resistant *Ea* strains in 1970, oxytetracycline and kasugamycin became the substitutes in chemical control of the disease (McGhee and Sundin, 2011; McManus and Jones, 1994). In Israel, fire blight was first reported in 1985 (Shabi and Zutra, 1987). Streptomycin resistance was subsequently detected in 1991, after which streptomycin was replaced with oxolinic acid (OA) (Manulis et al., 2003). OA is a quinolone antibiotic usually applied to control bacterial grain rot caused by *Burkholderia glumae* or *Pectobacterium carotovora* (Hikichi, 1993; Hikichi et al., 1994). The application of OA with 300 µg/ml during blooming season, in accordance with their decision support systems, significantly reduced the incidence of fire blight in pear trees in Israel (Shtienberg et al., 2001, 2003). Unfortunately, OA-resistance emerged in northern Israel 2 years after its application (Manulis et al., 2000, 2003).

OA inhibits Gram-negative bacterial pathogens by targeting type II topoisomerases (DNA gyrase and topoisomerase IV) involved in the maintenance of DNA topology. DNA gyrase is encoded by *gyrA* and *gyrB*, and mutations in either gene are responsible for OA-resistance in Gram-negative bacteria. Furthermore, the mutation of topoisomerase IV, encoded by *parC* and *parE* can increase resistance (Maeda et al., 2007; Ruiz, 2003). In *B. glumae*, a major causal pathogen of bacterial grain rot in rice plants, substitution of a serine at codon 83 in GyrA to an arginine or isoleucine conferred resistance to OA (Maeda et al., 2007). Amino acid substitutions in GyrA and GyrB are also known to confer quinolone resistance in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (Eaves et al., 2004; Feng et al., 2019; Maeda et al., 2007; Ruiz, 2003; Yonezawa et al., 1995). However, since few countries use OA to control fire blight (Stockwell and Duffy, 2012), the mechanisms underpinning OA-resistance in *Ea* have remained unexplored.

In South Korea, fire blight was first reported from Asian pear trees in Anseong in 2015 (Park et al., 2016). Since then, streptomycin, oxytetracycline, and OA have been recommended as antibiotics to control the disease during blooming season. In this study, to survey the occurrence of OA-resistant *Ea* strains in Korean orchards, we isolated pathogens from fire blight diseased apple and pear trees located at major production areas of South Korea. In addition, to characterize the underlying mechanisms of OA-resistance acquisition, we selected *in vitro* spontaneous OA-resistant *Ea* mutants and sequenced their *gyrA*, *gyrB*, and *parC* genes. We also compared growth rate and virulence between OA-resistant mutants and their wild-type (WT) strains. We were not able to find OA-resistant *Ea* strains in Korea. We demonstrated that mutations in *gyrA* con-

ferred OA-resistance in *Ea* and that OA-resistance incurs a significant fitness cost that may explain the low frequency of resistance. Future studies will explore the underlying mechanisms of OA resistance, and their influence on the physiology and ecology of *Ea*. In addition, control strategies to prevent the emergence of resistant mutants will be the focus of future studies.

Materials and Methods

Collection and identification of *Ea* isolates. A total of 516 *Ea* strains were isolated from lesions in leaves and twigs in fire blight-diseased apple or pear trees from 2020 to 2021 in Korea (Table 1, Supplementary Table 1). The diseased leaves or twigs were surface sterilized using 70% ethanol. The margin between the necrotic and healthy tissue was cut into 5 × 5 mm pieces, before macerating in sterile distilled water (SDW), vortexing, and incubating for 30 min at room temperature. Suspensions (10 µl) were streaked on tryptic soy agar (TSA; tryptone, 15 g; soytone, 5 g; NaCl, 5 g; agar, 15 g/l) plates and incubated at 27°C for 48 h. White colonies, a typical morphotype of *Ea*, were re-streaked on TSA to establish pure cultures. Colonies were confirmed as *Ea* using a targeted polymerase chain reaction (PCR) primer set developed by Bereswill et al. (1992).

Table 1. Oxolinic acid sensitivity of *Erwinia amylovora* isolates collected from apple and pear orchards of Korea

Province	City	Orchards	Isolates
Gyeonggi	Anseong	69	132
	Namyangju	5	11
	Yeoju	2	4
	Icheon	8	14
	Gwangju	1	1
	Pyeongtak	10	20
Gangwon	Pyeongchang	1	2
	Yeongwol	1	2
Chungbuk	Goesan	2	4
	Danyang	2	4
	Emseong	21	39
	Jecheon	25	53
	Chungju	59	117
Chungnam	Jincheon	1	1
	Dangjin	9	18
	Asan	3	5
Gyeongbuk	Cheonan	40	82
	Andong	4	6
Jeonbuk	Iksan	1	1
Total		264	516

Table 2. Oxolinic acid-resistant and wild-type *Erwinia amylovora* strains used in this study

Strain	Host	Year	Region	Note	Origin
TS3128	Asian Pear	2015	Anseong	Wild-type	Kang et al. (2021)
OX15				S83R mutant in <i>gyrA</i> of TS3128	This study
OX20				S83R mutant in <i>gyrA</i> of TS3128	This study
OX40				G81C mutant in <i>gyrA</i> of TS3128	This study
OX52				G81C mutant in <i>gyrA</i> of TS3128	This study

OA sensitivity assay. A total of 516 Ea isolates were screened for sensitivity to OA. Each isolate was streaked on TSA agar supplemented with or without 5 µg/ml OA before incubating at 27°C for 48 h (Supplementary Fig. 1). Ea strains TS3128 (OA-sensitive wild-type) and OX15 (a spontaneous OA-resistant mutants) were used as a negative and positive control, respectively. Isolates incapable of growth on TSA supplemented with 5 µg/ml OA were considered OA-sensitive.

Construction of OA-resistant mutants. The Ea strain TS3128, isolated from Anseong, Korea in 2015, was used to isolate OA-resistant mutants (Table 2). Spontaneous OA-resistant mutants were constructed using the progressive antibiotic exposure method described by Entenza et al. (2010). Briefly, strain Ea TS3128 was grown on tryptic soy broth (TSB) media supplemented with OA at 1.914 µM. Obtained colonies were sequentially passaged on plates supplemented with two-fold increasing OA concentrations. Strains surviving concentrations greater than 19.14 µM (5 µg/ml) of OA were named OX15, OX20, OX40, and OX52.

Sequencing of *gyrA*, *gyrB*, and *parC*. Genomic DNA was isolated from WT and OA-resistant Ea strains using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The target sequences were amplified using primer sets EagyrA (F: 5'-CACCGGTCAATATCGAAGAAGAGT-3', R: 5'-TACCCACGGC-GATCCCAGAAGAAC-3') and EagyrB (F: 5'-TCG-GCGGTTGAGCAGCAGATG-3', R: 5'-GCAGC-GTGGCACCGTCAAGAG-3'), encompassing 153-318 bp of *gyrA* and 1,116-1,449 bp of *gyrB*. And the target sequence of *parC* was amplified by EaparC1 (F: 5'-TGCGATGTCGGAAGTGGGGCTAAG-3', R: 5'-TCGGGTTCTCATGATTGACTC-3') and EaparC2 (F: 5'-CTGAATCATCGTCTGGAAAAAGTG-3', R: 5'-GACGAAACCATAACCGGCATCAGA-3') encompassing 153-895 bp and 1,096-1,827 bp regions of the gene (Supplementary Table 2). The primers were designed using the Lasergene PrimerSelect software (ver-

sion 7.2.1, DNASTAR Inc., Madison, WI, USA) and were synthesized by Bionics Corp. (Daejeon, Korea). PCR was performed with the C1000 Touch thermal cycler (Bio-Rad Inc., Hercules, CA, USA). A reaction mixture with a final volume of 25 µl (1× buffer, 0.2 mM of each dNTP, 4.0 mM MgCl₂) containing 1.25 U of GoTaq Flexi DNA polymerase (Promega), 25 ng of template DNA and a 0.2 µM final concentration of each primer was prepared. The amplification reaction involved an initial denaturation at 95°C for 5 min, 35 cycles of denaturation (95°C for 30 s), annealing (58°C, 58°C, 62°C, and 62°C for 30 s, respectively), extension (72°C for 1 min), and a final extension at 72°C for 10 min. PCR product sizes were confirmed by electrophoresis using a 1% agarose gel, stained using 6× LoadingSTAR (Dyn Bio, Seongnam, Korea). PCR products were sequenced by Bionics Corp. The resulting sequence files were aligned using ClustalV in Megalign software 5.05 (DNASTAR Inc.).

In vitro growth assay. OA spontaneous mutants (OX15, OX20, OX40, and OX52) and their parental WT strain (TS3128) were cultured overnight in TSB at 27°C, with 250 rpm shaking. The cell density of each strain was adjusted to 10⁶ cfu/ml in TSB, before placing the suspensions into 96-well culture plates and incubating at 27°C with shaking at 120 rpm. The OD₆₀₀ was measured every hour for 28 h using a microplate reader (Hidex F1/Sense, Turku, Finland). Each experiment was repeated twice with three technical replications.

Virulence assay using immature apple fruits. To test the virulence of spontaneous OA-resistant mutants, each strain (TS3128, OX15, OX20, OX40, and OX52) was used to infect immature apple fruits. The surface of apple fruits (cultivar Hongro; diameter, ~3 cm) was first sterilized with 70% ethanol. Each strain was cultured in TSB media for 24 h, and cell suspensions (10 µl) (containing 10⁸ cfu/ml) were used to inoculate apple fruits at a 2 mm depth using a sterile pipette tip. SDW was also inoculated as a negative control. Infected apples were incubated in a chamber with 95% relative humidity at 27°C. The infection of OA

mutants to the apple fruits was replicated twice with three technical replications. The infection area was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), using color threshold quantification after 3, 6, and 9 days post-inoculation, respectively.

Statistical analysis. For statistical analysis, a one-way ANalysis Of VAriance (ANOVA) and Duncan's multiple range test were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) at each time point.

Results

OA sensitivity of Ea strains collected from apple or pear orchards in Korea. To investigate the occurrence of OA-resistant Ea isolates in Korean orchards, we collected a total of 516 Ea isolates from diseased apple or pear trees sampled from May to June in various regions of Korea in 2020-2021 (Table 1). The resistance breakpoint for OA is defined as concentrations exceeding 5 µg/ml as described by Manulis et al. (2003) and Kleitman et al. (2005). The growth of all collected isolates was completely inhibited by addition of 5 µg/ml OA in TSA (Supplementary Fig. 1), indicating that all isolates were susceptible to OA.

GyrA and GryB amino acid polymorphisms in OA-resistant mutants. The dose incremental contact of the strain TS3128 with OA induced resistance to the bactericide. To define the mechanisms underlying OA-resistance in Ea, the quinolone resistance-determining regions (QRDR) of *gyrA* and *gyrB* (Ruiz, 2003) and mutation emerging regions of *parC* (Kumagai et al., 1996) reported to confer resistance in *E. coli* were amplified from the spontaneous OA-resistant isolates (OX15, OX20, OX40, and OX52) and sequenced. The sequences demonstrated a G to T substitution at nucleotide position 241 in *gyrA* (causing a glycine

to cysteine substitution at amino acid position 81), and a C to A substitution at nucleotide position 249 (causing an arginine to serine substitution at amino acid position 83) (Fig. 1). The amplified region of *gyrB* and *parC* showed no substitution in the region relative to the parental sequence. In addition, the sequences of *gyrA* and *gyrB* obtained from 18 isolates of Ea that were collected from orchards showed 100% homology to the WT sequence (Ea TS3128, NCBI accession No. GCA_013375015.1) (Supplementary Fig. 2), which indicating no nucleotide substitution has been occurred. Taken together, these results indicate that OA-resistance in Ea can be conferred by individual nucleotide substitutions at position 241 and 249 of *gyrA*, and that *gyrB* and *parC* are not influential in OA-resistance acquisition.

In vitro growth of OA-resistant mutants. To assess the changes of growth rate by nucleotide substitution, OA-resistant strains OX15, OX20, OX40, and OX52, and their parental strain TS3128 (WT) (Table 2) were inoculated in TSB media and their growth was monitored at regular intervals. The WT strain TS3128 reached an OD₆₀₀ of 1.02 at 28 h post incubation (Fig. 2). The OA-resistant mutants OX15, OX20, OX40, and OX52 showed reduced growth compared to TS3128 ($P \leq 0.001$), reaching an OD₆₀₀ of 0.54-0.74 after 28 h. The growth of OA-resistant mutants showed large variability with high standard deviations compared to the WT, and there was no significant difference in the overall growth rates of S83R (OX15 and OX20) and G83C (OX40 and OX52) mutants. These results indicate that spontaneous *gyrA* mutations can confer changes in the growth rate of mutant strains in nutrient rich conditions.

Virulence of OA-resistant mutants in apple fruits. To investigate the influence of OA-resistance acquisition on the virulence of Ea, we inoculated each strain into immature apple fruits. The fruits inoculated with WT Ea showed

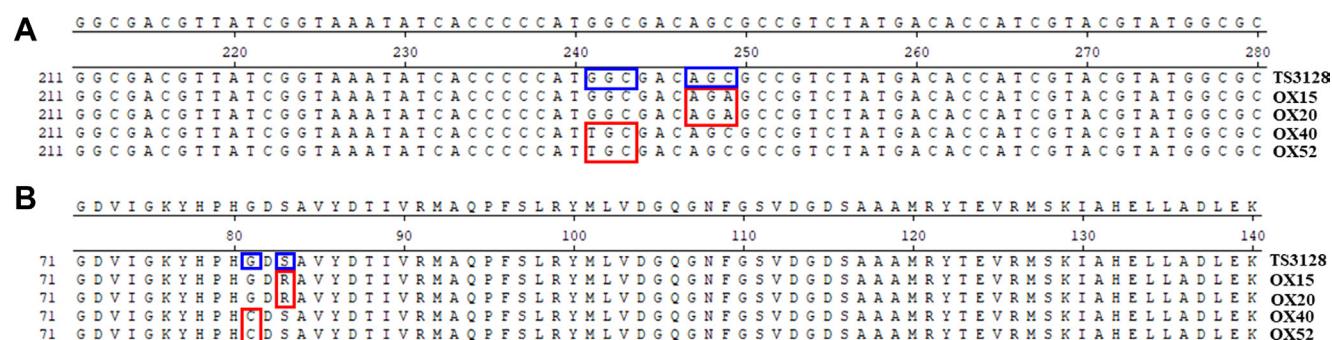


Fig. 1. Mutation sites in *gyrA* of oxolinic acid-resistant spontaneous mutants. Nucleotide (A) and amino acid (B) sequences were aligned between wild-type (TS3128) and mutant strains (OX15, OX20, OX40, and OX52). Blue and red rectangles indicate wild-type and mutant sequences, respectively.

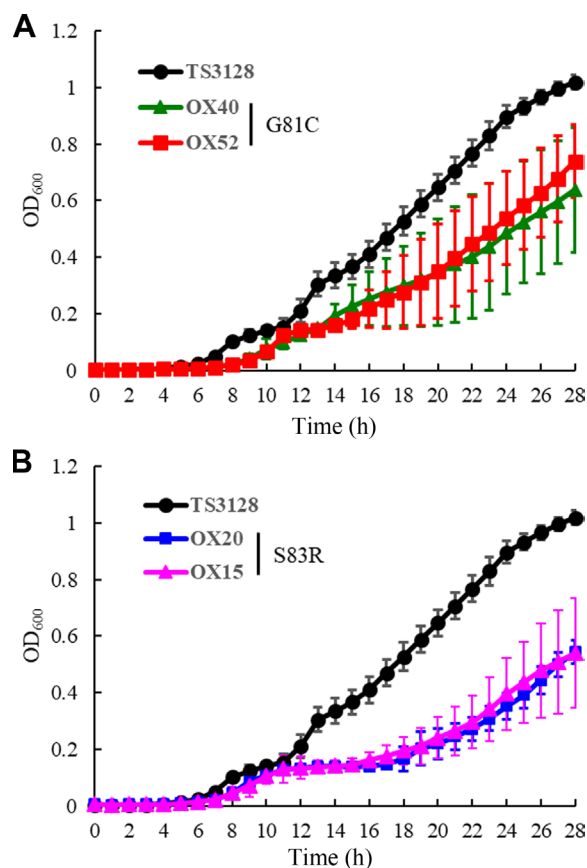


Fig. 2. Growth rate of oxolinic acid-resistant mutants. (A) Growth of *GyrA* mutants at codon 81 (OX40 and OX52). (B) Growth of *GyrA* mutants at codon 83 (OX20 and OX15). Growth was compared with the wild-type parental strain (TS3128). Error bars represented standard deviation from the mean.

necrotic lesions 6 days after inoculation, and the disease symptoms progressed until 9 days after inoculation (Fig. 3). The fruits inoculated with OX15 and OX20 showed smaller lesion sizes compared to the WT and delayed emergence of necrotic symptoms at 6 days post-inoculation ($P < 0.001$). Furthermore, the fruits inoculated with OX40 and OX52 showed no significant increases in necrotic lesions. These results indicate that OA-resistant mutants showed reduced or negligible virulence in an immature apple, which highlighting the role of *gyrA* mutations in pathogenesis or survival of *Ea* in the fruits.

Discussion

OA has been recommended for the control of fire blight in pear and quince (*Cydonia oblonga*) in Israel since 1998, and OA was sprayed 7 times during blooming season (Manulis et al., 2000, 2003) in some orchards. Consequently, OA-resistant *Ea* strains were detected 2 years after the

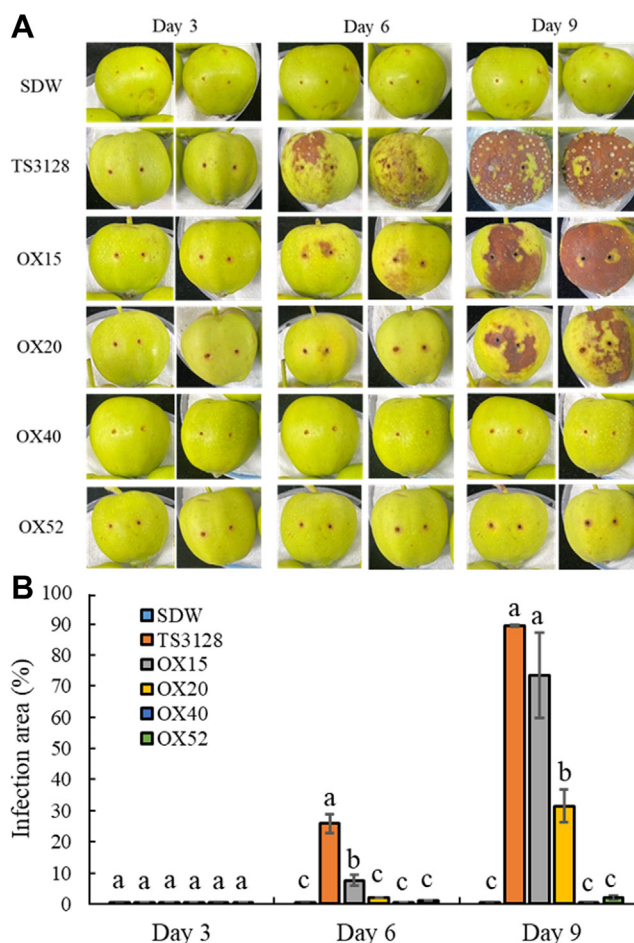


Fig. 3. Virulence of oxolinic acid-resistant mutants in immature apple fruits. (A) Immature apple fruits were inoculated with wild-type (TS3128) and oxolinic acid-resistant mutants (OX15, OX20, OX40, and OX52). The negative control was inoculated with sterilized distilled water (SDW). The inoculated fruits were stored at 95% relative humidity and photos were taken at 3, 6, and 9 days post-inoculation. (B) The infection area was measured using ImageJ using color threshold quantification. Statistical significance was determined with an ANOVA with Duncan's multiple range test at each time point. Common letter means not significantly different from each other ($P < 0.001$).

first application of OA in northern Israel. In South Korea, OA has been recommended for the fire blight control from 2015. We collected *Ea* isolates from diseased apple and pear orchards in Korea to monitor the emergence of OA-resistant *Ea* strains. The growth of all isolates in this study was inhibited by $5 \mu\text{g/ml}$ OA. The nationwide collection of *Ea* strains from apple and pear cultivation areas in this study, in addition to a previous report by Lee et al. (2018), demonstrate that OA-resistant *Ea* are not present in Korea. Only two applications of antibiotics are recommended during blooming season in Korea, which may explain no

occurrence of OA-resistance. In addition, multiple antibiotics including streptomycin, oxytetracycline, OA, and other biological control agents have been in use during the flowering season in Korea. Furthermore, many farmers are reluctant to use antibiotics during blooming due to the potential phytotoxic effect of chemicals when directly applied to flowers (Ham et al., 2020). However, continuous use of OA may readily induce emergence of OA-resistant mutants as has been observed in other bacterial pathogens. Inadequate spray coverage or slow environmental degradation of chemicals may facilitate pathogen exposure to low antibiotic concentrations, eventually leading to the evolution of resistance (McGhee and Sundin, 2011). Furthermore, antibiotic exposure acts to maintain the presence of resistance genes in the population, which can be transferred to previously susceptible organisms through horizontal gene transfer (Stockwell and Duffy, 2012). Despite the shortcomings of routine antibiotic use (McManus et al., 2002), they are still an attractive strategy because of high efficacy in bacterial pathogen control. To manage fire blight without the emergence of resistance, application of OA at adequate control time with several antibiotics having different modes of action is required.

The target of quinolone antibiotics including OA is DNA gyrase in Gram-negative bacteria. DNA gyrase catalyzes topological changes of DNA, especially negatively supercoils or relaxes DNA in the presence or absence of ATP, respectively (Horowitz and Wang, 1987). DNA gyrase comprises the two subunits GyrA and GyrB, forming tetrameric enzymes composed of two A and two B subunits (A₂B₂). GyrA contains a tyrosine active site for DNA cleavage and re-ligation at its N-terminus, and GyrB contains an ATPase active site for ATP binding and hydrolysis. The GyrA-GyrB-DNA interface introduces two negative supercoils into DNA at the cost of two ATPs. Quinolones usually bind to the enzyme-DNA complex and accelerate the rate of DNA cleavage (Collin et al., 2011; Levine et al., 1998). Amino acid substitutions, which are generally occurred in the QRDR of the GyrA N-terminus, confer OA-resistance in Gram-negative bacteria (Ruiz, 2003; Yoshida et al., 1990). Mutations at codons 81 or 83 in GyrA have been reported to confer quinolone resistance in many Gram-negative bacteria including *E. coli*, *B. glumae*, *P. aeruginosa*, and *S. enterica* (Supplementary Table 3). The OA-resistant *Ea* mutants described in this study harbored glycine to cysteine and serine to arginine substitutions at codons 81 and 83 in GyrA (Fig. 1), but no substitutions were observed in GyrB. In the phytopathogen *B. glumae*, the same G81C and S83R substitutions in GyrA were reported in Japan (Maeda et al., 2007), and G81D, D82G,

S83I, D87G, and D87N mutations in GyrA have also been reported to confer resistance in *B. glumae*. The substitution of glycine to cysteine at 81 position, and serine to arginine at 83 position of GyrA may alter the negative charge at the positions, reducing the binding activity of quinolones to DNA gyrase-DNA complex (Maeda et al., 2004, 2007). In GyrB, resistance-conferring amino acid substitutions were reported in *E. coli*, *P. aeruginosa*, and *S. enterica*, but not in *B. glumae* (Maeda et al., 2007). In this study, the OA-resistant *Ea* strains showed no mutations in the *gyrB* gene. Furthermore, *parC* and *parE*, which encode topoisomerase IV, confer quinolone resistance in Gram-positive bacteria and show high degrees of homology to *gyrA* and *gyrB*, respectively (Levine et al., 1998; Ruiz, 2003). Several mutations in *parC* were reported in quinolone-resistant *E. coli*, and a few mutants in *parE* were reported additionally (Kumagai et al., 1996; Ruiz, 2003). In this study, no mutations in *parC* were observed in our OA mutants. Taken together, further work is required to establish the role of mutations in conferring OA-resistance in *Ea*. A detailed analysis of OA-resistant *Ea* mutants may shed light on mechanisms of resistance in this major phytopathogen.

Fitness costs in resistant mutants emerge because antibiotics usually target essential biological functions in the cells. The fitness costs of antibiotic resistance acquisition typically manifest as reduced growth rate in several bacterial pathogens (Andersson and Hughes, 2010). Streptomycin-resistant *S. enterica* subsp. *enterica* serovar Typhimurium harboring K42N or P90S substitutions in RpsL show reduced growth in rich culture medium (Paulander et al., 2009). Fitness costs are also observed in kasugamycin-resistant *Ea* with mutations in the *ksgA* gene (McGhee and Sundin, 2011). *Ea ksgA* mutants showed reduced *in vitro* growth and decreased virulence in immature pear fruits. In this study, the growth and virulence of OA-resistant mutants were attenuated compared to the WT (Figs. 2 and 3). In some antibiotic resistant mutants, despite similar growth rates to the WT strain, we still observed reduced virulence to the hosts. The growth kinetics of an OA-resistant S83R mutant in *B. glumae* was similar to the WT strain, but their virulence was significantly reduced in rice spikelets (Maeda et al., 2007). In *Ea*, there was no significant difference in generation time in Luria-Bertani media between OA-resistant and susceptible *Ea* in Israel. However, when OA-resistant mutants were used to inoculate blossoms, annual shoots, and spurs of pear trees, their colonization efficiency (cfu/blossom or cfu/g plant tissue) was 5 to >50 times lower than that of susceptible *Ea*, indicating reduced potentials of virulence (Kleitman et al., 2005). These reports suggest that the virulence of OA-resistant GyrA mutants is attenuated.

ated. Conversely, in *Campylobacter jejuni*, GyrA mutations conferring ciprofloxacin resistance enhanced fitness in the host chicken (Luo et al., 2005). In isoniazid-resistant *Mycobacterium tuberculosis*, virulence was attenuated in strains harboring a T275P substitution in the catalase-peroxidase enzyme (KatG), but enhanced in those with S315T substitutions (Li et al., 1998; Pym et al., 2002). In *P. aeruginosa*, an I83T mutation in GyrA conferred resistance to nalidixic acid and the mutation also altered expression of the type III secretion system, leading to enhanced virulence (Wong-Beringer et al., 2008). Overall, our results and others indicate that growth and virulence are differently influenced depending on the nature of mutation. Mutants with reduced fitness may struggle to persist in the environment without antibiotic selection, leading to suppression of virulence (Andersson and Hughes, 2010; Melnyk et al., 2015). Bacteria can acquire compensatory mutations to mitigate the fitness defects imposed by resistance mutations (Andersson and Hughes, 2010). The continuous application of antibiotics may provide multiple rounds of selection for fitness compensatory mutations, accelerating the emergence of antibiotic resistant mutants with various growth or virulence phenotypes. Such mutants cannot be controlled under agricultural environments of which treating target antibiotics, eventually causing disease to the host plants.

In summary, OA-resistant Ea isolates were not detected from fire blight infected orchards in Korea in 2020-2021, which indicating no occurrence of OA-resistance in the fields. Substitution at codon 81 and 83 of *gyrA* conferred OA-resistance in Ea strains at the cost of growth and virulence defects. The influence of OA-resistance on the physiological and ecological characteristics of these strains requires further study. Studies of the underlying mechanisms for altered growth rate and virulence will broaden our knowledge on the function of antibiotic resistance in bacterial pathogens. This study mandates implementation of antimicrobial stewardship practices and monitoring programs to prevent the emergence of OA-resistant strains in apple and pear orchards.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This study was carried out with the support of Cooperative Research Programs (Project No. PJ01505902) from Rural Development Administration, Republic of Korea.

Electronic Supplementary Material

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

References

- Ácímović, S. G., Zeng, Q., McGhee, G. C., Sundin, G. W. and Wise, J. C. 2015. Control of fire blight (*Erwinia amylovora*) on apple trees with trunk-injected plant resistance inducers and antibiotics and assessment of induction of pathogenesis-related protein genes. *Front. Plant Sci.* 6:16.
- Andersson, D. I. and Hughes, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8:260-271.
- Bereswill, S., Pahl, A., Bellemann, P., Zeller, W. and Geider, K. 1992. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. *Appl. Environ. Microbiol.* 58:3522-3526.
- Bonn, W. G. and van der Zwet, T. 2000. Distribution and economic importance of fire blight. In: *Fire blight: the disease and its causative agent, Erwinia amylovora*, ed. by J. L. Van Neste, pp. 37-53. CAB International, Wallingford, UK.
- Calzolari, A., Finelli, F. and Mazzoli, G. L. 1999. A severe unforeseen outbreak of fire blight in the Emilia-romagna region. *Acta Hortic.* 489:171-176.
- Collin, F., Karkare, S. and Maxwell, A. 2011. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. *Appl. Microbiol. Biotechnol.* 92:479-497.
- Eaves, D. J., Randall, L., Gray, D. T., Buckley, A., Woodward, M. J., White, A. P. and Piddock, L. J. V. 2004. Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. *Antimicrob. Agents Chemother.* 48:4012-4015.
- Entenza, J. M., Giddey, M., Vouillamoz, J. and Moreillon, P. 2010. In vitro prevention of the emergence of daptomycin resistance in *Staphylococcus aureus* and enterococci following combination with amoxicillin/clavulanic acid or ampicillin. *Int. Antimicrob. Agents* 35:451-456.
- Feng, X., Zhang, Z., Li, X., Song, Y., Kang, J., Yin, D., Gao, Y., Shi, N. and Duan, J. 2019. Mutations in *gyrB* play an important role in ciprofloxacin-resistant *Pseudomonas aeruginosa*. *Infect. Drug Resist.* 12:261-272.
- Ham, H., Lee, K. J., Hong, S. J., Kong, H. G., Lee, M.-H., Kim, H.-R. and Lee, Y. H. 2020. Outbreak of fire blight of apple and pear and its characteristics in Korea in 2019. *Res. Plant Dis.* 26:239-249 (in Korean).
- Hikichi, Y. 1993. Antibacterial activity of oxolinic acid on *Pseudomonas glumae*. *Ann. Phytopathol. Soc. Jpn.* 59:369-374.
- Hikichi, Y., Okuno, T. and Furusawa, I. 1994. Susceptibility of rice spikelets to infection with *Pseudomonas glumae* and its population dynamics. *J. Pestic. Sci.* 19:11-17.
- Horowitz, D. S. and Wang, J. C. 1987. Mapping the active

- site tyrosine of *Escherichia coli* DNA gyrase. *J. Biol. Chem.* 262:5339-5344.
- Kang, I.-J., Park, D. H., Lee, Y.-K., Han, S.-W., Kwak, Y.-S. and Oh, C.-S. 2021. Complete genome sequence of *Erwinia amylovora* strain TS3128, a Korean strain isolated in an Asian pear orchard in 2015. *Microbiol. Resour. Announc.* 10:e00694-21.
- Kleitman, F., Shtienberg, D., Blachinsky, D., Oppenheim, D., Zilberstaine, M., Dror, O. and Manulis, S. 2005. *Erwinia amylovora* populations resistant to oxolinic acid in Israel: prevalence, persistence and fitness. *Plant Pathol.* 54:108-115.
- Kumagai, Y., Kato, J.-I., Hoshino, K., Akasaka, T., Sato, K. and Ikeda, H. 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. *Antimicrob. Agents Chemother.* 40:710-714.
- Lee, M. S., Lee, I., Kim, S. K., Oh, C.-S. and Park, D. H. 2018. *In vitro* screening of antibacterial agents for suppression of fire blight disease in Korea. *Res. Plant Dis.* 24:41-51 (in Korean).
- Levine, C., Hiasa, H. and Mariani, K. J. 1998. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochim. Biophys. Acta* 1400:29-43.
- Li, Z., Kelley, C., Collins, F., Rouse, D. and Morris, S. 1998. Expression of *katG* in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. *J. Infect. Dis.* 177:1030-1035.
- Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L. and Zhang, Q. 2005. Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proc. Natl. Acad. Sci. U. S. A.* 102:541-546.
- Maeda, Y., Kiba, A., Ohnishi, K. and Hikichi, Y. 2004. Implications of amino acid substitutions in *gyrA* at position 83 in terms of oxolinic acid resistance in field isolates of *Burkholderia glumae*, a causal agent of bacterial seedling rot and grain rot of rice. *Appl. Environ. Microbiol.* 70:5613-5620.
- Maeda, Y., Kiba, A., Ohnishi, K. and Hikichi, Y. 2007. Amino acid substitutions in *GyrA* of *Burkholderia glumae* are implicated in not only oxolinic acid resistance but also fitness on rice plants. *Appl. Environ. Microbiol.* 73:1114-1119.
- Manulis, S., Kleitman, F., Dror, O. and Shabi, E. 2000. Isolation of strains of *Erwinia amylovora* resistant to oxolinic acid. *IOBC WPRS Bull.* 23:89-92.
- Manulis, S., Kleitman, F., Shtienberg, D., Shwartz, H., Oppenheim, D., Zilberstaine, M. and Shabi, E. 2003. Changes in the sensitivity of *Erwinia amylovora* populations to streptomycin and oxolinic acid in Israel. *Plant Dis.* 87:650-654.
- McGhee, G. C. and Sundin, G. W. 2011. Evaluation of kasugamycin for fire blight management, effect on nontarget bacteria, and assessment of kasugamycin resistance potential in *Erwinia amylovora*. *Phytopathology* 101:192-204.
- McManus, P. S. and Jones, A. L. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. *Phytopathology* 84:627-633.
- McManus, P. S., Stockwell, V. O., Sundin, G. W. and Jones, A. L. 2002. Antibiotic use in plant agriculture. *Annu. Rev. Phytopathol.* 40:443-465.
- Melnyk, A. H., Wong, A. and Kassen, R. 2015. The fitness costs of antibiotic resistance mutations. *Evol. Appl.* 8:273-283.
- Momol, M. T., Norelli, J. L., Piccioni, D. E., Momol, E. A., Gustafson, H. L., Cummins, J. N. and Aldwinckle, H. S. 1998. Internal movement of *Erwinia amylovora* through symptomless apple scion tissues into the rootstock. *Plant Dis.* 82:646-650.
- Norelli, J. L., Jones, A. L. and Aldwinckle, H. S. 2003. Fire blight management in the twenty-first century: using new technologies that enhance host resistance in apple. *Plant Dis.* 87:756-765.
- Park, D. H., Lee, Y.-G., Kim, J.-S., Cha, J.-S. and Oh, C.-S. 2017. Current status of fire blight caused by *Erwinia amylovora* and action for its management in Korea. *J. Plant Pathol.* 99:59-63.
- Park, D. H., Yu, J.-G., Oh, E.-J., Han, K.-S., Yea, M. C., Lee, S. J., Myung, I.-S., Shim, H. S. and Oh, C.-S. 2016. First report of fire blight disease on Asian pear caused by *Erwinia amylovora* in Korea. *Plant Dis.* 100:1946.
- Paulander, W., Maisnier-Patin, S. and Andersson, D. I. 2009. The fitness cost of streptomycin resistance depends on *rpsL* mutation, carbon source and RpoS (σ). *Genetics* 183:539-546.
- Pym, A. S., Saint-Joanis, B. and Cole, S. T. 2002. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect. Immun.* 70:4955-4960.
- Ruiz, J. 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *J. Antimicrob. Chemother.* 51:1109-1117.
- Shabi, E. and Zutra, D. 1987. Outbreaks of fire blight in Israel in 1985 and 1986. *Acta Hort.* 217:23-32.
- Shtienberg, D., Shwartz, H., Oppenheim, D., Zilberstaine, M., Herzog, Z., Manulis, S. and Kritzman, G. 2003. Evaluation of local and imported fire blight warning systems in Israel. *Phytopathology* 93:356-363.
- Shtienberg, D., Zilberstaine, M., Oppenheim, D., Herzog, Z., Manulis, S., Shwartz, H. and Kritzman, G. 2001. Efficacy of oxolinic acid and other bactericides in suppression of *Erwinia amylovora* in pear orchards in Israel. *Phytoparasitica* 29:143-154.
- Stockwell, V. O. and Duffy, B. 2012. Use of antibiotics in plant agriculture. *Rev. Sci. Tech.* 31:199-210.
- Wong-Beringer, A., Wiener-Kronish, J., Lynch, S. and Flanagan, J. 2008. Comparison of type III secretion system virulence among fluoroquinolone-susceptible and -resistant clinical isolates of *Pseudomonas aeruginosa*. *Clin. Microbiol. Infect.* 14:330-336.
- Yonezawa, M., Takahata, M., Matsubara, N., Watanabe, Y. and Narita, H. 1995. DNA gyrase *gyrA* mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 39:1970-1972.
- Yoshida, H., Bogaki, M., Nakamura, M. and Nakamura, S. 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* 34:1271-1272.