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Amino Acid Substitutions in GyrA of *Burkholderia glumae* Are Implicated in Not Only Oxolinic Acid Resistance but Also Fitness on Rice Plants▽

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Oxolinic acid (OA) resistance in field isolates of *Burkholderia glumae*, a causal agent of bacterial grain rot, is dependent on an amino acid substitution at position 83 in GyrA (GyrA83). In the present study, among spontaneous in vitro mutants from the OA-sensitive *B. glumae* strain Pg-10, we selected OA-resistant mutants that emerged at a rate of 5.7×10^{-10}. Nucleotide sequence analysis of the quinolone resistance-determining region in GyrA showed that Gly81Cys, Gly81Asp, Asp82Gly, Ser83Arg, Asp87Gly, and Asp87Asn are observed in these OA-resistant mutants. The introduction of each amino acid substitution into Pg-10 resulted in OA resistance, similar to what was observed for mutants with the responsible amino acid substitution. In vitro growth of recombinants with Asp82Gly was delayed significantly compared to that of Pg-10; however, that of the other recombinants did not differ significantly. The inoculation of each recombinant into rice spikelets did not result in disease. In inoculated rice spikelets, recombinants with Ser83Arg grew less than Pg-10 during flowering, and growth of the other recombinants was reduced significantly. On the other hand, the reduced growth of recombinants with Ser83Arg in spikelets was compensated for under OA treatment, resulting in disease. These results suggest that amino acid substitutions in GyrA of *B. glumae* are implicated in not only OA resistance but also fitness on rice plants. Therefore, GyrA83 substitution is thought to be responsible for OA resistance in *B. glumae* field isolates.

Oxolinic acid (OA), a quinolone, shows antibacterial activity against gram-negative phytopathogenic bacteria, including *Burkholderia glumae*, *Erwinia amylovora*, and *Pectobacterium carotovora* subsp. *carotovora* (8). Foliar application of OA to heading rice plants has a high efficacy in the control of bacterial grain rot caused by *B. glumae* as well as bacterial rot of potato and onion caused by *P. carotovora* subsp. *carotovora* and fire blight of orchard plants caused by *E. amylovora* (9, 10, 11). Low et al. (24) demonstrated that antibiotic treatment in the long term might provoke the diversification of antibiotic resistance. In Japan, for the past 15 years, OA has been used for disease control three times per rice cultivation season (10). Recently, OA-resistant *B. glumae* was isolated from OA-treated rice plants (12, 25), and furthermore, OA-resistant strains of *E. amylovora* were isolated from pear orchards in Israel, where the number of OA applications since 1998 has been one to three sprays per season (27).

Quinolones act by inhibiting the action of type II topoisomerase, DNA gyrase, and topoisomerase IV (4, 13, 15, 20, 23). DNA gyrase is a tetrameric enzyme composed of two A subunits and two B subunits encoded by *gyrA* and *gyrB*, respectively (6, 34). Topoisomerase IV is also an A_2B_2 enzyme encoded by *parC* and *parE* (16, 17, 23), which are highly homologous to *gyrA* and *gyrB*, respectively. The main function of DNA gyrase is to catalyze the negative supercoiling of DNA (14), while topoisomerase IV seems to be associated with decatenation of the daughter replicons (4). The target of quinolones in gram-negative bacteria is different from that in gram-positive bacteria: in gram-negative bacteria, the target is DNA gyrase, whereas in gram-positive bacteria, the target is topoisomerase IV (31). Therefore, either *gyrA* or *gyrB* mutations are responsible for the resistance of gram-negative bacteria. In addition, topoisomerase IV mutations can further increase this level of resistance. Decreased quinolone uptake is also involved in quinolone resistance and is thought to be associated with one of two factors: increased membrane impermeability of bacteria to quinolones or the overexpression of efflux pumps (31). In gram-negative bacteria, the major target of mutations is the *gyrA* gene (41), and most of the mutations identified so far have been located in a small region of GyrA, the quinolone resistance-determining region (QRDR) (40).

In a previous study, we demonstrated that Ser83Arg and Ser83Ile substitutions, which are located in the QRDR, in GyrA are commonly responsible for OA resistance in *B. glumae* field isolates, suggesting that only OA-resistant isolates with Ser83Arg and Ser83Ile can retain their fitness on rice plants grown in paddy fields (25). Furthermore, OA-resistant *E. amylovora* strains reportedly have lower fitness than wild-type strains (19). However, the implications of OA resistance in the fitness of the bacteria on their host plants remain unclear.

In this study, OA-resistant mutants were selected among spontaneous in vitro mutants from the *B. glumae* strain Pg-10 and their OA-resistant mechanisms were investigated by nucleotide sequencing analysis of the QRDR in GyrA of the mutants and the introduction of amino acid substitutions responsible for the amino acid mutations in the OA-resistant mutants into the GyrA of Pg-10. Moreover, the growth of these GyrA recombinants on rice plants was also compared to that of

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference and/or source
E. coli strain		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17supE44* Δ(*lac*)*U169*(ϕ*80lac*Δ*M15*)	TaKaRa and this study
B. glumae strains		
Pg-10	Wild type	12
81C	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-81C Km^r	This study
81D	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-81D Km^r	This study
82G	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-82G Km^r	This study
83R	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-83R Km^r	This study
87G	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-87G Km^r	This study
87N	Recombinant of Pg-10 with 3.9-kb DNA fragment of pGY-87N Km^r	This study
Plasmids		
pBluescript KS	Amp^r	Stratagene
pGEM-T	Amp^r	Promega
pHSG398	Cm^r	TaKaRa
pCUD800	*sacB* Km^r	5
pHSG398RI	pHSG398 derivative	25
pGY-R2	1.2-kb DNA fragment from Pg-10 in pHSG398RI	25
pGY-81CsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-81C) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study
pGY-81DsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-81D) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study
pGY-82GsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-82G) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study
pGY-83RsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-83R) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study
pGY-87GsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-87G) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study
pGY-87NsacB	2.6-kb *sacB* and 1.2-kb DNA fragment (RI-Kpn-87N) amplified by recombinant PCR from Pg-10 in pGYRA-2	This study

Pg-10. The results suggest that only OA-resistant *B. glumae* isolates with a substitution of an amino acid at position 83 of GyrA (GyrA83) retain their fitness on rice plants, especially with OA application. Therefore, GyrA83 substitution is thought to be responsible for the OA resistance of *B. glumae* field isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. glumae* strains were routinely grown in peptone-yeast (PY) medium (peptone, 5 g; yeast extract, 2 g; deionized water, 1 liter) at 30°C (12). *Escherichia coli* strains were grown in LM medium (7) at 37°C. The following antibiotics were used in the selective media in the amounts indicated (μg/ml): ampicillin, 50; kanamycin, 50; chloramphenicol, 50.

Antibiotic susceptibility assay. OA was kindly gifted by Sumitomo Chemical Co. Ltd. The MICs of OA were determined using the sequential dilution method. Briefly, 1.0×10^2 freshly grown cells of the tested *B. glumae* strain were incubated on an OA-containing PY agar medium at 30°C for 2 days. OA was dissolved in NaOH solution, the concentration of which was 3/10 that of OA. The NaOH concentrations used in this study had no influence on bacterial growth. Bacterial populations grown in vitro were spectrophotometrically assayed in three independent experiments with an optical density at 580 nm using a biophotorecorder (Advantec, Tokyo, Japan). The lowest concentration of antibiotic that completely inhibited growth was defined as the MIC.

DNA manipulations. Plasmid and chromosomal DNA isolation, cloning, subcloning, and PCR were performed according to standard procedures (33). Restriction enzymes were obtained from TaKaRa (Otsu, Japan). *B. glumae* was transformed by electroporation as described by Allen et al. (1). DNA sequences were determined using an automated DNA sequencer (model 373; Applied Biosystems, Tokyo, Japan) and analyzed using DNASIS-Mac software (Hitachi Software Engineering, Yokohama, Japan).

Spontaneous in vitro mutants resistant to OA. To isolate spontaneous OA-resistant mutants from Pg-10, Pg-10 was incubated in PY medium at 30°C for 24 h and then washed three times with sterilized water by centrifugation at $12{,}000 \times g$ for 1 min. The pellet was suspended in sterilized water, and the bacterial density was adjusted to 1.0×10^{12} CFU/ml. The suspension was then spread onto PY agar media containing 1.0 μg/ml OA and incubated at 30°C for 3 days. Mutation rates (μ) to OA resistance were calculated according to the equation $\mu = \ln(P_0/N)$, where P_0 represents the number of cultures without mutants divided by the total number of cultures and N represents the total number of bacteria plated (22).

Determination of GyrA83 using MAMA PCR. To detect GyrA83 substitutions in the spontaneous OA-resistant mutants, 5′-CATCCGCACGGCGACAGC-3′ (gyrA-Ser), 5′-CATCCGCACGGCGACAGR-3′ (gyrA-Arg), and 5′-CATCCGCACGGCGACAT-3′ (gyrA-Ile) forward primers and the reverse gyrA2 primer 5′-GCGATCCCGGACGAGCC-3′ were used in the mismatch amplification mutation assay (MAMA) PCR (26). PCR amplification was performed with 1 cycle of 94°C for 4 min and 25 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 30 s. Nine-microliter aliquots of each PCR product were loaded onto horizontal 2% Tris-acetate-EDTA agarose gels and stained with ethidium bromide for the detection of 293-bp DNA fragments after electrophoresis.

DNA sequencing of QRDR in *gyrA*. To analyze the DNA sequences of the QRDRs in *gyrA* of the spontaneous in vitro mutants, a 338-bp DNA fragment was PCR amplified from chromosomal DNA using 5′-TACAAGAAGTCGGCGCG-3′ (gyrA1) and 5′-GCGATCCCGGACGAGCC-3′ (gyrA2) as primers (25). Each PCR product was purified by 2% Tris-acetate-EDTA gel electrophoresis and Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad, Hercules, CA). The DNA sequences of the PCR products were analyzed using gyrA1 and gyrA2.

Amino acid substitutions in the GyrA of Pg-10. The single point mutations corresponding to Gly81Cys, Gly81Asp, Asp82Gly, Ser83Arg, Asp87Gly, and Asp87Asn in the GyrA of Pg-10 were introduced into a 1.2-kb DNA fragment containing the *gyrA* QRDR from chromosomal Pg-10 DNA using recombinant PCR as follows: DNA fragments, RI-Kpn-81C, RI-Kpn-81D, RI-Kpn-82G, RI-Kpn-83R, RI-Kpn-87G, and RI-Kpn-87N, respectively, were synthesized by PCR using two pairs of primers, the forward primer 5′-GCGAATTCGGGGAGGGCGCG-3′ (RI−gyrA+) and reverse primer X and forward primer Y and the reverse primer 5′-GGGGTACCTCGCCGCGCTTG-3′ (Kpn−gyrA−). The X

TABLE 2. Primers used for recombinant PCR to introduce amino acid substitutions into the GyrA of *B. glumae* strain Pg-10

Amino acid substitution in GyrA	Primer	Sequence[a]
Gly81Cys	X	5′-TACCATCCGCACTGCGACAGCGCGGTC-3′
	Y	5′-GACCGCGCTGTCGCAGTGCGGATGGTA-3′
Gly81Asp	X	5′-TACCATCCGCACGACGACAGCGCGGTC-3′
	Y	5′-GACCGCGCTGTCGTCGTGCGGATGGTA-3′
Asp82Gly	X	5′-CATCCGCACGGCGGCAGCGCGGTCTAC-3′
	Y	5′-GTAGACCGCGCTGCCGCCGTGCGGATG-3′
Ser83Arg	X	5′-CCGCACGGCGACAGAGCGGTCTACGAC-3′
	Y	5′-GTCGTAGACCGCTCTGCCGCCGTGCGG-3′
Asp87Gly	X	5′-AGCGCGGTCTACGGCACGATCGTCCGG-3′
	Y	5′-CCGGACGATCGTGCCGTAGACCGCGCT-3′
Asp87Asn	X	5′-AGCGCGGTCTACAACACGATCGTCCGG-3′
	Y	5′-CCGGACGATCGTGTTGTAGACCGCGCT-3′

[a] Underlined letters indicate nucleotide changes.

and Y primers are presented in Table 2. PCR amplification was performed with 1 cycle of 94°C for 2 min and 5 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, followed by 20 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. Each PCR product was purified by agarose gel electrophoresis, then mixed, and PCR amplified using the RI−gyrA+ and Kpn−gyrA− primers. PCR amplification was performed with 1 cycle of 94°C for 2 min and 25 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. RI-Kpn-81C, RI-Kpn-81D, RI-Kpn-82G, RI-Kpn-83R, RI-Kpn-87G, and RI-Kpn-87N fragments were digested with EcoRI and KpnI and then substituted into EcoRI/KpnI-digested pGYRA-2 fragments (25) containing *gyrA* PCR amplified from genomic Pg-10 DNA to create pGY-81C, pGY-81D, pGY-82G, pGY-83R, pGY-87G, and pGY-87N, respectively. Next, a 2.6-kb PstI- and BamHI-digested DNA fragment containing *sacB* from pUCD800 (5) was blunt ended by T4 DNA polymerase and ligated into the blunt-ended pGY-81C, pGY-81D, pGY-82G, pGY-83R, pGY-87G, and pGY-87N KpnI sites, as described above, to create pGY-81CsacB, pGY-81DsacB, pGY-82GsacB, pGY-83RsacB, pGY-87GsacB, and pGY-87NsacB, respectively. These plasmids were electroporated into Pg-10 cells, and the chloramphenicol-resistant and sucrose-sensitive recombinants were selected. The recombinants were incubated in PY medium for 6 h, and then the following chloramphenicol-sensitive and sucrose-resistant recombinants were selected: 81C, 81D, 82G, 83R, 87G, and 87N with Gly81Cys, Gly81Asp, Asp82Gly, Ser83Arg, Asp87Gly, and Asp87Asn in GyrA, respectively.

Plant assays. Rice (*Oryza sativa* L. cv. Koshihikari) plants were grown in 1/5,000 Wagner pots in a greenhouse at 18 to 30°C. Rice plants at booting were inoculated with 1.0×10^8 CFU/ml suspension of *B. glumae* by spraying an application of 5 ml/pot, as described previously (9, 11), and then grown in a growth room at 25°C under 10,000 lx for 16 h per day. A 200-μg/ml solution of OA was applied to flowering rice spikelets by a spray application of 5 ml/pot. Diseased spikelets were observed at 14 days after flowering, and the percentage of diseased spikelets was calculated. Each assay was repeated in five successive trials, and within each trial, each strain was used to inoculate 12 plants.

Bacterial populations in rice spikelets. Rice spikelets at 5 days after flowering were washed with water, weighed, and ground with a mortar and pestle in distilled water. The original solution and 10-fold serial dilutions were then dispensed in 0.1-ml aliquots and spread onto S-PG media (37) or S-PG media plus OA at 2 μg/ml. Bacterial colonies were counted to estimate the pathogen population after 2 days of incubation at 42°C, as described previously (9, 11).

RESULTS

Spontaneous in vitro mutants resistant to OA. After incubation of Pg-10 on PY agar media containing 1.0 μg/ml OA, we isolated 328 OA-resistant spontaneous mutants that emerged at a rate of 5.7×10^{-10}. The MIC of OA in these spontaneous mutants was then determined. The spontaneous mutants were divided into five classes based on their OA MIC as follows: the MICs of OA in 3, 2, 47, 232, and 44 mutants, hereafter referred to as MIC5, MIC10, MIC20, MIC50, and MIC100 mutants, were 5.0, 10, 20, 50, and more than 100 μg/ml, respectively (Table 3). GyrA83 analysis by MAMA PCR showed that the GyrA83 of all MIC5, MIC10, and MIC20 mutants, 55 MIC50 mutants, and 24 MIC100 mutants was Ser. Furthermore, the GyrA83 of 177 MIC50 mutants and 18 MIC100 mutants was Arg, and the GyrA83 of two MIC100 mutants was Ile. These results indicate that not only the GyrA83 mutation but also additional amino acid mutations lead to the OA resistance of these spontaneous mutants.

TABLE 3. MICs of OA in spontaneous in vitro OA-resistant mutants from *B. glumae* strain Pg-10 and deduced amino acids at position 83 (GyrA83) in GyrA of the mutants

MIC of OA (μg/ml)	GyrA83	No. of isolates
5	Ser	3
10	Ser	2
20	Ser	47
50	Ser	55
	Arg	177
≥100	Ser	24
	Arg	18
	Ile	2

Deduced amino acid sequences of the QRDR in GyrA of the spontaneous in vitro OA-resistant mutants. To elucidate the relationship between OA resistance and the deduced amino acid sequences of the QRDR in GyrA of the spontaneous in vitro OA-resistant mutants, the DNA sequences of the QRDR in *gyrA* from 3 MIC5, 2 MIC10, 47 MIC20, and 184 MIC50 mutants were analyzed. The deduced amino acid at position 82 in the GyrA (GyrA82) of two MIC5 mutants was Gly, though that of Pg-10 was Asp (Fig. 1). Furthermore, the deduced amino acid at position 87 in the GyrA (GyrA87) of an additional MIC5 mutant was Gly instead of Asp. The GyrA87 of all MIC10 mutants was Asn instead of Asp. In the MIC20 mutants, the deduced amino acid at position 81 in GyrA (GyrA81) was Asp or Cys instead of Gly. Among the MIC50 mutants, 159 had Ser83Arg in GyrA, while an additional mutant had Gly81Asp. No other amino acid substitutions were observed in the QRDR of any of the mutants.

Involvement of amino acid substitutions in GyrA in OA resistance. To determine whether Gly81Cys, Gly81Asp, Asp82Gly, Ser83Arg, Asp87Gly, and Asp87Asn in GyrA result in OA-resistant bacteria, a point mutation was introduced into *gyrA* in Pg-10 by marker exchange. Next, 81C, 81D, 82G, 83R, 87G, and 87N with Gly81Cys, Gly81Asp, Asp82Gly, Ser83Arg, Asp87Gly, and Asp87Asn in GyrA, respectively, were created. The MICs of OA in 81C, 81D, 82G, 83R, 87G, and 87N were 20, 20, 5.0, 50, 5.0, and 10 μg/ml, respectively, suggesting that not only Ser83Arg but also Gly81Cys, Gly81Asp, Asp82Gly, Asp87Gly, and Asp87Asn in GyrA are implicated in OA resistance of the spontaneous in vitro mutants.

In vitro growth of GyrA recombinants. To analyze the influence of amino acid substitutions in GyrA on bacterial growth, the in vitro growth of the recombinants 81C, 81G, 82G, 83R, 87G, and 87N was analyzed. 81C, 81G, 83R, 87G, and 87N

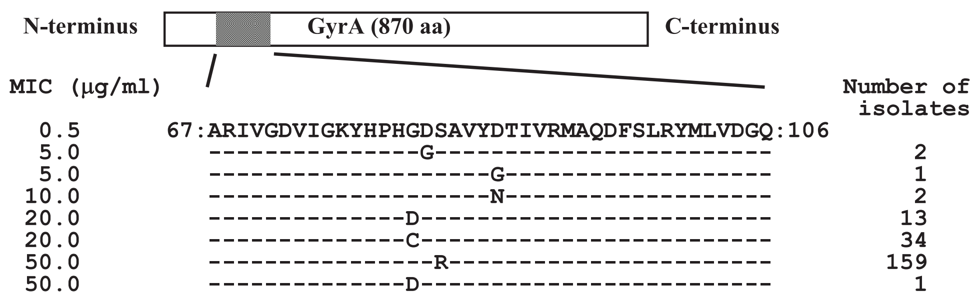

FIG. 1. MIC of OA in spontaneous in vitro mutants from *B. glumae* strain Pg-10 and their deduced amino acid sequences of the QRDR in GyrA. aa, amino acids.

grew vigorously, similar to the parent strain Pg-10, while the growth of 82G, on the other hand, was delayed significantly compared to that of Pg-10 (Fig. 2).

Ability of GyrA recombinants to grow in rice spikelets and their virulence on rice plants. After invading flowering spikelets of rice plants, *B. glumae* grows vigorously for 5 days, resulting in bacterial grain rot (9, 11). Therefore, the growth ability of the bacteria in rice spikelets affects their virulence. To analyze the influence of amino acid substitutions in GyrA on bacterial growth in rice spikelets and on bacterial virulence, rice plants at booting were inoculated with GyrA recombinants and their populations in spikelets at 5 days after flowering were investigated. The Pg-10 population reached 7.0×10^6 CFU/g (Fig. 3), and the percentage of diseased spikelets was 82.4% (Table 4). The population of 83R was 3.8×10^5 CFU/g (Fig. 3), and only 5.8% of spikelets were diseased (Table 4). On the other hand, the populations of 81C, 81D, 87G, and 87N were less than that of Pg-10 at 1.9×10^4 to 4.2×10^4 CFU/g (Fig. 3). Moreover, the population of 82G was 9.2×10^4 CFU/g, significantly reduced compared to that of Pg-10. No diseased spikelets were observed in rice plants inoculated with 81C, 81D, 82G, 87G, and 87N (Table 4). These results suggest that among the recombinants, only 83R retains its ability to grow in rice spikelets, but at a lower rate than the parent strain Pg-10.

Foliar application of OA prevents *B. glumae* growth in rice spikelets, resulting in high control efficacy of the disease (9, 11). When OA was applied to flowering spikelets, the population of Pg-10 at 5 days after flowering was 1.0×10^3 CFU/g (Fig. 3) and no spikelets were diseased (Table 4). The populations of all GyrA recombinants after treatment of rice spikelets with OA were more abundant than those in nontreated rice spikelets. Especially, the population of 83R reached 3.2×10^6 CFU/g (Fig. 3), and 58.1% of spikelets were diseased (Table 4). The percentages of diseased spikelets inoculated with other GyrA recombinants were less than 3.0%. These results suggest that OA application may lead to an increase in the ability of GyrA recombinants, especially those with Ser83Arg in GyrA, to grow in rice spikelets.

DISCUSSION

In gram-negative quinolone-resistant bacteria such as *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium, the majority of mutations described to date have been found within the QRDR, which is located in the N terminus (Ala67-Gln106) of the GyrA, situated close to the catalytic cleavage residue Tyr at position 122, which interacts with the broken DNA strand during the topoisomerase reaction (4, 13, 15, 20, 23). The high-resolution structure of the

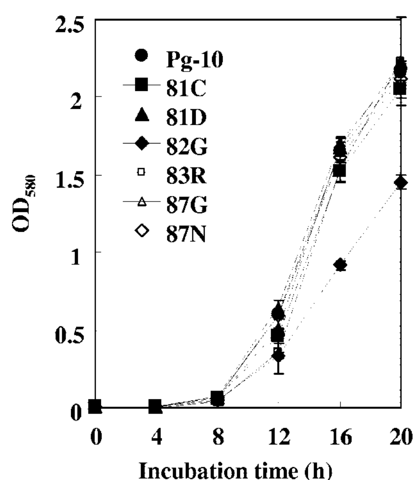

FIG. 2. In vitro growth of *B. glumae* recombinants with amino acid substitutions in GyrA. Values represent the means ± standard deviations for three separate experiments. OD_{580}, optical density at 580 nm.

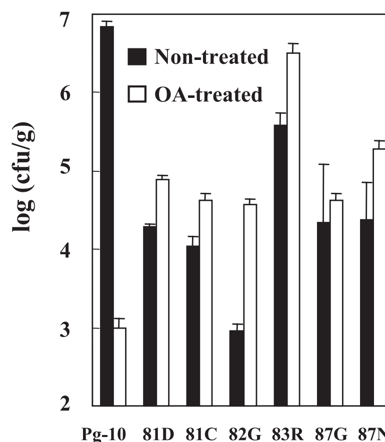

FIG. 3. Populations of *B. glumae* recombinants with amino acid substitutions in GyrA on rice spikelets at 5 days after flowering. Rice spikelets were inoculated with the recombinants at 10 days before flowering. Values represent the means ± standard deviations for three separate experiments.

TABLE 4. Percentage of diseased spikelets at 5 days after flowering in rice plants inoculated with *gyrA* recombinants of *B. glumae*

Group	% of diseased spikelets in rice plants inoculated with the indicated strain or recombinant						
	Pg-10	81C	81D	82G	83R	87G	87N
Nontreated rice spikelets	82.4	0	0	0	5.8	0	0
OA-treated rice spikelets	0	2.9	1.8	0.7	58.1	1.1	1.4

breakage-reunion domain of DNA gyrase suggests that QRDR residues, which are solvent exposed, may be involved in DNA binding (29). Our previous study showed that Ser83Arg and Ser83Ile in GyrA are, respectively, implicated in moderate and high OA resistance of *B. glumae* field isolates, with no mutations other than GyrA83 being observed in the QRDR of *B. glumae* field isolates (25). On the other hand, the results of the present study show that not only Ser83Arg but also Gly81Cys, Gly81Asp, Asp82Gly, Asp87Gly, and Asp87Asn in the QRDR of GyrA confer OA resistance in this bacterium. The MICs of OA in recombinants with Asp82Gly and Asp87Gly, Asp87Asn, and Gly81Cys and Gly81Asp substitutions in GyrA were 5, 10, and 20 μg/ml, respectively, showing that the OA resistance conferred by these amino acid substitutions is lower than that conferred by Ser83Arg and Ser83Ile. The presence of high-level resistance to quinolones in clinical *E. coli* isolates was associated with mutations at hot spots, codons 83 and 87 in GyrA (3). Gly81Cys, Gly81Asp, Asp82Gly, Asp87Gly, and Asp87Asn substitutions on their own reportedly result in low-level resistance to fluoroquinolones (4, 28, 30). Furthermore, Asp87Gly is associated with lower ciprofloxacin resistance than Asp87Asn (32). The present results show that OA resistance conferred by Asp87Gly is lower than that conferred by Asp87Asn. Though Asp87Gly and Asp87Asn involve the loss of a negatively charged amino acid, Gly and Asn are polar and nonpolar residues, respectively. The Gly81Cys and Gly81Asp substitutions involve substitution from a neutral-polar amino acid to a nonpolar amino acid and negatively charged amino acid, respectively. The Asp82Gly substitution also involves the loss of a negatively charged amino acid. Therefore, these changes may influence the ability to form hydrogen bonds and the negative charge at these positions, which is important for quinolone interactions with the DNA gyrase-DNA complex, leading to OA resistance.

By repeating the cycle of mutagenesis and selection, the *E. coli dnaQ49* mutator acquires high ofloxacin resistance (36). Moreover, under the strong pressure of ofloxacin, *dnaQ49* also follows the history of mutations in the gyrase and topoisomerase IV genes, as previously observed in clinical isolates of quinolone-resistant *E. coli*; this is dependent on nucleotide mutation, a main cause of evolution that accompanies DNA replication. In addition, Yasuda et al. (38) reported that the clinical emergence of fluoroquinolone-resistant strains may be due to the in vivo stepwise selection of strains with genetic alterations in GyrA and ParC, as observed in the in vitro selection of fluoroquinolone-resistant mutants. The results of the present study suggest that not only GyrA83 substitution but also Gly81Cys, Gly81Asp, Asp82Gly, Asp87Gly, and Asp87Asn in GyrA are involved in the OA resistance of spontaneous in vitro mutants from Pg-10. Therefore, it is thought that serial OA treatment of rice plants growing in paddy fields will result in selection of OA-resistant isolates with Gly81Cys, Gly81Asp, Asp82Gly, Asp87Gly, and Asp87Asn substitutions in GyrA as well as isolates with Ser83Arg and Ser83Ile. The solubility of OA in water is 3.2 μg/ml (10). Here, populations of GyrA recombinants in rice spikelets treated with OA were greater than in nontreated spikelets, suggesting that OA-resistant *B. glumae* isolates existing on rice plants grown in paddy fields might be theoretically exposed to OA solutions at concentrations below the MIC of OA. Nevertheless, it remains to be elucidated why only Ser83Arg and Ser83Ile in GyrA are commonly responsible for OA resistance in *B. glumae* field isolates.

The assessment of DNA supercoiling in a GyrA mutant with single and double alanine substitutions at GyrA83 and GyrA87 showed that mutation of GyrA87 leads to a loss of supercoiling activity, whereas mutation of GyrA83 is largely without effect on enzyme activity (2). Moreover, slow-growing quinolone-resistant mutants from *P. aeruginosa* with a *gyrA* mutation showed decreased DNA supercoiling (21). Furthermore, in the present study, the in vitro growth ability of recombinants with an Asp82Gly substitution was significantly reduced. We selected only two mutants with Asp82Gly among the 236 OA-resistant mutants. These findings suggest that amino acid substitutions in GyrA may be implicated in not only OA resistance but also in vitro growth of the bacteria.

Kleitman et al. (19) reported that OA-resistant *E. amylovora* strains have lower fitness than wild-type strains under no OA treatment. Here, analysis of bacterial populations in rice spikelets showed that the Ser83Arg recombinants grew less than Pg-10, while the growth abilities of the other OA-resistant recombinants were significantly reduced. Since in vitro growth of recombinants with Gly81Cys, Gly81Asp, Ser83Arg, Asp87Gly, and Asp87Asn substitutions was similar to that of Pg-10, these amino acid substitutions are thought to result in a reduction of fitness with respect to the ability to proliferate in rice spikelets. Therefore, amino acid substitutions in GyrA are responsible for not only OA resistance but also the reduction of fitness in rice plants. However, the reduction of fitness by GyrA83 substitution may be less than that by other amino acid substitutions in GyrA. Interestingly, the population of 83R in OA-treated rice spikelets was similar to that of Pg-10 in nontreated rice spikelets; that is, in spikelets, the reduced growth of recombinants with Ser83Arg was compensated for under OA treatment. There are three possible factors thought to be related to the fitness of 83R in OA-treated rice spikelets: interactions between OA and the DNA gyrase-DNA complex, the involvement of OA in the association between the mutated GyrA and GyrB subunits, and the contribution of other environmental factors in rice spikelets to DNA gyrase activity, leading to bacterial growth.

Toxoflavin is a phytotoxin, produced by *B. glumae*, with a broad host range and is involved in the development of bacterial grain rot (35, 39). The production of toxoflavin in *B. glumae* and its transportation into plant cells are regulated by quorum sensing through *N*-octanoyl homoserine lactone (18). The disease development thus depends on vigorous growth of *B. glumae* in flowering spikelets (9, 11). These evidences support that the reduced fitness of 83R in spikelets is compensated

for under OA treatment, resulting in disease. In Japan, OA is used for disease control three times per rice cultivation season (10). Therefore, in rice plants cultivated in paddy fields, *B. glumae* with Ser83Arg and Ser83Ile in GyrA may be selected among OA-resistant bacteria by nucleotide mutations caused during DNA replication, and serial pressure of OA may allow these OA-resistant bacteria to compensate for their reduced fitness in rice plants. Taken together, the present findings suggest that GyrA83 substitution is commonly responsible for OA resistance of *B. glumae* field isolates.

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