New method to detect oxolinic acid-resistant *Burkholderia glumae* infesting rice seeds using a mismatch amplification mutation assay polymerase chain reaction

**Abstract**  Bacterial seedling rot and grain rot of rice caused by *Burkholderia glumae* are seed-borne diseases, traditionally controlled with oxolinic acid (OA). Ser83Arg and Ser83Ile substitutions in GyrA protein are commonly responsible for moderate and high resistance to OA, respectively, in field isolates of *B. glumae*. To detect OA-resistant *B. glumae* infesting rice seeds, a mismatch amplification mutation assay polymerase chain reaction protocol was developed using DNA from bacteria incubated in modified S-PG medium and primers based on the amino acid substitutions at position 83 in GyrA.

**Key words**  *Burkholderia glumae* · Oxolinic acid · MAMA PCR

Bacterial grain rot and seedling rot of rice caused by *Burkholderia glumae* Kurita et Tabei cause serious yield losses of rice in Japan. Bacterial seedling rot in growing seedlings is caused by a rapid increase in the *B. glumae* population in the epidermis of plumules when seed germination is artificially hastened (Hikichi 1993a,b). After the rice seedlings are transplanted into paddy fields, the pathogen colonizes the upper leaf sheath containing the flag leaf sheath, invades the flowering spikelets, multiplies rapidly, and eventually rots the grain (Hikichi et al. 1994). To control the disease, farmers in Japan have treated seeds and heading rice plants with oxolinic acid (OA) (Hikichi et al. 1989). The chemical, which inhibits bacterial growth in the plumules and spikelets, controls bacterial seedling rot and bacterial grain rot of rice with high efficacy (Hikichi 1993a,b; Hikichi et al. 1994).

Recently, OA-resistant *B. glumae* was isolated from rice seedlings grown from OA-treated seeds (Hikichi et al. 1998). Maeda et al. (2004) later reported that, based on the minimum inhibitory concentration (MIC) of OA, OA-resistant field isolates of *B. glumae* could be grouped into moderately resistant isolates (MRs) (50 µg/ml) and highly resistant isolates (HRs) (>100 µg/ml). Ser83Arg and Ser83Ile substitutions in GyrA are commonly responsible for moderate and high resistance to OA in field isolates of *B. glumae*, respectively (Maeda et al. 2004). Because the conditions for raising seedlings are also suitable for growth of the seed-borne bacteria, the growth of OA-resistant bacteria on rice plants can eventually lead to bacterial seedling rot and grain rot, with subsequent significant losses in production.

In this article we report a new method to detect OA-resistant *B. glumae* in rice seeds. We used a mismatch amplification mutation assay (MAMA) polymerase chain reaction (PCR) (Cha et al. 1992; Zirnstein et al. 1999) based on the amino acid at position 83 in GyrA (*GyrA*83) of *B. glumae* field isolates.

Genomic DNA was isolated from *B. glumae* Pg-10 susceptible to OA, Pg-7 moderately resistant to OA, and Pg-15 highly resistant to OA using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s manual. To discriminate whether *GyrA*83 of the bacteria was Ser, Arg, or Ile, forward primers 5′-CATCCGCACGGCGACAGC-3′ (*gyrA*-Ser), 5′-CATCCGCACGGCGACAGR-3′ (*gyrA*-Arg), and 5′-CATCCGCACGGCGACAT-3′ (*gyrA*-Ile) and the reverse primer 5′-GCGATCCCGGACGAGCC-3′ (named *gyrA*-R4) were used for the MAMA PCR. The PCR amplification was performed by 1 cycle of 94°C for 4 min; 25 cycles of 94°C for 1 min; 70°C, 67°C, or 65°C for 1 min; and 72°C for 30 s, respectively. A 9-μl sample of each PCR product was loaded onto horizontal 2.0% Tris-acetate-EDTA (TAE) agarose gels and stained with ethidium bromide to detect a specific 293-bp DNA fragment corresponding to the nucleotide sequences of *gyrA* of the isolates after electrophoresis. The 293-bp DNA fragment was amplified from genomic DNAs of Pg-10, Pg-7, and Pg-15 using *gyrA*-Ser, *gyrA*-Arg, and *gyrA*-Ile, respec-
Rice seeds (*Oryza sativa* L. cv. Koshihikari) were inoculated with Pg-10 susceptible to OA, Pg-7 moderately resistant to OA, or Pg-15 highly resistant to OA by immersion in a bacterial solution at 1.0 × 10⁶ cfu/ml for 4 h and then were dried at room temperature. To extract genomic DNA from *B. glumae* infesting rice seeds, 30 g of rice seeds (cv. Koshihikari), including one seed inoculated with Pg-10, Pg-7, or Pg-15, were vortexed in 50 ml of water. The water was filtered through mixed cellulose ester membranes (0.2µm) (Advantec, Tokyo, Japan). The membrane filters that had been infiltrated with water in which 30 g of noninoculated rice seeds (about 1,000 seeds) had been vortexed (Fig. 2), suggesting that *B. glumae* had not infected the seeds. When 30 g of rice seeds containing one Pg-10-inoculated seed was used, the 293-bp DNA fragment was amplified in MAMA PCR using gyrA-Ser as a forward primer, but it was not amplified using gyrA-Arg and gyrA-Ile (Fig. 2). Similarly, when rice seeds containing either a Pg-7- or Pg-15-inoculated seed were used, the DNA fragment was only amplified using gyrA-Arg or gyrA-Ile, respectively. Moreover, when 30 g of rice seeds containing one seed each of the Pg-10-, Pg-7-, and Pg-15-inoculated seeds was used, the 293-bp DNA fragment was amplified using any of the primers as a forward primer. These results suggest that the PGSLM MAMA PCR allows discrimination of GyrA83 of the bacteria in rice seeds.

Rice seeds (cv. Nipponbare) produced from rice plants inoculated with *B. glumae* Y2 (Miyagawa 2000) at flowering were dipped in a solution of OA (20% wettable powder; Sumitomo Chemical, Tokyo, Japan) at 1.000µg/ml for 24 h. The seeds were soaked in water for 2 days at 25°C, germinated at 30°C for 1 day, and then sown in nursery soil (Toku No. 2; UBE Industries, Tokyo, Japan) using a serial procedure as described previously (Hikichi 1993b). For each trial, 50 g of rice seeds were sown in each of three nursery boxes. The seedlings were then cultivated in a greenhouse at 25°C for 2 weeks. The index to rate bacterial seedling rot of rice was calculated 16 days after sowing as follows.

Disease severity = 100 × (2A + B)/2N

where A is the number of decayed seedlings, B is the number of seedlings with other symptoms, and N is the number examined.

When untreated seeds were sown, the percentage of diseased seedlings and disease severity were 99.52 [standard error (SE), 0.83] and 82.9 (SE 23.0), respectively. When rice seeds were treated with OA, the percentage of diseased seedlings and disease severity were 17.59 (SE 19.01) and 10.73 (SE 12.39), respectively, suggesting that not only OA-susceptible but also OA-resistant *B. glumae* were present.