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Detection of quinolone-resistant genes in *Photobacterium damselae* subsp. *piscicida* strains by Targeting-Induced Local Lesions in Genomes (TILLING)

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Introduction

Photobacterium damselae subsp. piscicida is the causative agent of pseudotuberculosis, a common disease of marine fish in Japan. Oxolinic acid (OA) has been used to prevent this infection, but its broad use has been followed by the emergence of quinolone resistance. (Takashima *et al.*, 1985; Kim & Aoki, 1993; Morii *et al.*, 2003). Most of the acquired resistance by quinolones can be attributed to mutation in the genes encoding DNA gyrase or topoisomerase IV (Reece & Maxwell, 1991; Kato *et al.*, 1990). To study the quinolone resistance mechanism, mutations in *gyrA* (DNA gyrase) and *parC* (topoisomerase IV) genes were analyzed. Furthermore, we attempted to detect a point mutation using Targeting Induced Local Lesions IN Genomes (TILLING), which is a general strategy used for the detection of a variety of induced point mutations and naturally occurring polymorphisms (Colbert *et al.*, 2001). We developed a new detection method for the rapid and large scale identification of quinolone-resistant strains of *P. damselae* subsp. *piscicida* using TILLING.

Material and Methods

Minimum inhibitory concentrations (MICs) of 3 quinolones (nalidixic acid, OA and orbifloxacin) against 76 strains of *P. damselae subsp. piscicida* were examined. One sensitive, three intermediate level and three high level resistance strains were selected on the basis of quinolones MICs for identifying *gyrA* and *parC* genes. Quinolone resistance-determining region (QRDR) were amplified with degenerate primers, followed by cassette ligation-mediated PCR.

In addition, to detect the point mutation of the *gyrA* gene, we performed a TILLING assay with CEL I for high throughput detection of chemically induced point mutations

and natural polymorphisms with the LI-COR gel analysis system.

Results

Open reading frames encoding proteins of 875 and 755 amino acid residues were detected in the *gyrA* and *parC* genes, respectively. GyrA and ParC sequences were most closely related to *Vibrio parahaemolyticus* GyrA (83% identity) and *Photobacterium profundum*ParC (86% identity) sequences, respectively. The deduced amino acid sequences of the QRDR of the intermediate and high level resistance strains were compared with the sensitive strain. A Ser to Ile mutation (AGT→ATT) was detected in the *gyrA* QRDR of all intermediate level and high level resistance strains while no mutations were found in the *parC* gene. A mutation in the *gyrA* gene was also detected in nalidixic acid-resistant mutants of strain SP96002 obtained from agar medium containing increased levels of quinolone. These results suggest that GyrA, like other gram-negative bacteria, is a target of quinolone in *P. damselae* subsp. *piscicida*.

Furthermore, we attempted to detect a point mutation using TILLING. We developed a new detection method for the rapid and large scale identification of quinolone-resistant strains of *P. damselae* subsp. *piscicida* using TILLING. This method can also be used to screen the presence of resistant strains in other bacterial species.

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