Development of PCR and TaqMan PCR Assays to Detect *Pseudomonas coronafaciens*, a Causal Agent of Halo Blight of Oats

Ji-Hye An¹, Young-Hee Noh¹, Yong-Eon Kim¹, Hyok-In Lee² and Jae-Soon Cha¹*

¹Department of Plant Medicine, Chungbuk National University, Cheongju 361-763, Korea
²Animal and Plant Quarantine Agency, Anyang 430-016, Korea

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*Corresponding author.
Phone) +82-43-261-2554, FAX) +82-43-271-4414
E-mail) jscha@cbnu.ac.kr

**Pseudomonas coronafaciens** causes halo blight on oats and is a plant quarantine bacterium in many countries, including the Republic of Korea. Using of the certificated seed is important for control of the disease. Since effective detection method of *P. coronafaciens* is not available yet, PCR and TaqMan PCR assays for specific detection of *P. coronafaciens* were developed in this study. PCR primers were designed from the draft genome sequence of *P. coronafaciens* LMG 5060 which was obtained by the next-generation sequencing in this study. The PCR primer set Pc-12-F/Pc-12-R specifically amplified 498 bp from the 13 strains of *P. coronafaciens* isolated in the seven different countries (Canada, Japan, United Kingdom, Zimbabwe, Kenya, Germany, and New Zealand) and the nested primer set Pc-12-ne-F/Pc-12-ne-R specifically amplified 298 bp from those strains. The target-size PCR product was not amplified from the non-target bacteria with the PCR and nested primer sets. TaqMan PCR with Pc-12-ne-F/Pc-12-ne-R and a TaqMan probe, Pc-taqman, which were designed inside of the nested PCR amplicon, generated Ct values which in a dose-dependent manner to the amount of the target DNA and the Ct values of all the *P. coronafaciens* strains were above the threshold Ct value for positive detection. The TaqMan PCR generated positive Ct values from the seed extracts of the artificially inoculated oat seeds above 10 cfu/ml inoculation level. PCR and TaqMan PCR assays developed in this study will be useful tools to detect and identify the plant quarantine pathogen, *P. coronafaciens*.

**Keywords**: detection, halo blight, oat, PCR, TaqMan PCR

Halo blight, an important disease of the oats is caused by *Pseudomonas coronafaciens* (Elliott, 1920; Young et al., 1978) and occurs in relatively cool and moist climates leading to substantial economic losses (Marten et al., 1984). It produces light green, oval spots on the leaves of the plant with dark water-soaked centers (Marten et al., 1984; Harder and Haber, 1992; Wallwork, 1992). *P. coronafaciens* survives on plant debris, soil and seeds (Martens et al., 1984). One of the effective control measures of the disease is prevention of pathogen transfer to the other plants using of the certificated seed (Collins, 2010). Also, *P. coronafaciens* is a plant quarantine bacterium in many countries, including the republic of Korea. For the certificated seed program and plant quarantine, the specific and effective detection method must be available.

Various tests can be used to detect the seed-borne pathogens, such as plating on selective media, ELISA, seedling grow-out tests, PCR, real-time PCR and DNA microarrays (Walcott, 2003). Among these techniques, plating on selective media and seedling grow-out tests are laborious and time-consuming and the PCR assay is more sensitive and time-saving than ELISA (Cho et al., 2010). In particular, the TaqMan PCR assay, which detects microorganisms quantitatively with a TaqMan probe, has been shown to be successful in detection and identification of seed-borne pathogens (Bella et al., 2008; Schena et al., 2004; Finetti-Sialer and Ciancio, 2005). For *P. coronafaciens*, however, effective detection method from oat seeds is not available. Any PCR assay for the specific detection of *P. coronafaciens* has not been published yet.

In this study highly specific PCR and TaqMan PCR assays for detection of *P. coronafaciens* have been developed. For development of the *P. coronafaciens* - specific PCR assays, information for the unique nucleotide sequence of *P. coronafaciens* must be available. Since there are not many gene sequences of *P. coronafaciens* available in the GenBank, in the present study, next-generation sequencing analysis was used to obtain a whole draft genome
sequence of *P. coronafaciens*. From the genome sequence, PCR primers were designed for the PCR and TaqMan PCR assays which were specific for the *P. coronafaciens*.

### Materials and Methods

#### Bacterial strains and cultures.

Strains of *P. coronafaciens* isolated in Canada, Japan, United Kingdom, Zimbabwe, Kenya, Germany and New Zealand were obtained from LMG (Belgian Coordinated Collection of Microorganisms, Laboratory of Microbiology, University of Gent, Belgium) and from KACC (Korean Agricultural Culture Collection, Rural Development Administration, the Republic of Korea). For the non-target bacteria, strains related to *P. coronafaciens* in genus *Pseudomonas* or the representative strains of common plant pathogenic bacteria in genus *Acidovorax*, *Pectobacterium*, *Ralstonia*, *Rhodococcus*, *Xanthomonas*, and *Clavibacter* were collected from the various culture collections (Table 1). The bacteria were routinely grown in nutrient agar media containing 8 g of

| Table 1. List of *Pseudomonas coronafaciens* strains, the non-target bacterial strains of *Pseudomonas* spp. and other plant-pathogenic bacterial strains used in this study |
|---|---|---|---|
| Strainab | Hostc | Originc | Yearc |
| **Acidovorax** |  |
| avenae subsp. avenae | NCPPB 1011 | Zea mays | USA | 1958 |
| **Clavibacter** |  |
| michiganensis subsp. incidiosus | NCPPB 1020 | Medicago sativa | Canada | nk |
| michiganensis subsp. michiganensis | NCPPB 1064 | Lycopersicon esculentum | Italy | 1961 |
| michiganensis subsp. sepedonicus | NCPPB 2137 | Solanum tuberosum | Canada | nk |
| **Pectobacterium** |  |
| carotovorum subsp. carotovorum | NCPPB 312 | Solanum tuberosum | Denmark | nk |
| **Pseudomonas** |  |
| coronafaciens | KACC 12133 | nk | nk | nk |
| | KACC 13262 | nk | nk | nk |
| | LMG 2170 | Bromus sp. | Canada | 1962 |
| | LMG 2330 | nk | nk | 1966 |
| | LMG 5030 | Lolium multiflorum | Japan | 1967 |
| | LMG 5060b | Avena sativa | United Kingdom | 1958 |
| | LMG 5061 | Secale cereale | Canada | 1962 |
| | LMG 5081 | Avena sativa | Zimbabwe | 1971 |
| | LMG 5380 | Avena sativa | Kenya | 1970 |
| | LMG 5449 | Avena sativa | Germany | 1959 |
| | LMG 5452 | Avena sativa | New Zealand | 1969 |
| | LMG 5536 | Avena sativa | United Kingdom | 1965 |
| | LMG 13190 | Avena sativa | nk | nk |
| **savastanoi pv. glycinea** | NCPPB 1134 | Glycine javanica | Zimbabwe | 1961 |
| **savastanoi pv. phaseolicola** | KACC 10575 | Phaseolus vulgaris | Poland | nk |
| **savastanoi pv. savastanoi** | NCPPB 639 | Olea europaea | Yugoslavia | nk |
| **syringae pv. actinidiae** | KACC 10582 | Actinidia chinensis | Rep. of Korea | 1999 |
| **syringae pv. antirrhini** | ICMP 4303 | Antirrhinum majus | United Kingdom | 1965 |
| **syringae pv. aptata** | DSM 5025 | Beta vulgaris | nk | nk |
| **syringae pv. atrofaciens** | LMG 5095 | Triticum aestivum | New Zealand | 1968 |
| **syringae pv. berberidis** | NCPPB 2724 | Berberis sp. | New Zealand | 1972 |
| **syringae pv. ciccaronei** | NCPPB 2355 | Ceratonia siliqua | nk | 1969 |
| **syringae pv. delphini** | ICMP 529 | Delphinium sp. | New Zealand | 1957 |
| **syringae pv. dysoxylili** | ICMP 545 | Dysosyllum spectabile | New Zealand | 1949 |
| **syringae pv. eriobotryae** | NCPPB 2331 | Eriobotrya japonica | United Kingdom | 1970 |
| **syringae pv. helianthi** | NCPPB 1229 | Helianthus annuus | Zambia | 1962 |
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nutrient broth and 15 g of agar per liter.

**Genome sequencing and PCR primer design.** Whole-genome shotgun sequencing of *P. coronafaciens* LMG 5060 was performed using the Roche/454 pyrosequencing method on a genome sequencer with FLX titanium (Margulies et al., 2005). ORF prediction was performed with Glimmer v. 3.02 (Delcher et al., 2007), Prodigal (Hyatt et al., 2010), and GeneMark.hmm-P (Lukashin and Borodovsky, 1998). ORFs from the draft genome sequence of *P. coronafaciens* LMG 5060 with more than 500 base pairs were BLASTed in the gene bank. To design the primers, candidate ORFs were selected from the ORFs with lower than 80% nucleotide homology to any known genes. The PCR primers were designed with Primer 3 (v. 0.4.0). The specificity of the primers for *P. coronafaciens* was analyzed by PCR. Nested

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**Table 1. Continued**

| Strainab                        | Hostc | Originc | Yeard |
|---------------------------------|-------|---------|-------|
| *syringae* pv. *japonica*       | ICMP 6305 | *Hordeum vulgare* | Japan | 1951 |
| *syringae* pv. *lachrymans*     | ATCC 11965 | *Cucumis sativus* | nk   | nk   |
| *syringae* pv. *lapsa*          | ATCC 10859 | *Triticum aestivum* | nk  | 1978 |
| *syringae* pv. *maculicola*     | ICMP 3935 | *Brassica oleracea* | New Zealand | 1965 |
| *syringae* pv. *mellea*         | ICMP 5711 | *Nicotiana tabacum* | Japan | 1968 |
| *syringae* pv. *mori*           | ICMP 4331 | *Morus alba* | Hungary | 1958 |
| *syringae* pv. *mosprunorum*    | ICMP 5795 | *Prunus domestica* | nk   | nk   |
| *syringae* pv. *myricae*        | ICMP 7118 | *Myrica rubra* | Japan | 1978 |
| *syringae* pv. *panici*         | NCPPB 1498 | *Panicum sp.* | nk   | nk   |
| *syringae* pv. *papulans*       | ICMP 4040 | *Malus domestica* | USA  | nk   |
| *syringae* pv. *passiflorae*    | NCPPB 1386 | *Passiflora edulis* | New Zealand | 1962 |
| *syringae* pv. *persicae*       | NCPPB 2761 | *Prunus persica* | France | 1974 |
| *syringae* pv. *pisi*           | ICMP 4433 | *Pisum sativum* | Canada | 1946 |
| *syringae* pv. *ribicola*       | NCPPB 963 | *Ribes aureum* | nk   | nk   |
| *syringae* pv. *sesame*         | NCPPB 1016 | *Sesamum indicum* | Yugoslavia | nk |
| *syringae* pv. *syringae*       | NCPPB 388 | *Oryza sativa* | Hungary | nk |
| *syringae* pv. *tabaci*         | ICMP 2835 | *Nicotiana tabacum* | Hungary | 1959 |
| *syringae* pv. *tagetis*        | ICMP 4091 | *Tagetes erecta* | Zimbabwe | 1972 |
| *syringae* pv. *tomato*         | NCPPB 2683 | *Lycopersicon esculentum* | New Zealand | 1972 |
| *syringae* pv. *ulmi*           | NCPPB 632 | *Ulmus sp.* | Yugoslavia | 1958 |

**Ralstonia**

*Solanacearum*

| Solanacearum | NCPPB 339 | *Solanum tuberosum* | Israel | nk |

**Rhizobium**

| radiobacter | DSM 30205 | *Malus sp.* | nk | 1972 |
| rhizogenes  | ATCC 11325 | *Malus domestica* | nk | nk |
| rubi        | NCPPB 1854 | *Rubus ursinus var. loganobaccus* | USA | 1942 |
| viti        | NCPPB 3554 | *Vitis vinifera* | Australia | 1977 |

**Rhodococcus**

| fascians | LMG 3601 | *Lilium speciosum cv. Rubrum* | Belgium | nk |

**Xanthomonas**

| campestris pv. campestris | KACC 10377 | *Brassica oleracea var. capitata* | Rep. of Korea | nk |
| campestris pv. vesicatoria | KACC 11157 | *Capsicum annuum* | Rep. of Korea | 1999 |
| oryzae pv. oryzae | KACC 10331 | *Oryza sativa* | Rep. of Korea | nk |

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a ATCC, American Type Culture Collection, USA; DSM, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; ICMP, International Collection of Microorganisms from Plants, Landcare Research, New Zealand; KACC, Korean Agricultural Culture Collection, Rural Development Administration, Rep. of Korea; LMG, Collection of the Laboratorium voor Microbiologie en Microbiële Genetica, Ghent University, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom.

b This strain was used for genome sequencing by NGS.

c nk, not known.
primers and the nucleotide sequence for the TaqMan probe were designed from amplicon sequence of *P. coronafaciens*-specific primers. All the primers and the TaqMan probe used in the study were synthesized by Bioneer Co., Ltd (Daejeon, Korea) or NeoProbe Co., Ltd. (Daejeon, Korea). The TaqMan probe was labeled at the 5’ end with FAM and at the 3’ end with TAMRA.

**PCR and TaqMan PCR assays.** The genomic DNA was extracted from the bacterial cells using the GeneAll Exgene™ Cell SV kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer’s protocol. DNA concentrations were determined with a Qubit™ fluorometer (Invitrogen®, Carlsbad, CA, USA).

The PCR assays used 1 µl of template DNA in a 25 µl reaction mixture containing 2 mM of Tris-HCl (pH 8.0), 10 mM of KCl, 10 uM of EDTA, 100 uM of DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 2.5 mM of dNTP, 5 units of Ex Taq DNA (TaKaRa Bio Inc., Shiga, Japan), and 10 pM of each primer. The reactions were performed in a T Gradient Thermal Cycler (Biometra, Göttingen, Germany) programmed for one cycle of 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension step for 7 min at 72°C. The amplicons were analyzed by electrophoresis in a 2% agarose gel in TAE buffer.

The TaqMan PCR assay was carried out using 2 µl of DNA template, 8.5 µl of Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), 1 µl of TaqMan probe (10 pmoles/µl), 0.5 µl (10 pmoles/µl) of each nested primer, and 12.5 µl of DEPC-DW (Bioneer, Daejeon, Korea). Real-time PCR amplifications were performed in a Smart Cycler™ System (Cepheid, Sunnyvale, CA, USA) with 40 cycles at 95°C for 30 sec and 60°C for 20 sec after initial incubation for 30 sec at 95°C.

**Oat seed preparation and extraction.** Prior to inoculation, the oat seeds (cultivar Samhan, provided by the National Honam Agricultural Experiment Station in Korea) were surface sterilized by soaking in 70% ethanol for 30 minute and 1.2% sodium hypochlorite solution for 1 hour. The oats seeds were washed 3 times with sterilized water and dried completely in clean bench. Twelve grams of the surface-sterilized oat seeds were artificially inoculated by shaking (120 rpm) in 45 ml of *P. coronafaciens* LMG 5060 suspension for 24 h. The seeds were dried by leaving them in a clean bench for 20 h. The bacterial pathogen was extracted from the artificially inoculated oat seeds as described previously (Maes et al., 1996). The seeds (12 g) were shaken in 12 ml of cold aqueous saline (0.85% NaCl) containing a drop of Tween 20 for 5 min on a rotator shaker (180 rpm) at room temperature. The extracts were treated at 95°C for 7 min and used in the PCR assay or stored at −20°C.

**Results**

**Primer and PCR specificity.** To obtain specific primers of the *P. coronafaciens*, the draft genome sequence of *P. coronafaciens* LMG 5060 was constructed by genome shotgun sequencing in this study. The draft genome sequence was deposited at NCBI GenBank (accession number: JSED00000000). The 36 primer sets were designed from the ORFs in total 2028 contigs which showed low homology to that of known genes in GenBank and the primer specificities for *P. coronafaciens* were checked by PCR assays with target and non-target bacterial strains. Among the primers tested, the Pc-12-F (5’-GATTGCGTCATATGCAACAT-3’) and Pc-12-R (5’-AATAGCAGATCCAGCCAAAT-3’) primer sets amplified a 498 bp in all 13 strains of *P. coronafaciens* (Fig. 1), whereas target-size DNA was not amplified in non-target bacteria, including 30 *P. syringae* pathovars, 3 *P. savastanoi* pathovars, and 16 other pathogenic bacteria (Fig. 2). The nested primers, Pc-12-ne-F (5’-AACAGCGGCGTGCAGGATGG-3’) and Pc-12-ne-R (5’-AACGTGATAGCAGGCCAAAG-3’) were designed from the Pc-12-F/Pc-12-R amplicon. The PCR assay was conducted with the Pc-12-ne-F/Pc-12-ne-R nested primer set and genomic DNA amplified the 298 bp in all 13 strains of *P. coronafaciens* (supplement Fig. 1), whereas target-size DNA was not amplified in any non-target bacteria, including 30 *P. syringae* pathovars, 3 *P. savastanoi* pathovars, 3 *P. savastanoi* pathovars.

![Fig. 1. Gel electrophoresis of the polymerase chain reaction products formed with primer Pc-12-F/Pc-12-R and bacterial DNA of *Pseudomonas coronafaciens* strains. Lanes 1~13, *P. coronafaciens* LMG 5060, KACC 13262, KACC 12133, LMG 2170, LMG 5030, LMG 5061, LMG 5081, LMG 5380, LMG 5449, LMG 5452, LMG 5536, LMG 13190, LMG 2330; lane 14, water as a negative control.](image-url)
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Ovars, and 16 other pathogenic bacteria (Supplement Fig. 2).

**TaqMan PCR specificity.** To reduce the rate of false-positives and quantitatively detect *P. coronafaciens*, a TaqMan probe, Pc-taqman (5'-TGAAACCGCCGAAACGGTCT-3'), was designed from the sequence of the Pc-12-ne-F/Pc-12-ne-R amplicon. The real-time PCR with Pc-12-ne-F/Pc-12-ne-R and Pc-taqman generated Ct values in a dose-dependent for 10 ng-10 pg of *P. coronafaciens* LMG 5060' genomic DNA. The $r^2$ of the linear regression was 0.994 (Fig. 3A).

The specificity of the TaqMan PCR was checked with the target and non-target bacteria. In the TaqMan PCR assays, 10-fold diluted DNAs (10 ng to 10 pg) of *P. coronafaciens* LMG 5060 were used as standard, and 10 ng of DNAs of the other bacterial strains were used. The Ct values of the 13 *P. coronafaciens* strains were 22–28 cycles (Fig. 3B and Table 2), whereas the average Ct values of the DNA of 49 non-target bacterial strains were more than 37 cycles (Table 2). The Ct values of the target and non-target bacteria were well separated into two different groups.

**TaqMan PCR detection of *P. coronafaciens* from the artificially inoculated oat seeds.** The TaqMan PCR assay developed in this study was applied to detect *P. coronafaciens* from the oat seeds. Since the seeds naturally infested

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Fig. 2. Gel electrophoresis of the polymerase cain reaction products formed with primer Pc-12-F/Pc-12-R and total DNA of lane 1, *Pseudomonas coronafaciens* LMG 5060, lanes 2–31, *P. syringae* pvs, *actinidiae* KACC 10582, *antirhini* ICMP 4303, *aptata* DSM 50252, *atrofaciens* ICMP 4394, *berberidis* NCPPB 2724, *ciccaronei* NCPPB 2355, *delphhini* ICMP 529, *dysoxyli* ICMP 545, *eriobotiae* NCPPB 2331, *helianthi* NCPPB 1229, *japonica* ICMP 6305, *lachrymans* ATCC 11965, *lapsa* ATCC 10859, *maculicola* ICMP 3935, *mellea* ICMP 5711, *mori* ICMP 4331, *morsprunorum* ICMP 5795, *myricae* ICMP 7118, *panici* ICMP 1498, *papulans* ICMP 4040, *passiclaceae* NCPPB 7261, *pisi* ICMP 4433, *ribicola* NCPPB 963, *sesami* NCPPB 1016, *syringae* NCPPB 388, *tabaci* ICMP 2835, *tagetis* ICMP 4091, *tomato* NCPPB 2683, *ulini* NCPPB 632; lanes 32–34, *P. savastanoi* pvs. *glycinea* NCPPB 1134, *paleroligochloro* KACC 10575, and *savastanoi* NCPPB 639; lane 35, *Acidovorax avenae* subsp. *insidiosus* NCPPB 1020, *michiganensis* NCPPB 1064, *sepedonicus* NCPPB 2137; lane 39, *Pectobacterium carotovorum* subsp. *carotovorum* NCPPB 312; lanes 40–43, *Rhizobium radiobacter* DSM 30205, *R. rhizogenes* ATCC 11325, *R. rubi* NCPPB 1854, *R. vitis* NCPPB 3554; lane 44, *Rhodococcus fascians* LMG 3601; lane 45, *Ralstonia solanacearum* NCPPB 339; lanes 46–47, *Xanthomonas campestris* pvs. *campestris* KACC 10377, *vesicatoria* KACC 11157; lane 48, *X. oryzae* pv. *oryzae* KACC 10331; lane 1, *P. coronafaciens* LMG 5060, as a positive control; lane 49, water as a negative control.

Fig. 3. Sensitivity and specificity of the TaqMan real-time PCR with primer, Pc-12-ne-F/Pc-12-ne-R and TaqMan probe, Pc-taqman. (A) The linear regression generated by ten-fold dilution of DNA of *Pseudomonas coronafaciens* LMG 5060 and (B) TaqMan PCR with DNAs (circle dot) of *P. coronafaciens* LMG 5060, and *P. coronafaciens* strains (square dot): *P. coronafaciens* KACC 13262, KACC 12133, LMG 2170, LMG 5030, LMG 5061, LMG 5081, LMG 5380, LMG 5449, LMG 5452, LMG 5536, LMG 13190, and LMG 2330.
Table 2. The Ct values of *Pseudomonas coronafaciens* strains, the non-target bacterial strains and the seed extracts from artificially inoculated oat seeds with *Pseudomonas coronafaciens* LMG 5060 which were obtained by the TaqMan PCR assays

| Samples                                      | DNA conc. | Mean Ct  
|----------------------------------------------|-----------|-----------
| *Pseudomonas coronafaciens*                  |           |           |
| LMG 5060 (STD)                               | 10 ng     | 22.8 ± 0.1|
| 1 ng                                         | 28.7 ± 2.5|
| 100 pg                                       | 31.3 ± 1.5|
| 10 pg                                        | 36.5 ± 3.3|
| KACC 12133                                   | 10 ng     | 22.5 ± 0.1|
| KACC 13262                                   | 10 ng     | 22.7 ± 0.2|
| LMG 2170                                      | 10 ng     | 23.3 ± 0.1|
| LMG 2330                                     | 10 ng     | 27.1 ± 0.7|
| LMG 5030                                     | 10 ng     | 22.8 ± 0.3|
| LMG 5061                                     | 10 ng     | 28.0 ± 1.0|
| LMG 5081                                     | 10 ng     | 25.8 ± 2.1|
| LMG 5380                                     | 10 ng     | 24.7 ± 0.4|
| LMG 5449                                     | 10 ng     | 25.1 ± 0.4|
| LMG 5452                                     | 10 ng     | 25.5 ± 2.0|
| LMG 5536                                     | 10 ng     | 23.5 ± 0.6|
| LMG 13190                                    | 10 ng     | 27.9 ± 1.5|
| *Non-target bacterial strains*                |           |           |
| *Acidovorax avenae* subsp. avenae*           | NCPPB 1011| 10 ng     | 39.1 ± 1.7|
| *Clavibacter michiganensis* subsp. incidiosus| NCPPB 1020| 10 ng     | > 40       |
| *Clavibacter michiganensis* subsp. michiganensis| NCPPB 1064| 10 ng     | > 40       |
| *Clavibacter michiganensis* subsp. sepedonicus| NCPPB 2137| 10 ng     | > 40       |
| *Pectobacterium carotovorum* subsp. carotovorum| NCPPB 312 | 10 ng     | > 40       |
| *Pseudomonas savastanoi* pv. glycinea*       | NCPPB 1134| 10 ng     | > 40       |
| *Pseudomonas savastanoi* pv. phaseolicola*   | KACC 10575| 10 ng     | 39.5 ± 0.9|
| *Pseudomonas savastanoi* pv. savastanoi*    | NCPPB 639 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. actinidiae*       | KACC 10582| 10 ng     | > 40       |
| *Pseudomonas syringae* pv. antirrhini*       | ICMP 4303 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. aptata*           | DSM 50252 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. atrofaciens*      | LMG 5095  | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. berberidis*       | NCPPB 2724| 10 ng     | 37.8 ± 1.9|
| *Pseudomonas syringae* pv. ciccaronei*       | NCPPB 2355| 10 ng     | 39.6 ± 0.8|
| *Pseudomonas syringae* pv. delphini*         | ICMP 529  | 10 ng     | 39.5 ± 0.9|
| *Pseudomonas syringae* pv. dysoxyl*          | ICMP 545  | 10 ng     | 39.3 ± 1.3|
| *Pseudomonas syringae* pv. eriobotryae*      | NCPPB 2331| 10 ng     | > 40       |
| *Pseudomonas syringae* pv. helianthi*        | NCPPB 1229| 10 ng     | 39.9 ± 0.2|
| *Pseudomonas syringae* pv. japonica*         | ICMP 6305 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. lachrymans*       | ATCC 11965| 10 ng     | > 40       |
| *Pseudomonas syringae* pv. lapsa*            | ATCC 10859| 10 ng     | > 40       |
| *Pseudomonas syringae* pv. maculicola*       | ICMP 3935 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. mellea*           | ICMP 5711 | 10 ng     | 38.7 ± 1.3|
| *Pseudomonas syringae* pv. mori*             | ICMP 4331 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. morsprunorum*     | ICMP 5795 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. myricae*          | ICMP 7118 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. panici*           | NCPPB 1498| 10 ng     | > 40       |
| *Pseudomonas syringae* pv. papulans*         | ICMP 4040 | 10 ng     | > 40       |
| *Pseudomonas syringae* pv. passiflorae*      | NCPPB 1386| 10 ng     | 39.7 ± 0.6|
| *Pseudomonas syringae* pv. persicace*        | NCPPB 2761| 10 ng     | > 40       |
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with the pathogen were not available, the artificially inoculated oat seeds were used. TaqMan PCR with the seed extracts generated a range of mean Ct values from 21.2 for $10^8$ cfu/ml-inoculated seed extracts to 32.2 for 10 cfu/ml-inoculated seed extracts (Table 2). Since Ct values of all the non-target bacteria used in this study were larger than 37 cycles, the TaqMan PCR can detect *P. coronafaciens* from the seed extract of the artificially inoculated oat seeds above 10 cfu/ml inoculation level.

Estimation of the recovery number of *P. coronafaciens* from the artificial inoculated seed extract was tried with several different ways, but the accurate enumeration of *P. coronafaciens* from the seed extract failed because the many non-target-shape colonies were cultured on the culture plate and the selective medium for *P. coronafaciens* was not available. Surface sterilization of the oat seeds prior to artificial inoculation could not prevent growth of some non-target bacteria on the recovery culture plates.

**Discussion**

Highly specific PCR and TaqMan PCR assays have been developed in this study to detect *P. coronafaciens*, the halo blight pathogen of oats. Since *P. coronafaciens* is a plant quarantine bacterium in many countries and using of the certificated seed is important for the disease control, developments of these assays are significant for the management of halo blight of oats.

To obtain specific primers of the *P. coronafaciens*, a genome-wide search was conducted from the draft genome sequence of *P. coronafaciens* which was constructed by genome shotgun sequencing in this study. A specific primer, Pc-12-F/Pc-12-R, was designed from ORF 7 in contig 7 of the draft genome sequence of *P. coronafaciens* (NCBI GenBank accession number: JSED00000000). ORF 7 was 1341 nucleotides long, and no significant homologous gene was found in the NCBI GenBank database. PCRs with Pc-
12-F/Pc-12-R and Pc-12-ne-F/Pc-12-ne-R generated the target size DNA from all 13 strains of *P. coronafaciens* isolated in seven countries. The target-size DNA was not amplified in 49 strains of non-target bacteria. The Ct values of the target and non-target bacteria were well separated in the TaqMan PCR. The homology of the ORFs in which PCR was designed and the results of the PCR and TaqMan PCR indicate that the primer sequences and PCR assays developed in this study are highly specific to *P. coronafaciens*.

TaqMan PCR was applied to detect the target pathogen from artificially inoculated oat seeds. TaqMan PCR generated the *P. coronafaciens*-positive Ct values in the seed extracts obtained from oat seeds inoculated in 10 cfu/ml and above. Although detection sensitivity of this TaqMan PCR cannot compared to the previously published results because detection of *P. coronafaciens* from oat seeds has not been published, positive detection of the seed extracts obtained from oat seeds inoculated in 10 cfu/ml *P. coronafaciens* LMG 5060 suspension and above is thought to be a quite high sensitivity and can apply to seed test and plant quarantine service.

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