

## Molecular diagnosis of the bacteria causative of tomato canker disease

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**Abstract.** Tomato canker disease is caused by a Gram-positive actinomycete, *Clavibacter michiganensis* subsp. *michiganensis*, and emerging in various districts. We tried molecular identification of the causative pathogen upon the frequent occurrence of this disease in Fukui Prefecture in 2005. We showed that the comparison of the nucleotide sequences is useful for accurate diagnosis of pathogenic bacteria of tomato.

**Key words:** tomato canker disease, *Clavibacter michiganensis*, diagnosis

Tomato canker disease is caused by a Gram-positive actinomycete, *Clavibacter michiganensis* subsp. *michiganensis*<sup>1-4)</sup> and symptomatically characterized by canker and wilting of leaflet and petioles, brown discoloration of xylem vessels, collapse of pith, and bird eye-like white spots on the fruit (EPPO website, <http://www.eppo.org/>). In Japan, this malady first occurred in Hokkaido in 1958, and then spread through the country. After temporary diminution, tomato canker disease is again emerging in various districts. More recently, putative canker disease extensively seized tomato seedlings reared in Fukui Prefecture. Upon this occurrence, attempts were made to identify the causative pathogen by nucleotide sequencing, in addition to conventional methods.

From tomato plants, fresh affected tissue and healthy control portion were cut out, immersed, for sterilization of tissue surface, in 70% ethanol for 30 sec and then in 0.5% hypochlorite for 1-3 min. After washing with sterilized water, about 5 mm cube was cut off, homogenized in 1 ml of sterilized water, diluted and streaked on plates containing SMCMM agar medium 5) or Heart Infusion agar medium (Difco Laboratories, Detroit, Mich., USA). Plates were incubated at 28°C for 1-2 weeks (SMCC med.) or 1 week (HI med.) As a standard strain, *C. michiganensis* subsp. *michiganensis* N6302 (MAFF 301039) was used which had been isolated in 1963 from Fukui Pref. and deposited in Microorganisms Section of the NIAS Gene bank ([http://www.nias.affrc.go.jp/index\\_e.html](http://www.nias.affrc.go.jp/index_e.html)). DSM 463646) was also used for reference.

Bacterial colonies independently isolated from 13 lesions were all smooth, shining, round and yellow. In the appearance of colonies, these bacilli indistinguishably resembled the standard strain N6302. In addition, a colony bearing similar surface features was isolated also from a healthy tissue. Therefore,

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experiments were performed with each colony, to extract chromosomal DNA and sequence 16S ribosomal RNA gene (rDNA). Each isolated sample was cultured overnight in Antibiotic medium 3 (Difco. Lab.), and one ml of the culture was centrifuged at 8000 x g for 5 min at 4°C. The bacterial pellet was suspended in 200 µl of 20% sucrose-50 mM EDTA-25 mM Tris·HCl (pH8.0). After addition of 5 mg/ml lysozyme, the mixture was incubated at 37°C for 30 min, then centrifuged at 5000 x g, for 5 min at 4°C, and chromosomal DNA was extracted from the pellet, using ISOPLANT kit (Nippon gene, Toyama) which contained benzyl chloride and a detergent.

The 16S rRNA gene was amplified by PCR (Fig. 1), using synthetic primers Cm-UP1 (5'-gctcaggacgaacgctggc-3') and Cm-LP1 (5'-atgacttgacggcggtgtg-3'). This universal primer pair was designed based on comparative analysis of the *Clavibacter michiganensis* subsp. *michiganensis* 16S rRNA gene sequences (DDBJ/GenBank/EMBL databases). The PCR products were directly sequenced using synthetic degenerated primers Cm-UP2 (5'-gatcagtggcgaacgggtgag-3'), Cm-UP4 (5'-ctgctgtgaaatcccgaggct-3'), and r11 7). The PCR enzyme and buffer were used according to suppliers' recommendation. The total volume of the PCR mixtures was 50 µl. PCR enzyme (Takara Ex-Taq) was a product from Takara Shuzo Co., Ltd. Kyoto. Usually, 35 cycles of amplification were carried out, under the conditions of initial denaturation (60 sec at 94°C), strand denaturation (30 sec at 94°C), annealing (30 sec at 50°C), and elongation (90 sec at 72°C). The nucleotide sequence was determined by the dideoxy method, with ABI PRISMTM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence data were analyzed using GENETYX computer software (Software development Co. Ltd., Tokyo). The chromosomal DNA sequence database was searched using BLASTN search algorithms (<http://www.ncbi.nlm.nih.gov/BLAST/>).

PCR profile of each 16S rRNA was shown in Fig. 1. Nucleotide sequences of about 1.0 kb were determined with the amplified DNA, and compared with that of the standard strain N6302 (No. 1 of Fig. 1, and Fig. 2), and a reference strain DSM 46364 (Fig. 2). The sequences of 11 samples (No. 2 and No. 4-13) were identical throughout the 1054 bp region, whereas differed from N6302 only in 833 th nucleotide (C vs. T), and from DSM 46364 in 84 th residue (T vs. G). High homology (99.9%) between these samples isolated this time from tomato lesions and the standard (No. 1) or reference strain indicated that all of them belong to *C. michiganensis* subsp. *michiganensis*. On the other hand, homology between No. 3 and the standard strain was 93.3%, and BLASTN search exhibited that No. 3 was homologous (99.9%) with *Microbacterium* which was relative to *Clavibacter*. In addition, No. 14 showed 91.4% homology with the standard strain N6302, and 99.9% homology with *Kocuria*, respectively.

Diversity of symptoms makes conventional diagnosis of tomato canker disease considerably difficult. As shown above, discrimination of *Clavibacter* from *Microbacterium* or *Kocuria* is hardly possible by morphological examination or PCR analysis of each bacterial strain isolated from the affected plant tissue. Thus DNA sequencing is essential for final diagnosis of this disease.

Although isolated in Fukui, origin of N6302 was unknown, whereas the newly detected *michiganensis* strain was parasitic on tomato plants reared from the

seeds imported in 2005. Difference in the single nucleotide excluded reemergence of the original strain. Similarly, the newly detected transversion type change (T vs. G) excluded invasion of DSM 46364 strain into Fukui district. Moreover, various bacteria were isolated in 2006, from 19 tomato seedling samples bearing brown discoloration in vessel or crack in stalk, and their 16S rRNA genes were successfully amplified by PCR, using the primers described above. Sequence analysis has revealed that 7 bacterial samples belong to *Microbacterium* sp., five cases to *Curtobacterium* sp., two specimens to *Mycobacterium* sp., and remaining 5 strains are assigned to *Cellulosimicrobium* sp., *Clavibacter michiganensis*, *Methylobacterium* sp., *Ralstonia solanacearum*, *Stenotrophomonas* sp., respectively. Field soil of Fukui Prefecture has considerably been contaminated by *Ralstonia solanacearum*, causative of tomato wilt disease, and this pathogen is frequently occurring in the summer of 2006 as well.

Using newly designed primers for PCR amplification and sequencing, 32 bacterial strains isolated from tomato lesions were examined for their assortment. Thus, nucleotide sequences of each 16S rRNA gene were effectively determined. Owing to shortage of skilled experts, definitive diagnosis, from symptoms, of such plant diseases as tomato canker or wilt is rather difficult, in these days. On the other hand, PCR technique and nucleotide sequencing are available also in phytopathology laboratories. The primers and methods described above are useful for accurate and rapid diagnosis of pathogenic bacteria of tomato and related plants.

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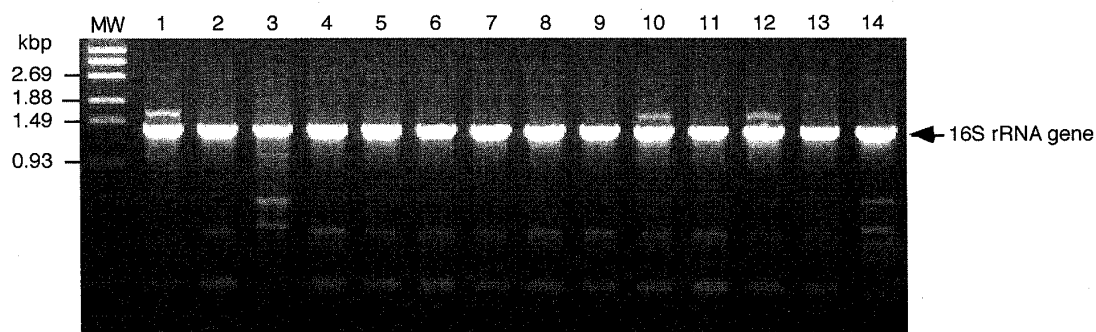


Fig. 1. PCR amplification of 16S rRNA sequence from various samples with primer pair Cm-UP1 and Cm-LP1. Lane MW, molecular weight marker; lane 1, *C. michiganensis* subsp. *michiganensis* N6302 (standard strain); lane 2-14, Bacterial colonies independently isolated from lesions or healthy tissues.

common	1	ACTGCTAGAAATGGTAGCTAATACCGGATATGACGATTGGCCGCATGGTCTGGTCGTGGA	60
DSM 46364	61	AAGAATTTTCGGTTGGGGATGGACCGCGGCCTATCAGGTTGTTGGTGAGGTAATGGCTCA	120
1		.....	
2,4-13		.....	
common	121	CCAAGCCTACGACGGTAGCCGGCTGAGAGGGTGACCGGCCACACTGGGACTGAGACAC	180
common	181	GGCCAGACTCCTACGGGAGGAGCAGTGGGGAATATTGCACAATGGCGAAAGCCTGAT	240
common	241	GCAGCAACGCCCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTAGTAGGGAAG	300
common	301	AAGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCG	360
common	361	TAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGCGTAAAGAGCTCGTAGGCGGTT	420
common	421	TGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGCTCTGAGTGGGTACGGGCAGA	480
common	481	CTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAG	540
common	541	GAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACGACGCTGAGGAGCGAAAGC	600
common	601	ATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGTTGGGAAC TAGA	660
common	661	TGTGGGACCATTCCACGGTCTCCGTGTCGAGCTAACGCATTAAGTCCCCGCCTGGGG	720
common	721	AGTACGGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGCGGAGC	780
DSM 46364	781	ATGCGGATTAATTTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGAAAC	840
1		.....	
2,4-13		.....	
common	841	ATGCAGAAATGTGTGCCCCGAAGGTCGGTATACAGGTGGTGCATGGTTGTCGTGAGCTC	900
common	901	GTGTCGTGAGATGTTGGTTAAGTCCCACAAGCGCAACCCCTCGTTCTATGTTGCCAG	960
common	961	CACGTAATGGTGGAACTCATAGGAGACTGCCGGGTCAACTCGAGGAAGTGGGGATG	1020
common	1021	ACGTCAAATCATCATGCCCTTATGTCTTGGGCT	1054

Fig. 2. Comparison of 16S ribosomal RNA genes among *C. michiganensis* subsp. *michiganensis* strains. DSM 46364, *C. michiganensis* subsp. *michiganensis* isolated in Germany; No. 1, *C. michiganensis* subsp. *michiganensis* N6302 (standard strain); No. 2 and No. 4-13 isolated this time. Common: identical throughout the strains.

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