Recently, crop seeds have been produced and traded internationally. Seeds can be carriers of foreign phytopathogens, thereby spreading diseases to new areas under suitable environmental conditions. To avoid the seed transmission of plant diseases, techniques for detecting specific pathogens are important. Various techniques including direct inspection, agar testing, and biochemical and molecular methods are currently recommended by official organizations such as the International Seed Health Initiative-Vegetable (ISHI-Veg, 2017; Kido et al., 2018).

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Clavibacter michiganensis subsp. michiganensis is a seedborne pathogen and the causal agent of bacterial canker, one of the most serious diseases of tomato (de Léon et al., 2011; Sen et al., 2015). C. michiganensis subsp. michiganensis is frequently transmitted by seeds, both internally within the seed and on the seed surface. Therefore, the most efficient strategy for controlling bacterial canker is prevention based on the use of pathogen-free seeds. To avoid seed transmission of this pathogen, quarantine regulations have been adopted by many countries to guarantee that commercial seed lots are free of contamination by C. michiganensis subsp. michiganensis or that the level of contamination is below an acceptable threshold. In most official diagnostic protocols, the pathogen is detected using semi-selective medium. Despite the development of protocols for detecting target bacteria using molecular techniques, the isolation of viable target bacteria colonies is needed for seed health testing (ISHI-Veg, 2017). Several variations of the agar test have been developed. In addition, some semi-selective media such as CMM1T (C. michiganensis subsp. michiganensis Tris-buffered semi-selective medium), SCM (semi-selective medium for C. michiganensis), SCMF (SCM Fast), BCT (bacterial canker of tomato), and SMSMM [selective medium for Corynebacterium michiganense pv. michiganense (presently C. michiganensis subsp. michiganensis)], have been used recently (EPPO, 2016; Ftayeh et al., 2011; ISHI-Veg, 2017; Shirakawa and Sasaki, 1988). Semi-selective media failed to facilitate the detection of low numbers of pathogens in seed, producing
false-negative results, and these media sometimes permit the detection of different bacteria such as saprophytes and nontarget bacteria (Franken et al., 1993; de Léon et al., 2011). However, whether nontarget bacteria from tomato seeds can grow on semi-selective media has not been clarified. In the present study, we detected nontarget bacteria from tomato seeds that can grow on representative media considered semi-selective for C. michiganensis subsp. michiganensis.

Five representative semi-selective media for the detection of C. michiganensis subsp. michiganensis, namely CMM1T, SCM, SCMF, BCT, and SMSMM, were used in this study. The composition of each medium per 1 L were as follows: CMM1T (10 g sucrose, 3.32 g Tris base, 11.44 g Tris-HCl, 0.25 g MgSO4·7H2O, 5 g LiCl, 2 g yeast extract, 1 g NH4Cl, 4 g casamino acids, 15 g agar, 10 mg polymyxin B sulfate, 28 mg nalidixic acid, 100 mg nystatin), SCM (10 g sucrose, 0.1 g yeast extract, 1.5 g H2BO3, 0.25 g MgSO4·7H2O, 2 g K2HPO4·0.5 g KH2PO4·18 g agar, 30 mg nalidixic acid, 50 g nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite, SCMF (10 g sucrose, 2 g yeast extract, 1.5 g H2BO3, 0.25 g MgSO4·7H2O, 2 g K2HPO4, 0.5 g KH2PO4, 18 g agar, 20 mg nalidixic acid, 50 mg nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite, 80 mg trimethoprim), BCT (2.5 g mannitol, 2 g yeast extract, 1 g K2HPO4·0.1 g KH2PO4, 0.05 g NaCl, 0.1 g MgSO4·7H2O, 0.015 g MnSO4·H2O, 0.015 g FeSO4·7H2O, 0.6 g H2BO3, 15 g agar, 20 mg nalidixic acid, 100 mg trimethoprim, 20 mg polyoxin B, 4.2 mg epoxiconazole, 12.5 mg fenpropimorph), and SMMM (2 g glycerol, 5 g peptone, 3 g yeast extract, 2 g K2HPO4, 0.5 g KH2PO4·0.25 g MgSO4·7H2O, 5 g LiCl, 15 g agar, 20 mg nalidixic acid, 40 mg cycloheximide, 80 mg K2Cr3O7, 2 mg NaNO3, 2.1 mg 2,4,5,6-tetrachloroisophthalonitrile).

All tested semi-selective media contained nystatin or cycloheximide. Both antibiotics inhibit the growth of fungi. Moreover, it is considered that other antimicrobial agents influence selectivity for C. michiganensis subsp. michiganensis in bacteria. Fifty milliliters of medium agar were poured into Petri dishes (90 mm in diameter). R2A (per 1 L: 0.5 g glucose, 0.5 g soluble starch, 0.5 g yeast extract, 0.5 g proteose peptone no. 3, 0.5 g casamino acid, 0.3 g K2HPO4, 0.05 g MgSO4·7H2O, 0.3 g sodium pyruvate, 15 g agar) medium containing nystatin at 100 μg mL−1 was used to detect total cultural bacteria in seed samples (Reasoner and Geldreich, 1985; Someya et al., 2013). Colonies on R2A medium was considered cultivable bacterial populations in the present study.

Fifteen lots of commercial tomato seeds, namely sample Nos. 1–15 (containing 10 cultivars produced at 6 different locations), were used. One gram of seeds was surface-sterilized in 70% ethanol for 30 s and 2.5% sodium hypochlorite for 1 min and then rinsed with sterile water three times. Seeds were then ground with 9 mL of sterile 15 mM phosphate buffer (pH 7.0) using sterilized pestle. Serial dilutions were spread on each semi-selective medium or R2A medium and incubated at 25°C in the dark for 7 days. Five replicate plates were used in the assay. After culturable bacterial colonies were observed on semi-selective media, sample Nos. 2, 8, and 9 were inoculated onto CMM1T, SCM and SCMF media, repetitively. C. michiganensis subsp. michiganensis MAFF 301038 was obtained from NARO Genebank and used as the positive control for semi-selective media.

When R2A medium was used for bacterial isolation, approximately 1×10⁴–1×10⁷ CFU of bacterial colonies were obtained from 1 g of tomato seeds (data not shown). Fungal colonies were not observed on tested media. Bacterial colonies from seed samples were only observed on three semi-selective media, namely CMM1T, SCM, and SCMF (FIG. 1). Bacteria from No. 2 were observed on CMM1T and SCMF, those from No. 8 were observed on SCM and SCMF, and those from No. 9 were observed on SCM. Bacteria from other seed samples were not observed on BCT and SMMM. Growth of the positive control (C. michiganensis subsp. michiganensis) was observed on all semi-selective media. However, in the positive control, growth and pigment production were slightly inhibited on BCT and SMMM (data not shown).

Yellowish colonies were detected on CMM1T for bacteria cultured from seed sample No. 2 at approximately 1.5 × 10³ CFU g⁻¹ seed. Grey or black colonies were observed on SCM or SCMF medium for bacteria cultured from seed sample Nos. 2, 8, and 9 at approximately 3.5 × 10³–3.4 × 10⁴ CFU g⁻¹ seed. No bacterial colonies were detected from other seed samples on any semi-selective medium. We randomly isolated 50 colonies from sample No. 2 grown on CMM1T and SCMF, No. 8 grown on SCM and SCMF, and No. 9 grown on SCM medium, respectively.

Bacterial DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 16S rRNA genes were amplified from genomic DNA obtained from bacterial isolates by PCR using Premix Taq (Takara Bio, Otsu, Japan). The primers 27F (‘-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (‘-AAGGAGGTGWTCCARCC-3′) were used for PCR. Sequencing and clustering analyses were performed using previously described methods (Someya et al., 2020). The nucleotide sequences of the partial 16S rRNA genes of the bacteria isolated from tomato seeds have been deposited in the DNA Data Bank of Japan under the accession numbers LC534328–LC534343.
Clustering analysis (>99% identity) revealed the presence of 16 operational taxonomic units (OTUs) among isolates detected on semi-selective media (TABLE 1). Regarding samples, three OTUs (AC1, AC2, and FC1) were detected from No. 2 on CMM1 and SCMF. Bacteria detected from Nos. 8 and 9 on SCM and SCMF were diverse, exhibiting 14 OTUs. Six OTUs (FC2, FC5, FC6, FC8, FC10, and FC12) were detected from both No. 8 and No. 9 samples, and four OTUs were only detected from Nos. 8 (FC1, FC3, FC7, and FC11) and 9 (AC3, FC4, FC9, and FC13).

Three OTUs belonged to the phylum Actinobacteria, and 13 OTUs belonged to the phylum Firmicutes. OTUs AC1 and AC2 were related to Micrococcus and Dermacoccus, and AC3 was related to Curtobacterium (FIG. 1). All 13 Firmicutes OTUs were related to the order Bacillales. OTUs FC1–FC13 belonged to Firmicutes, and they were prevalent within the order Bacillales including the families Bacillaceae, Paenibacillaceae, and Planococcaceae (FIG. 1).

Our results revealed the frequent presence of nontarget bacteria on media considered semi-selective for C. michiganensis subsp. michiganensis. The detected bacteria belonged to either Actinobacteria or Firmicutes. Proteobacteria were not detected on the tested tomato seeds. Although Clavibacter is a genus in the phylum

![FIG. 1](image-url)
Actinobacteria, the detected bacteria were from the genera *Micrococcus* and *Dermacoccus*, which are not closely related to *Clavibacter* (FIG. 1). Culturable bacteria were also detected from sample Nos. 8 and 9 on SCM and SCMF. Most bacteria detected on these media were from the phylum Firmicutes (TABLE 1). OTUs FC1–FC13 belonged to the families *Bacillaceae*, *Planococcaceae*, and *Paenibacillaceae*. These bacteria are not closely related to *Clavibacter* (FIG. 1). Nontarget bacteria were not detected on BCT and SMCM. Therefore, BCT and SMCM exhibited better elimination efficacy for nontarget bacteria than other semi-selective media in this study. Additionally, the phylogenetic distribution of nontarget bacteria differed among CMM1T, SCM, and SCMF. Actinobacteria was not detected on SCM and SCMF, and Firmicutes was not detected on CMM1T. It is believed that the results reflect differences in the combination and quantity of antibiotics in each medium. Thus, improvement of the combination and quantity of antibiotics may improve the efficacy of each semi-selective medium.

Selective medium for target bacteria was developed by varying the nutrient and antibiotic composition to eliminate nontarget bacteria. CMM1T, SCM, and SCMF, the representative semi-selective media for *C. michiganensis* subsp. *michiganensis*, contain selective factors such as polymyxin B, potassium tellurite, and trimethoprim (EPPO, 2016; ISHI-Veg, 2017). The aforementioned antibiotics primarily inhibit the growth of Gram-negative bacteria (Summers and Jacoby, 1977; Steinbuch and Fridman, 2016). However, most bacteria belonging to Actinobacteria and Firmicutes are Gram-positive. Therefore, most semi-selective media used in this study appear to be selective for certain Gram-positive bacteria. The OTUs for the same sample differed among the semi-selective media (TABLE 1). It is believed that the differences of OTUs for the same sample were linked to the different antibiotics in the semi-selective media. BCT and SMCM exhibited better elimination efficacy for nontarget bacteria than other semi-selective media in this study. Additionally, the phylogenetic distribution of nontarget bacteria differed among CMM1T, SCM, and SCMF. Actinobacteria was not detected on SCM and SCMF, and Firmicutes was not detected on CMM1T. It is believed that the results reflect differences in the combination and quantity of antibiotics in each medium. Thus, improvement of the combination and quantity of antibiotics may improve the efficacy of each semi-selective medium.

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| Sample No. | Isolate (%)<sup>a</sup> | Closest known species<sup>c</sup> | Acc. No. | Identity (%) |
|------------|-----------------|---------------------|---------|--------------|
| OTU<sup>b</sup> | CMM1T | SCM | SCM | SCM |
| AC1 | 11.4 | - | - | - | *Micrococcus antarcticus* | NR_025285 | 99 |
| AC2 | 88.6 | - | - | - | *Dermacoccus nishinomiyaensis* | NR_044872 | 99 |
| AC3 | - | - | 9.3 | 8.3 | *Curtobacterium luteum* | NR_026157 | 99 |
| FC1 | - | 100.0 | - | - | *Bacillus nealsonii* | NR_044546 | 99 |
| FC2 | - | 7.0 | - | 2.3 | *Bacillus gottheilii* | NR_108491 | 99 |
| FC3 | - | 4.7 | 91.7 | - | *Bacillus kochii* | NR_117050 | 99 |
| FC4 | - | - | - | 18.6 | *Bacillus simplex* | NR_114919 | 99 |
| FC5 | - | 9.3 | - | 9.3 | *Psychrobacillus lasiacapitis* | NR_159144 | 99 |
| FC6 | - | 4.7 | - | 9.3 | *Lysinibacillus macroides* | NR_114920 | 99 |
| FC7 | - | 2.3 | - | - | *Lysinibacillus louembei* | NR_145586 | 99 |
| FC8 | - | 9.3 | - | 9.3 | *Solibacillus silvestris* | NR_028865 | 99 |
| FC9 | - | - | - | 4.7 | *Rummelibacillus stabekisii* | NR_114270 | 99 |
| FC10 | - | 41.9 | - | 37.2 | *Terribacillus saccharophilus* | NR_041356 | 99 |
| FC11 | - | 9.3 | - | - | *Terribacillus saccharophilus* | NR_041356 | 99 |
| FC12 | - | 2.3 | - | 2.3 | *Terribacillus saccharophilus* | NR_041356 | 99 |
| FC13 | - | - | - | 4.7 | *Paenibacillus odorifer* | NR_028887 | 99 |
| Total | 100 | 100 | 100 | 100 | 100 |

<sup>a</sup>Relative abundance (%) of isolates belonging to each OTU.
<sup>b</sup>AC: OTUs belonged to the phylum Actinobacteria, FC: OTUs belonged to the phylum Firmicutes.
<sup>c</sup>The results of a pairwise BLAST analysis between a representative sequence and its closest type strain.
michiganensis subsp. michiganensis (data not shown). It is believed that semi-selective media are needed to improve for culturability of C. michiganensis subsp. michiganensis, as noted for the elimination of nontarget bacteria. To develop efficient selective media, it is important to use the proper combination and quantity of antibiotics to eliminate nontarget bacteria and enhance the culturability of target bacteria.

In recent years, the number of reports concerning microbial community analyses of plant tissues including seeds has increased (Bergna et al., 2018; Thomas and Shaik, 2020; Tuyrens et al., 2015). These reports indicated that Firmicutes and Actinobacteria are distributed on tomato seeds. Although their colonization routes, localization inside seeds, and roles are not well identified, some bacteria can improve the health and development of tomato (Bergna et al., 2018). However, Gram-positive bacteria present in these endophytes also have the potential to be selected as nontarget culturable bacteria in seed health tests using semi-selective media aiming to detect C. michiganensis subsp. michiganensis. To decrease the likelihood of false-positive results in seed health tests, novel methods of bacterial elimination and the development of more selective methods are needed. We will focus on the development of techniques aiming to increase the selectivity for the target bacterium C. michiganensis subsp. michiganensis in the presence of nontarget bacteria in future research.

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