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Short communication

Rapid identification of *Clavibacter michiganensis* subspecies *sepedonicus* based on the stable low molecular weight RNA (LMW RNA) profiles

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Abstract

Clavibacter michiganensis subsp. *sepedonicus* causes potato ring rot disease. The identification process for this bacterium is complex and long. This work demonstrates that the stable low-molecular-weight (LMW) RNA profiles allow their rapid identification. Staircase electrophoresis in polyacrylamide gels was used to analyze the LMW RNA profiles of 54 strains of *C. michiganensis* subsp. *sepedonicus* from different geographic origins. The profiles of several strains of other subspecies of *C. michiganensis* and other pathogens of potatoes were also analyzed. All the strains of *C. michiganensis* subsp. *sepedonicus* had the same LMW RNA profile. They had a band in class 2 of tRNA that was absent in the other subspecies of the species *C. michiganensis*. Also, the LMW RNA of *C. michiganensis* subsp. *sepedonicus* was different with respect to the LMW RNA profiles of other pathogens of potato. The results indicate the possible utilization of LMW RNA profiles in identification of the bacteria causing potato ring rot disease.

Abbreviations: LMW RNA – low molecular weight RNA; SCE – staircase electrophoresis

Clavibacter michiganensis subsp. *sepedonicus* causes potato ring rot disease, a disease which causes significant losses in potato crops in the USA and Canada. The suspicion of the presence of *C. michiganensis* subsp. *sepedonicus* stops importations in the countries which are found free of this disease. In Europe, a directive has been established to prevent the introduction of *C. michiganensis* subsp. *sepedonicus* into the territory of member states. This includes the Official Method for detection of this pathogen and the measures to prevent its spread with the aim of eradication (Council of the European Communities, 1993). In the Official Method of diagnosis, the presumptive identification test is based on indirect immunofluorescence and must be confirmed by the isolation and identification of the pathogens using biochemical and physiological tests and also infectivity tests in *Solanum melongena*. This long process leads to important economic losses and therefore it would be desirable to have rapid techniques for identification of this bacterium.

Several techniques based on PCR were proposed for diagnosis of this bacterium (Li and de Boer, 1995; Lows et al., 1998; Pastrik and Rainey, 1999; Pastrik, 2000), but the results can be unclear, because they are based only on the presence or absence of a characteristic DNA band that might be absent due to an inhibition of DNA amplification.

LMW RNA (stable low molecular weight RNAs) profiles allow the differentiation of genera based on the 5S rRNA zone, and of species, based on the tRNA profiles. They provide a molecular fingerprint that is characteristic for each microbial species studied (Velázquez et al., 1998a,b, 2000). A new technique for separation of these molecules in unidimensional gels

of polyacrylamide, staircase electrophoresis (SCE) (Cruz-Sánchez et al., 1997), enables the analysis of a large number of samples in a short time frame, allows the study of extensive bacterial populations, permits the analysis of their genetic diversity and the rapid identification of any isolate. Moreover, the results obtained in yeasts indicate that LMW RNA profiles are not intraspecific variations (Velázquez et al., 2000). This fact is very important because only techniques that offer no intraspecific variability can be used to differentiate microbial species.

The aim of this work was to analyze the LMW RNA profiles of a wide number of strains of *C. michiganensis* subsp. *sepedonicus* isolated from different geographical locations and determine the feasibility of using the profiles for the identification of this bacterium.

All the strains used in this study are listed in Table 1. Several strains of *C. michiganensis* subsp. *sepedonicus* were isolated from *Solanum tuberosum* affected with potato ring rot disease in several locations in Spain. The strains of *C. michiganensis* were grown on NBY medium (Vidaver, 1967) for two days at 24 °C and 180 rpm. The strains of *Erwinia* and *Ralstonia* were grown in nutrient broth for 24 h.

RNA was extracted (Höfle, 1988) and LMW RNA profiles were obtained using SCE in 14% polyacrylamide gels under denaturing conditions in steps of

Table 1. Strains used in this study

Strain	Host plant	Location	Source
Clavibacter michiganensis subsp. *sepedonicus*			
94-C3, 95-C19, 95-457, 96-81, 96-100	*Solanum tuberosum*	Spain/Palencia	This study
95-336	*S. tuberosum*	Spain/Valladolid	This study
95-C6	*S. tuberosum*	Spain/Zamora	This study
95-S18, 95-S29, 95-AS18, 95-AS47, 95-AS48, 95-AS51	*S. tuberosum*	Spain/Burgos	This study
95-AS52, 95-AS78, 95-AS79, 95-AS103, 95-AS115	*S. tuberosum*	Spain/Burgos	This study
95-M6, 95-M207, 96-S49, 96-AS5, 96-M255	*S. tuberosum*	Spain/Burgos	This study
96-BA5	*S. tuberosum*	Spain/Cáceres	This study
97-GAL1, 97-GAL2	*S. tuberosum*	Spain/Pontevedra	This study
C-R2, C-R12, C-R13, C-BRR7	*S. tuberosum*	Canada	S.H. de Boer
N-87-5, N-89-4, N-92–5	*S. tuberosum*	Norway	A. Sletten
D-221, D-282, D-285, D-288, D-298, D-316	*S. tuberosum*	Denmark	K. Mansfeld
D-320, D-326	*S. tuberosum*	Denmark	K. Mansfeld
D-294	*S. tuberosum*	Germany	K. Mansfeld
S-175, S-189, S-237, S-247, S-318, S-375, S-379	*S. tuberosum*	Sweden	P. Persson
S-403, S-BD132	*S. tuberosum*	Sweden	P. Persson
2140	*S. tuberosum*	USA	NCPPB
1792	*S. tuberosum*	USA	CFBP
ATCC33113^T	*S. tuberosum*	Canada	NCPPB
C. michiganensis subsp. *nebraskensis*			
CECT5040^T, CECT4209	*Zea mays*	USA	CECT
C. michiganensis subsp. *tessellarius*			
CECT4263	*Triticum aestivum*		CECT
C. michiganensis subsp. *insidiosus*			
CFBP5041	*Medicago sativa*	United Kingdom	CFBP
CFBP5042^T	*M. sativa*	USA	CFBP
C. michiganensis subsp. *michiganensis*			
121.1, 093.3F	*Lycopersicon lycopersicon*	Spain	IVIA
Erwinia carotovora subsp. *carotovora*			
194	*S. tuberosum*	Scotland	IVIA
163	*S. tuberosum*	Spain	IVIA
Ralstonia solanacearum			
NCPPB1493	*L. lycopersicon*	Puerto Rico	NCPPB
1580-7	*S. tuberosum*	Spain	This study

CFBP: Collection Nationale de Bactéries Phytopathogènes INRA: (France); NCPPB: National Collection of Plant Phytopathogenic Bacteria (UK); CECT: Spanish Type Culture Collection; IVIA: Instituto Valenciano de Investigaciones Agrarias (Spain).

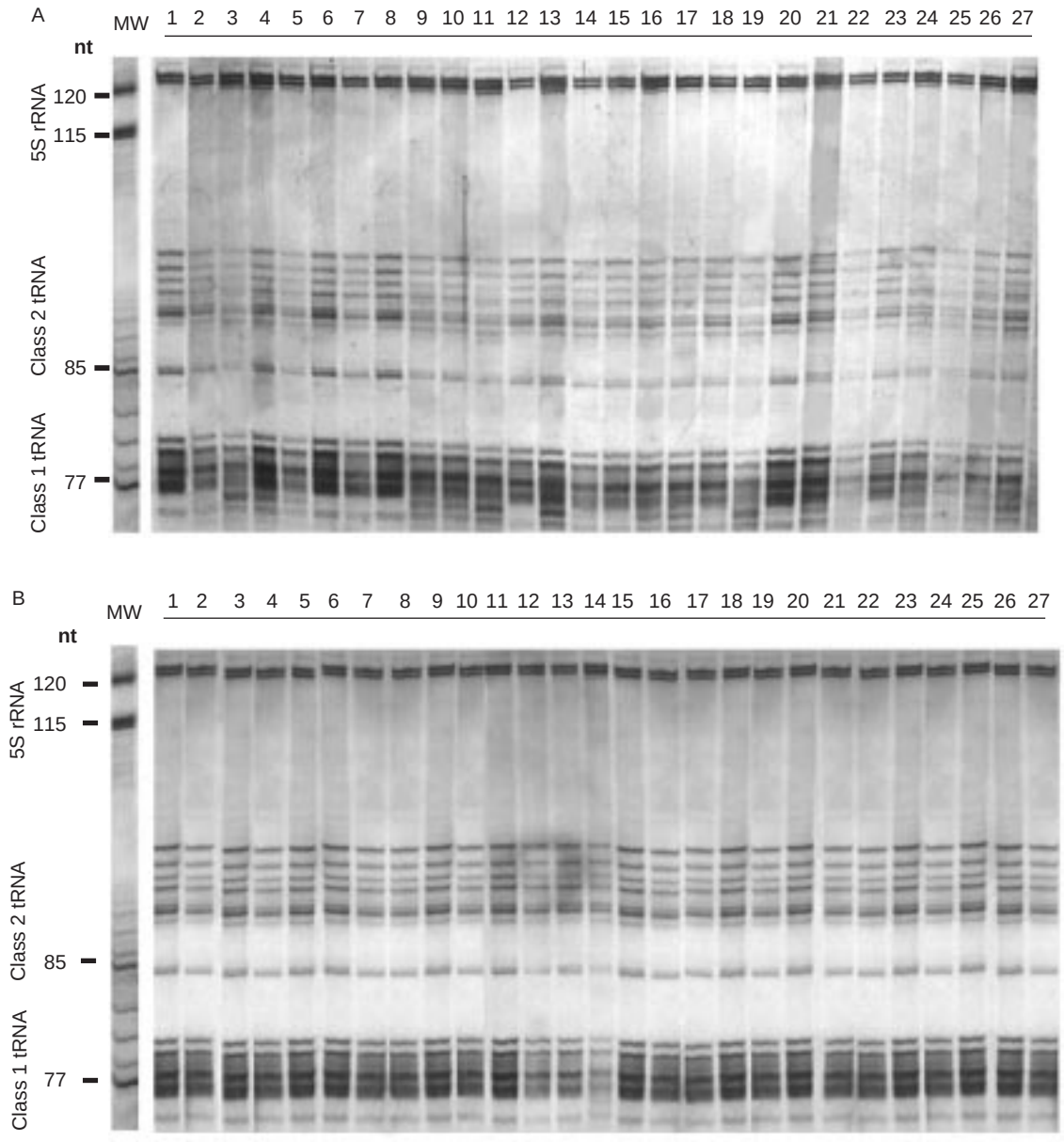

Figure 1. LMW RNA profiles of the strains of *C. michiganensis* subsp. *sepedonicus*: (A) lane (1) 95-S29, lane (2) S-175, lane (3) 96-100, lane (4) 96-AS5, lane (5) 96-81, lane (6) 96-S49, lane (7) 96-BA5, lane (8) 95-M207, lane (9) 95-AS103, lane (10) 97-GAL2, lane (11) 95-AS51, lane (12) S-189, lane (13) 2049, lane (14) 1154, lane (15) S-237, lane (16) 1792, lane (17) S-375, lane (18) S-403, lane (19) S-247, lane (20) D-326, lane (21) 95-S18, lane (22) 95-C19, lane (23) D-320, lane (24) 95-457, lane (25) 95-AS47, lane (26) 95-M6. (B) lane (27) 95-AS115, lane (28) D-288, lane (29) N-875, lane (30) 94-C3, lane (31) 2140, lane (32) D-298, lane (33) 96-M255, lane (34) D-294, lane (35) 95-336, lane (36) D-285, lane (37) C-BRR7, lane (38) S-BD132, lane (39) D-221, lane (40) C-R2, lane (41) D-282, lane (42) 95-AS79, lane (43) N-894, lane (44) 97-GAL1, lane (45) C-R12, lane (46) S-379, lane (47) C-R13, lane (48) S-318, lane (49) 95-AS48, lane (50) 95-C6, lane (51) 2137, lane (52) 95-AS52, lane (53) D-316, lane (54) 95-AS18, lane (55) 95-AS78, lane (56) N-925.

10 min, rising through a constant ramp with 50 V increases from 100 V to 2300 V (Cruz-Sánchez et al., 1997).

The following molecules from Boehringer Manheim (Manheim, Germany) and Sigma (St. Louis, MO, USA) were used as reference: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (Bidle and Fletcher, 1995), tRNA specific for tyrosine from *E. coli* (85 nucleotides) and tRNA specific for valine from *E. coli* (77 nucleotides) (Sprinzl et al., 1985). Samples were prepared (Cruz-Sánchez et al., 1997) and after electrophoresis, the gels were silver-stained (Haas et al., 1994).

The LMW RNA profiles of the strains of *C. michiganensis* subsp. *sepedonicus* are shown in Figure 1. As in other species of Gram negative or positive bacteria (Velázquez et al., 1998a,b) the LMW RNA profiles show three zones: 5S rRNA, class 1 tRNA and class 2 tRNA. Figure 1 shows that all strains from *C. michiganensis* subsp. *sepedonicus* displayed the same LMW RNA profile (Figure 1A,B) independently of their geographical origin.

As can be seen in Figure 2, the other subspecies of *C. michiganensis*: *insidiosus*, *nebraskense*, *michiganensis* and *tessellarius* show the same LMW RNA profile (Figure 2, lanes 2–8) among them. However, Figure 2 also shows, that the LMW RNA profile of subspecies *sepedonicus* (lane 1) has a single band (marked in the figure with an arrow) which was not present in class 2 tRNA of the other *C. michiganensis* subspecies.

Although this difference in LMW RNA profiles does not have relevance for the diagnosis of potato ring root disease because the subspecies *insidiosus*, *nebraskense*, *michiganensis* and *tessellarius* are not pathogens of potatoes, the results have potential taxonomic implications. Currently the classification of this species is not based on DNA–DNA relatedness and the LMW RNA suggests that *C. michiganensis* might be separated into two species. One of them includes the strains currently classified as *Clavibacter* subsp. *sepedonicus* and a second species includes the remaining subspecies. This hypothesis must be confirmed by DNA–DNA hybridization that according to the current taxonomic criteria on bacterial species description is the superior method with respect to the other molecular techniques, including 16S rRNA sequences (Stackebrandt and Goebel, 1994).

Besides *C. michiganensis* subsp. *sepedonicus*, *Erwinia carotovora* and *Ralstonia solanacearum* are the most important genera of non-filamentous bacterial pathogens of potato. Thus, several strains of these species were included in this study, although these species can be differentiated from *C. michiganensis* by other techniques. Figure 3 shows the LMW RNA profiles of *C. michiganensis* subsp. *sepedonicus* (lane 1),

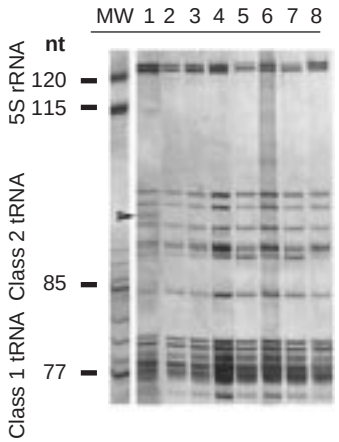

Figure 2. LMW RNA profiles of the strains of *C. michiganensis*: lane (1) *C. michiganensis* subsp. *sepedonicus* strain ATCC33113^T, lane (2) *C. michiganensis* subsp. *nebraskensis* CECT5040^T, lane (3) *C. michiganensis* subsp. *nebraskensis* CECT4209, lane (4) *C. michiganensis* subsp. *tessellarius* CECT4263, lane (5) *C. michiganensis* subsp. *insidiosus* CFBP5042^T, lane (6) *C. michiganensis* subsp. *insidiosus* CFBP5041, lane (7) *C. michiganensis* subsp. *michiganensis* 121.1, lane (8) *C. michiganensis* subsp. *michiganensis* 093.3F.

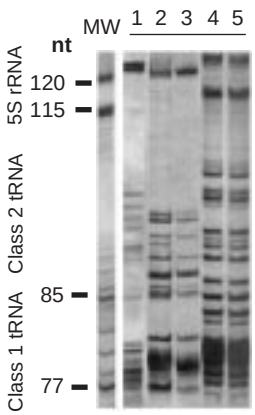

Figure 3. LMW RNA profiles of *C. michiganensis* subsp. *sepedonicus* strain ATCC33113^T (lane 1), *E. carotovora* subsp. *carotovora* 194 (lane 2), *E. carotovora* subsp. *carotovora* 163 (lane 3), *R. solanacearum* NCPPB1493 (lane 4) and *R. solanacearum* 1580-7 (lane 5).

E. carotovora subsp. *carotovora* (lanes 2 and 3) and *R. solanacearum* (lanes 4 and 5). As can be observed in this figure, the 5S rRNA zone is different among the three genera and the tRNA profiles are different among these species. As can be expected for strains from the same species, the LMW RNA profiles of two strains of *E. carotovora* were identical and the same results were obtained for the two strains of *R. solanacearum*. According to these results, *C. michiganensis* subsp. *sepedonicus* can be differentiated from the other non-filamentous species of pathogens of potatoes by LMW RNA profiling.

Thus, the results of this work show that each genus represented in this study has a unique 5S rRNA zone. Further, each species displays a characteristic tRNA profile, except in the case of *C. michiganensis* subsp. *sepedonicus* which can be differentiated from other subspecies of the same species by a single band present in class 2 tRNA. This result has a great utility for identification of this subspecies and suggests the need of a study of DNA–DNA hybridization to establish its taxonomic category.

The results obtained in this study are in agreement with those obtained in previous studies in other bacterial groups (Velázquez et al., 1998a,b) and show that SCE LMW RNA can be used in the rapid and sensitive identification of *C. michiganensis* subsp. *sepedonicus*.

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