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Genomic Fingerprinting of Virulent and Avirulent Strains of *Clavibacter michiganensis* subspecies *sepedonicus*

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Abstract. Genomic fingerprints of *C. michiganensis* subsp. *sepedonicus* were generated by CHEF gel electrophoresis of restriction digested high-molecular weight DNA. Low levels of intra-subspecific variation were detected by cluster analysis of the fingerprints. Four haplotypes were identified by genomic fingerprinting with *Hind*III, and eight were identified with *Eco*RI. Haplotypes generated with *Hind*III were less similar than those generated by *Eco*RI. Haplotypes generated with *Hind*III formed groups that corresponded well with plant reactions of the strains, but similar types of groupings were less apparent with haplotypes generated with *Eco*RI. When disease severity in eggplant and potato, population size in potato, and ability to induce a hypersensitive response (HR) in tobacco were overlaid onto dendograms of genetic similarity, avirulent HR-negative strains clustered separately from virulent HR-positive strains in both *Eco*RI and *Hind*III profiles. Avirulent HR-positive strains that lack pCS1 clustered with avirulent HR-negative strains in a *Eco*RI dendogram, but clustered with virulent HR-positive strains in a *Hind*III dendogram. Genomic fingerprinting of high-molecular weight DNA fragments provided a means for detecting genomic variability associated with virulence in *C. michiganensis* subsp. *sepedonicus*.

The genus *Clavibacter* is an agriculturally important member of the high G+C coryneforms impacting the health of several monocots and dicots [3]. *Clavibacter* is in the family *Microbacteriaceae* and a relative of the industrially important genera *Brevibacterium* and *Microbacterium* and the plant pathogenic genera *Curtobacterium* and *Rathayibacter* [17, 35]. It is also distantly related to the *Mycobacterium–Nocardia* group of bacteria and *Rhodococcus*, which are distinct from the *Microbacteriaceae* in containing mycolic acids in their cell walls [36].

Clavibacter michiganensis contains several economically important plant pathogens, which are classified into subspecies on the basis of host specificity and biochemical and genetic markers [24]. *C. michiganensis* and its subspecies constitute an uncontested monophyletic cluster possessing B2γ type cell wall peptidoglycan with diaminobutyric acid, MK-9 as the major menaquinone, and high GC% (70 ± 5) content [2, 3]. Subspecies of *C. michiganensis* are differentiated by their ability to cause disease on specific crops [3]. *Clavibacter michiganensis* subsp. *sepedonicus*, the cause of bacterial ring rot of potato, is of particular interest because of its status as a quarantine organism in North America and Europe [9]. Strains of *C. michiganensis* subsp. *sepedonicus* vary in disease-causing ability, colony morphology on nutrient rich media, production of extracellular polysaccharides and extracellular enzymes, and ability to elicit a hypersensitive response (HR) in tobacco [1, 4, 10, 30, 39]. The molecular basis for these differences is largely unknown; however, cellulase encoded on the native plasmids pCS1 and pCM1 was shown recently to be a virulence factor in *C. michiganensis* subsp. *sepedonicus* and *C. michiganensis* subsp. *michiganensis*, respectively [13, 16, 24].

Although phenotypic variability exists within *C. michiganensis* subsp. *sepedonicus* previous molecular genetic studies indicate the taxon is genetically uniform. Most strains of *C. michiganensis* subsp. *sepedonicus* contain a single circular plasmid (pCS1) and several copies of a 1.3 kb repetitive sequence (IS*1121*), which is present on pCS1 and in high copy number in the chromosome [26, 28, 37]. Mogen *et al.* evaluated the utility

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of IS*1121* for detecting restriction fragment length polymorphisms (RFLPs) in *C. michiganensis* subsp. *sepedonicus* and found low levels of polymorphisms among strains [28]. More recently, Louws et al. applied a repetitive-sequence-based (rep)-PCR genomic fingerprinting approach to classify subspecies of *C. michiganensis* [20]. Several DNA fragments in the range of 200–2,000 bp were present in *C. michiganensis* subsp. *sepedonicus*. The rep-PCR fingerprints of all strains of *C. michiganensis* subsp. *sepedonicus* examined were identical. Consistent with these findings, the rRNA gene sequences and low-molecular weight RNA profiles of *C. michiganensis* subsp. *sepedonicus* are also very uniform [17, 32].

The purpose of our study was to develop protocols for genomic fingerprinting of high-molecular weight DNA fragments from *C. michiganensis* subsp. *sepedonicus* and determine whether or not these types of genomic fingerprints substantiated previous reports of homogeneity or revealed new associations with virulence or avirulence. We present evidence of genomic homogeneity among some frequently cited strains of *C. michiganensis* subsp. *sepedonicus* and provide an estimate of their minimal genome size. We also present evidence for an association between genomic fingerprints and avirulence in *C. michiganensis* subsp. *sepedonicus*.

Materials and Methods

Strains. A set of previously cited *C. michiganensis* subsp. *sepedonicus* strains were selected for study (Table 1). In addition, spontaneous derivatives of Cs3 were included, as these vary in virulence or production of extracellular polysaccharides (EPS). Cs3NM is a nonmucoid derivative of Cs3. When grown in culture, Cs3NM produced a mucoid and a nonmucoid colony, which were purified and maintained at CSU as CIC242 and CIC243, respectively. Bacterial cultures were grown on NBY or DM medium from glycerol stocks stored at −80°C, as previously described [30]. Cells were incubated at 24–26°C for 5–7 days and subcultured on NBY medium for 4–6 days prior to preparation of inoculum.

Plant inoculations. Potato plants (*Solanum tuberosum* L. cv. Sangre) were micropropagated by nodal stem culture and inoculated as described previously [11]. Roots of three-week-old plants were soaked in a cell suspension (OD_{640} = 0.1) prepared in 20 mM potassium phosphate buffer, pH 7.2. Control plants were treated with phosphate buffer alone. Inoculated plants were transplanted to soil (MetroMix 350, Grace Sierra Horticultural Products Company, CA, USA), kept in a mist chamber for three days after inoculation, and then grown in a greenhouse (12 h daylight/day at 20–25°C days and 18–20°C nights.) Eggplants (*Solanum melanogena*) were germinated from seeds, grown in soil until the two-leaf stage, and transplanted into six in pots containing MetroMix 350 without supplements. The first true leaf was removed at the stem with a sterile scalpel. A 20 μl bacterial suspension containing about 10^8 cfu/ml in phosphate buffer was injected into the stem through the petiole stump. Induction of a hypersensitive response (HR) in the nonhost tobacco was evaluated according to a previously described method [30].

Bacterial population sizes and disease severity ratings. Bacterial population sizes were estimated from stems of inoculated potato plants. Stems were collected 60 days post inoculation (dpi). Basal sections one cm in length were removed at the soil line and stored at −80°C until processing. The number of cells of *C. michiganensis* subsp. *sepedonicus* was determined by the immunofluorescent antibody staining (IFAS) procedure with monoclonal antibody 9A1 supplied by Agdia Inc., Elhart, IN, according to manufacturer's recommendations. Bacterial population sizes were calculated by taking the average of ten microscopic views per sample and multiplying by the dilution factor and number of microscope views per slide.

Disease severity ratings were obtained on inoculated eggplant and potato plants at 30 and 60 dpi for eggplant and potato, respectively, using the Horsfall-Barratt scale of 1 to 12. One indicates no symptoms present and a score of 12 indicates symptoms on 100% of the plant, as previously described [30].

Data analysis of *in planta* experiments. Greenhouse experiments were arranged in a randomized complete block design with five replications. Each experiment was performed three times. Differences in the number of IFAS units between treatment means were determined on logarithmically transformed values combined over experiments. The mixed procedure of analysis of variance and covariances and T tests for least square differences (LSD) were computed by using SAS version 8.1 (SAS Institute Inc., Cary, NC).

Preparation of high-molecular weight DNA. High-molecular weight genomic DNA of *C. michiganensis* subsp. *sepedonicus* was prepared in agarose blocks. Cells were grown on NBY or DM [23] agar for 4–6 days at 26°C. Five ml of Pett IV buffer (10 mM Tris-HCl pH 7.6; 1 M NaCl) was added to the plate and the cells were scraped into the buffer. Cells were pelleted and resuspended in fresh PettIV buffer at a concentration of 2×10^9 cells/ml. The cell suspension was added and mixed with an equal value of 1% low melting point agarose at 42°C. The mixture was dispensed into 100 μl plug molds and solidified on ice. The hardened plugs were transferred to a tube containing two

Table 1. Strains of *Clavibacter michiganensis* subsp. *sepedonicus* used in this study

Strain designation	Description	Source/reference
ATCC 33113	Type strain, wild type (also known as LMG 2889)	ATCC/[27]
ATCC 33111	Wild type	ATCC/[27]
ATCC 9850	Wild type	ATCC/[27]
NCPPB 2140	Wild type (also known as LMG-2899)	M. Harrison/[6]
CIC31	Spontaneous rifampicin-resistant mutant of CSU#2539-69	M. Harrison/[12]
Cs3	Mucoid wild type strain from Vancouver, B.C.	M. Metzler/[30]
Cs3NM	Spontaneous nonmucoid derivative of CS3 isolated by S. De Boer	S. De Boer [27]
CIC242	Mucoid derivative of Cs3NM	This study
CIC243	Nonmucoid derivative of Cs3NM	This study
P45	Does not contain pCS1	A. Oleson/[27]
BCP45	Spontaneous spectinomycin-resistant mutant of P45.	C. Orser/[38]

volumes of sterile lysis buffer (6 mM Tris-HCl, pH 7.6; 1 M NaCl; 0.1 M EDTA, pH 7.5; 0.2% deoxycholate; 0.5% N-lauroylsarcosine) containing 1 mg/ml lysozyme and incubated overnight at 37°C. After decanting the supernatant, plugs were rinsed twice with cold H_2O and two volumes of ESP buffer (0.5 M EDTA, pH 9-9.5; 1% N-lauroylsarcosine) containing 1 mg/ml proteinase K was added. The samples were incubated for two days with gentle shaking and one buffer change. The plugs were stored in ESP at 4°C.

Restriction endonuclease digestion. Prior to restriction digestion of DNA, plugs were washed with distilled water three times. To inactivate proteinase K, plugs were incubated for 2 h at 37°C in five volumes of TE buffer and Pefabloc Sc (Roche Biochemicals) (10 mM Tris-HCl, pH 7.0; 1 mM EDTA pH 8.0; Pefabloc 1–5 mM). The plugs were rinsed with TE, pH 8.0, three times for 20 min and transferred to microfuge tubes containing 200 μl restriction enzyme buffer with 100 IU of enzyme. The samples were restricted overnight at 37°C. The enzyme was stopped with EDTA (final concentration 0.5 M) and SDS (final concentration 1%). The samples were stored at 4°C.

Genomic fingerprinting. DNA restriction fragments in agarose plugs were separated by CHEF gel electrophoresis using a CHEF Mapper (BioRAD Life Science Groups, Richmond, CA). The plugs were loaded into wells of 1% agarose gels prepared in 0.5 × TBE cooled to 14°C. A lambda PFG molecular weight marker (New England Biolab, Beverly, MA) was used as a standard. Electrophoresis conditions were 6 V/cm for 17 h; 120° angle; initial switch time of 5 s, final switch time of 12 s, and a linear ramp. Digital images of gels were made, and processed with imaging software (Image, The Sanger Center) to yield band sizes. Genomic fingerprinting was repeated at least once for each strain.

Analysis of genomic fingerprints. Pairwise similarities of genomic fingerprints were determined by generating a binary data set for each strain based on the absence or presence of all possible bands represented in the fingerprints of the 11 strains. A similarity coefficient (F) was calculated for each pairwise comparison, as $F = 2Nxy/(Nx + Ny)$, according to the method of Nei and Li [29]. Nx is the number of bands present in strain X, Ny is the number of bands present in strain Y, and Nxy is the number of bands in common. A dissimilarity matrix (1–F) was generated and analyzed by the NEIGHBOR Program of the PHYLIP (Phylogeny Inference Package) software package using the unweighted pair group methods with arithmetic means (UPGMA) option (version 3.5c; J. Felsenstein, University of Washington, Seattle). Graphical representations of the cluster analysis were obtained by plotting the output of the NEIGHBOR analysis as a dendogram using the DRAWGRAM program of PHYLIP.

PCR amplification of *C. michiganensis* subsp. *sepedonicus* genomic DNA. DNA from each test strain of *C. michiganensis* subsp. *sepedonicus* was amplified with the three *Cms*-specific primer pairs (Cms 50, Cms 72, and Cms 85), as described by Mills *et al.* [25], and by the pCS1-specific primer pair (Cms 6 and Cms 7), as described by Schneider *et al.* [38].

Results

Identification, virulence, and *in planta* population sizes of *C. michiganensis* subsp. *sepedonicus* strains. The identity of all strains used in this study was verified immunologically and by PCR amplification using the three pairs of *C. michiganensis* subsp. *sepedonicus*-specific primers developed by Mills *et al.* [25]. All strains, including Cs3NM, CIC243, and CIC242 reacted with a *C. michiganensis* subsp. *sepedonicus*-specific antibody and, when amplified with PCR primer pairs 50, 72, and 85, yielded DNA fragments of the appropriate sizes (data not shown). In addition, all strains except the plasmidless, cellulase-deficient strains P45 and BCP45 were amplified by the pCS1-specific primers of Schneider *et al*. [38] (Table 2).

Strains of *C. michiganensis* subsp. *sepedonicus* varied in virulence in eggplant and potato and population sizes in planta (Table 2). Three disease classes were identified. Class 1, which included CIC243 and Cs3NM, grew poorly in potato (less than 3×10^4 IFAS units/sample), caused none or very slight symptoms in eggplant and potato, and did not induce the HR in tobacco. Class 2, which included the cellulase-negative strains BCP45 and P45, grew in potato to a greater extent than class 1 (4.6×10^6–1.2×10^7 IFAS units/sample) and induced the HR. Class 3, which included most of the strains and the type strain ATCC33113, caused severe symptoms in eggplant and potato, grew in potato to the greatest extent ($>1 \times 10^8$ IFAS units/sample), and induced the HR. Covariate analysis of disease severity and population size *in planta* revealed significant differences due to strain ($p < .0001$), and a significant interaction between disease severity and strain ($p = .008$). Thus the number of bacterial cells needed to produce the same degree of disease severity differed significantly among strains.

Genomic fingerprints of *C. michiganensis* subsp. *sepedonicus* strains. CHEF gel electrophoresis of *Eco*RI and *Hind*III digested high-molecular weight DNA produced several fragments suitable for genomic fingerprint analysis. The majority of bands were in the size range of 45–400 Kb. Several similarly sized fragments appeared in all strains, but intrasubspecific variation was evident, especially among the *Hind*III profiles (Fig. 1). *Hind*III profiles differentiated four haplotypes and gave similarity coefficients in the range of 0.70 to 1.00 (Table 3). *Eco*RI profiles differentiated eight haplotypes with similarity coefficients ranging from 0.86 to 1.00 (Table 4). The relatively greater degree of similarity among strains detected by *Eco*RI digestion as compared with *Hind*III digestion was evident in dendograms generated by PHYLIP (Fig. 2).

When disease class information was overlaid onto the dendograms of genomic fingerprints, several associations became evident. The type strain, ATCC33113, clustered together with other class 3 (virulent and HR-positive) strains in both the *Hind*III and *Eco*RI dendograms. In both the *Eco*RI and *Hind*III dendograms, strain CIC31 was separated from other class 3 strains.

Table 2. Biology of *Clavibacter michiganensis* subsp. *sepedonicus* strains used for genomic fingerprint analyses

Strain	Population size in potato[a]	Disease severity—potato[b]	Disease severity—eggplant	Tobacco HR	Disease class[d]	PCR amplification: Primer pairs *Cms* 50, 72, and 85	PCR amplification: Primers *Cms* 6 and 7[e]
CIC243	1.5×10^4a	1.55 a	1.90 ab	−	1	+	+
Cs3NM	2.8×10^4a	1.43 a	3.77 c	−	1	+	+
BCP45	4.6×10^6b	1.40 a	3.40 bc	+	2	+	−
P45	1.2×10^7b	1.67 a	3.73 bc	+	2	+	−
ATCC33113	1×10^8c	6.00 bc	7.40 de	+	3	+	+
CIC242	1.2×10^8c	8.33 de	5.93 d	+	3	+	+
Cs3	1.7×10^8c	7.47 cd	6.47 d	+	3	+	+
ATCC9850	2.3×10^8c	9.92 e	9.80 f	+	3	+	+
CIC31	2.5×10^8c	6.52 bcd	7.47 de	+	3	+	+
NCPPB2140	3.3×10^8c	4.77 b	7.27 de	nt[c]	nd	+	+
ATCC33111	3.3×10^8c	9.70 e	8.53 ef	+	3	+	+

[a] IFAS units/sample. Statistical analyses performed with log transformed values. Numbers followed by the same letter are not significantly different (LSD = 0.94 log IFAS units/sample).
[b] LSD = 2.18 for disease severity in potato; LSD = 1.85 for disease severity in eggplant.
[c] nt = not tested.
[d] Disease class: see text and Fig. 2 legend (nd = not determined).
[e] Primers 6 and 7 amplify plasmid-specific sequences found on pCS1.

Based on the *Hind*III dendogram, Cs3 and CIC242 clustered with each other and were distinguished from other class 3 strains (Fig. 2A). In contrast, Cs3 and CIC242 clustered together with other class 3 strains in the *Eco*RI dendogram. Class 1 strains (avirulent and HR-negative) clustered separately from class 3 strains in both *Eco*RI and *Hind*III profiles. Class 2 strains (avirulent and HR-positive) clustered with class 1 strains in the *Eco*RI dendogram, but clustered with class 3 strains in the *Hind*III dendogram.

Estimation of minimal genome size. Genomic fingerprinting of large DNA fragments provided a means for estimating the minimal genome size of *C. michiganensis* subsp. *sepedonicus*. Minimal genome estimates were calculated by summing the restriction fragment sizes greater than 45 kb (the lower limit of size resolution in the gel) obtained for each strain and then averaging the total size over all strains. The minimal genome size estimates based on *Hind*III and *Eco*RI fragment sizes were 2.50 Mb (sd = 0.15) and 2.64 Mb (sd = 0.36), respectively.

Discussion

In this study we present a protocol for obtaining high-molecular weight DNA fragments suitable for genomic fingerprinting of *C. michiganensis* subsp. *sepedonicus*. DNA prepared in this manner consisted primarily of high-molecular weight fragments that were resolved by the CHEF conditions described. To obtain genomic fingerprint profiles, the DNA was restricted with various enzymes and separated by CHEF gel electrophoresis. We determined that *Not*I, which is often used for genomic fingerprinting of other bacteria, was not suitable for genomic analysis of *C. michiganensis* subsp. *sepedonicus*. *Not*I cuts very frequently in the >70% G+C DNA of *C. michiganensis* subsp. *sepedonicus*, yielding primarily small sized fragments less than 50 kb (data not shown). Instead, we found that *Eco*RI and *Hind*III were ideally suited for our purposes, as these cut the genome relatively infrequently. These enzymes were also preferable because they do not digest pCS1, the 50 kb circular plasmid present in most strains of the pathogen. Using *Eco*RI and *Hind*III thus provided a relatively inexpensive means of assessing genetic variability within non-pCS1 genomic regions of *C. michiganensis* subsp. *sepedonicus*.

The minimal genome size of *C. michiganensis* subsp. *sepedonicus* was estimated to be 2.5 to 2.6 Mb. This is a minimal genome size because only fragments greater than 45 kb were included in the estimate. Previous estimates used in the study of *C. michiganensis* subsp. *sepedonicus* assumed a genome size of 2.5 Mb [14] or 6 Mb [27]. Even if there were as many as 10 bands of about 45 kb not included in our estimate, the genome size would still be within 0.5 Mb of the estimated 2.5–2.6 Mb, making it one of the smaller bacterial genomes [7]. Analyses of undigested genomic DNA examined under different CHEF conditions are needed to

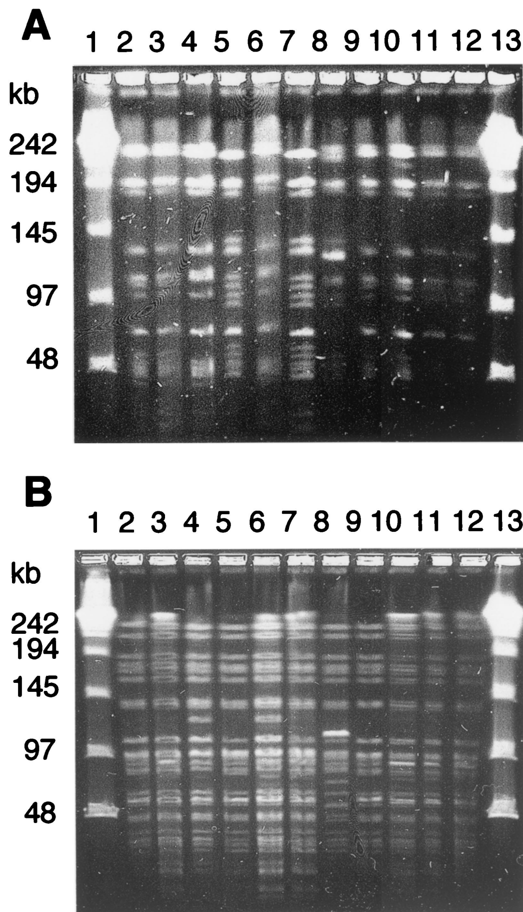

Fig. 1. Genomic fingerprints of *Clavibacter michiganensis* subsp. *sepedonicus* obtained by CHEF gel electrophoresis of high-molecular weight genomic DNA digested with *Hind*III (**A**) or *Eco*RI (**B**). Lanes 1 and 13 contain λ markers; lanes 2–12 represent strains BCP45, P45, CIC243, CIC242, Cs3NM, Cs3, CIC31, NCPPB2140, ATCC9850, ATCC33111, ATCC33113, respectively.

provide further evidence of genome size and organization for this subspecies [19, 34].

The best fit dendograms of the similarity coefficients from both *Hind*III and *Eco*RI CHEF genomic fingerprints confirmed that virulent HR-positive strains of *C. michiganensis* subsp. *sepedonicus* belong to the same or closely related haplotypes. The genetic similarity values provided by heirarchical analysis of the similarity coefficients were quite high (>88%). This finding is completely consistent with previous conclusions that *C. michiganensis* subsp. *sepedonicus* is a monophyletic cluster [2, 3, 17]. The homogeneity of *C. michiganensis* subsp. *sepedonicus* stands in contrast to the marked heterogeneity of the closely related *Curtobacterium flaccumfaciens* [21] or the more distantly related *Rhodococcus fascians* [34].

CHEF genomic fingerprints varied among strains of *C. michiganensis* subsp. *sepedonicus*, even though genetic similarity values were high. CHEF analysis of high-molecular weight DNA appears at least as powerful as RFLP or randomly amplified polymorphic DNA (RAPD) analysis of low-molecular weight DNA for detecting differences at the level of strain. In general, values of genetic similarity detected by CHEF gel analysis were less than or equal to those detected by RFLP analyses of either the repetitive element from *C. michiganensis* subsp. *sepedonicus* [28] or 16S rDNA [17]. Results from CHEF analysis were also consistent with those recently reported by Pastrik and Rainey [33], in which RAPD fingerprints were used to differentiate strains within subspecies of *C. michiganensis*. We found that the ability to detect differences among strains depended on the choice of restriction enzyme used to generate the CHEF profile. *Hind*III revealed greater dissimilarity between certain strains, and was useful for distinguishing between HR-negative strains and HR-positive strains. *Eco*RI gave more haplotypes, but fingerprints were more similar than ones obtained with *Hind*III. The most dissimilar strain used in this study was CIC31. This strain is a spontaneous rifampicin-resistant mutant of CSU#2539-69 and has been used routinely in field, greenhouse, and laboratory experiments in Colorado [11, 12, 30]. The genetic dissimilarity detected between CIC31 and other strains might have been present in the original wild-type strain, which is no longer in culture, or might reflect mutations associated with acquisition of antibiotic resistance.

We were able to detect differences between the genomic fingerprints of virulent and avirulent strains of *C. michiganensis* subsp. *sepedonicus*. Differences in genome size and organization have been reported recently for virulent and avirulent strains of the related phytopathogen, *R. fascians* [34]. DNA fingerprints of class 1 (avirulent and HR-negative) strains of *C. michiganensis* subsp. *sepedonicus* differed from those of the class 3 (virulent and HR-positive) strains (Fig. 1 and 2). The presence of an approximately 330-kb band and a 115-kb band in the *Hind*III profiles of avirulent HR-negative strains, Cs3NM and CIC243, might indicate that these bands resulted from mutations leading to the loss of both virulence and HR induction. Similarly, the presence of a 124-kb band in the *Eco*RI fingerprint of Cs3NM and CIC243 correlates with the loss of the mucoid phenotype and virulence. While these band differences can provide clues into a molecular basis for virulence in *C. michiganensis* subsp. *sepedonicus*, conclusive localization of genes for extracellular polysaccharide production and virulence requires much further study.

The differences in high-molecular weight genomic fingerprints detected among strains were independent of

Table 3. Similarity coefficients for pairwise comparisons of *Hind*III genomic fingerprints of selected *Clavibacter michiganensis* subsp. *sepedonicus* strains

Strain	BC-P45	P45	CIC243	CIC242	CS3NM	CS3	CIC31	NCPPB 2140	ATCC 9850	ATCC 33111	ATCC 33113
BC-P45	1.00										
P45	1.00	1.00									
CIC243	0.89	0.89	1.00								
CIC242	0.80	0.80	0.70	1.00							
Cs3NM	0.89	0.89	1.00	0.70	1.00						
Cs3	0.80	0.80	0.70	1.00	0.70	1.00					
CIC31	0.84	0.84	0.74	0.76	0.74	0.76	1.00				
NCPPB2140	1.00	1.00	0.89	0.80	0.89	0.80	0.84	1.00			
ATCC9850	1.00	1.00	0.89	0.80	0.89	0.80	0.84	1.00	1.00		
ATCC33111	1.00	1.00	0.89	0.80	0.89	0.80	0.84	1.00	1.00	1.00	
ATCC33113	1.00	1.00	0.89	0.80	0.89	0.80	0.84	1.00	1.00	1.00	1.00

Table 4. Similarity coefficients for pairwise comparisons of *Eco*RI genomic fingerprints of selected *Clavibacter michiganensis* subsp. *sepedonicus* strains

Strain	BC-P45	P45	CIC243	CIC242	CS3NM	CS3	CIC31	NCPPB 2140	ATCC 9850	ATCC 33111	ATCC 33113
BC-P45	1.00										
P45	0.98	1.00									
CIC243	0.98	0.95	1.00								
CIC242	0.98	0.95	0.95	1.00							
Cs3NM	0.95	0.98	0.98	0.93	1.00						
Cs3	0.95	0.98	0.93	0.98	0.95	1.00					
CIC31	0.90	0.88	0.88	0.93	0.86	0.91	1.00				
NCPPB2140	0.98	0.95	0.95	1.00	0.93	0.98	0.93	1.00			
ATCC9850	0.93	0.95	0.91	0.95	0.93	0.98	0.89	0.95	1.00		
ATCC33111	0.95	0.98	0.93	0.98	0.95	1.00	0.91	0.98	0.98	1.00	
ATCC33113	0.95	0.98	0.93	0.98	0.95	1.00	0.91	0.98	0.98	1.00	1.00

pCS1, since pCS1 is not digested by *Eco*RI or *Hind*III and open-circular plasmids in agarose plugs rarely enter into gels during CHEF electrophoresis [18]. The clustering of P45 in the *Hind*III profile with other HR-inducing strains is consistent with previous conclusions that P45's reduced virulence is primarily due to its loss of pCS1 and concomitant loss of cellulase production [16, 31]. On the other hand, its clustering with class 1 strains in the *Eco*RI dendogram suggests there may be additional differences between P45 and class 3 strains other than just the loss of pCS1.

The apparent correlation between the loss of virulence and loss of EPS in Cs3 derivatives CIC243 and CS3NM needs to be interpreted cautiously. Previous studies have shown that virulence in *C. michiganensis* subsp. *sepedonicus* is not strictly correlated with the mucoid phenotype [10, 30, 39]. In fact, a mucoid avirulent derivative of Cs3, designated 3M, has been reported [30]. Determining the molecular mechanism that yields spontaneous derivatives of Cs3 may provide for a better understanding of the relationship between EPS and virulence in *C. michiganensis* subsp. *sepedonicus*.

The reliability and reproducibility of genomic fingerprints obtained by CHEF gel electrophoresis has made it an attractive method for typing of other bacterial species and may be useful for studies with *C. michiganensis* subsp. *sepedonicus* [7, 8]. Some strains of *C. michiganensis* subsp. *sepedonicus* do not appear to change significantly even after continual subculture [28], while others like Cs3 appear to be unstable, producing spontaneous avirulent and nonmucoid mutants. Nonetheless, it may be feasible to use CHEF genomic fingerprinting to track strains associated with bacterial ring rot outbreaks, since we found that CHEF genomic fingerprints of *C. michiganensis* subsp. *sepedonicus* were very reproducible between experiments and reliably represented a strain's lineage. Fingerprints of two different forms of the plasmidless strain P45 obtained from different sources gave the same fingerprints. Furthermore,

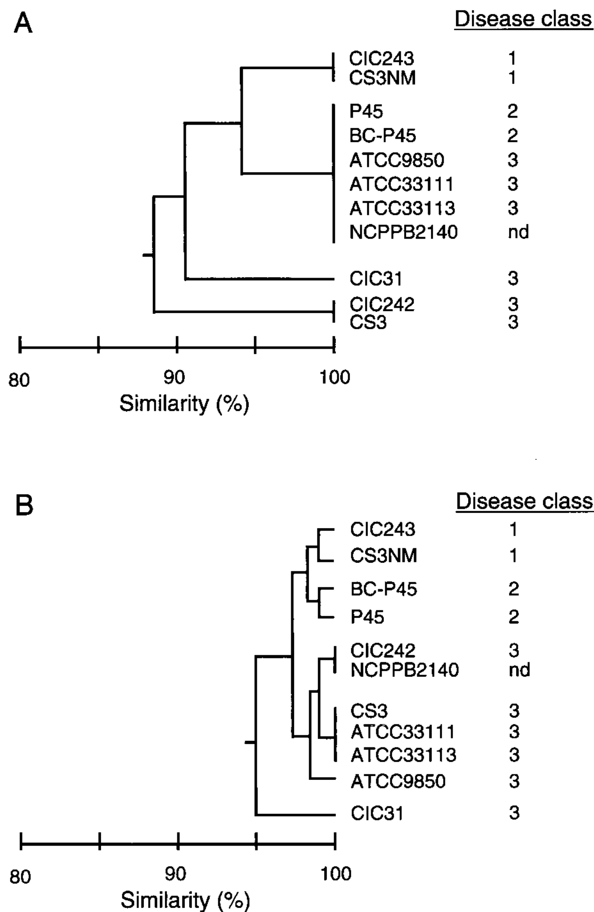

Fig. 2. Dendograms of genomic fingerprints obtained with *Hind*III (**A**) or *Eco*RI (**B**) digested high-molecular weight DNA of strains of *C. michiganensis* subsp. *sepedonicus* representing different disease classes. Class 1 indicates low population size in potato ($\leq 3 \times 10^4$ IFAS units/sample), none or slight symptom expression in eggplant and potato, and no induction of tobacco HR; class 2 indicates moderate population size in potato (between 4×10^6 and 2×10^7 IFAS units/sample), none or slight symptom expression in eggplant and potato, and induction of tobacco HR; class 3 indicates extensive multiplication in potato ($\geq 1 \times 10^8$ IFAS units/sample), severe symptom expression in eggplant and potato, and induction of tobacco HR.

the fingerprints of Cs3 and its mucoid derivative, CIC242, were reproducible, regardless of their sources.

Plant pathogenic members of the high G+C Gram-positive coryneform group present several challenges to scientists investigating molecular plant-microbe interactions. Most species in the group grow slowly *in vitro* and are recalcitrant to genetic manipulation by methods developed for the study of Gram-negative phytopathogens [24]. Nonetheless, the development of transformation systems for *C. michiganensis* [15, 22] and the recent identification of genes associated with virulence in two of its subspecies [5, 13, 16] are positive indicators that the molecular biology of *C. michiganensis* is indeed tractable. The isolation and manipulation of high-molecular weight DNA fragments, as presented here, offers a further means for advancing the molecular biology of *C. michiganensis* subsp. *sepedonicus*.

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