

**Genetic Variation in Strains of *Clavibacter michiganense* subsp. *sepedonicum*:
Polymorphisms in Restriction Fragments Containing a Highly Repeated Sequence**

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ABSTRACT

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Plasmid pCSI, a widely distributed plasmid in the potato pathogen *Clavibacter michiganense* subsp. *sepedonicum*, contains two copies of a 1.3-kilobase repeated sequence that is present in high copy number on the bacterial chromosome. A *Bam*HI restriction fragment containing one of the plasmid copies of the repeated sequence has been cloned into transcription vector pGEM4. The transcript of this cloned fragment has been used as a molecular probe to detect polymorphisms in restriction fragments that contain the repeated sequence. Eleven different restriction enzymes were used to generate restriction fragment length polymorphism (RFLP) patterns from chromosomal DNA of 10 strains of the bacterium.

Under the conditions employed in this study, restriction enzymes *Bam*HI, *Bcl*II, *Bst*XI, and *Sma*I were particularly effective in yielding RFLP patterns that permit discrimination of strains. Restriction enzyme *Nhe*I was useful for differentiating pathogen strains that contain autonomously replicating pCSI from those in which the plasmid is integrated into the bacterial chromosome. The RFLP patterns produced by *Nhe*I also provided evidence that integration of pCSI into the chromosome is a site-specific process. No alterations in RFLP patterns were observed upon repeated subculture of selected strains. Numerical analysis of the RFLP data indicated that the strains are of high similarity.

Additional keywords: potato bacterial ring rot, *Solanum tuberosum*.

Clavibacter michiganense subsp. *sepedonicum* (4) is the causal agent of potato bacterial ring rot. Strains of this microorganism exhibit little morphological, physiological, or pathogenic variation, which currently makes differentiation and identification of isolates difficult (5). However, the development of modern molecular biological methods (18) has provided a new approach to differentiation of closely related organisms. Genetic microheterogeneity of related organisms often results in varying genomic locations for nucleotide sequences sensitive to cleavage by specific restriction endonucleases. These variations can be detected as restriction fragment length polymorphisms (RFLPs) (17) upon hybridization of the fragments with appropriate molecular probes. Genetic characterization of the differences between individuals, strains, species, or genera by use of RFLPs has several advantages over the older methods of examination of variant isozymes or serotypes, and the resolution of this technique is limited primarily by the number of restriction enzymes and unique molecular probes that one is willing to employ (1,36). In the past few years, the existence of RFLPs has been exploited for basic and applied genetics of animal and plant systems (1,36). Studies of RFLPs in microbes are much less extensive, and most of the microbial studies have been done with enterobacteria (11,31,37). However,

a few recent reports have described the analysis of genomes from plant pathogenic fungi (13,15,19) and bacteria (6,7) by this method.

Previous studies in this laboratory have demonstrated the common occurrence of integrative plasmid pCSI in strains of *C. m. sepedonicum* and the existence of a 1.3-kilobase (kb) repeated sequence in the plasmid and in high copy number in the chromosomal DNA of all tested strains of this organism (24,25). In this paper, we describe the preparation of a subclone of the repeated sequence from pCSI and demonstrate the utility of this cloned sequence as a hybridization probe for RFLP studies directed at genetic characterization and differentiation of strains of *C. m. sepedonicum*.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Strains of *C. m. sepedonicum* were chosen to represent a diverse cross section of the pathogen population. They were obtained from the American Type Culture Collection (ATCC), Rockville, MD; Plant Disease Division Culture Collection (PDDCC), Auckland, New Zealand; S. De Boer, Agriculture Canada, Vancouver, B.C.; F. Manzer, University of Maine, Orono, ME; D. Clarke, Idaho Crop Improvement Association, Idaho Falls, ID; and the culture

collection of the Plant Pathology Department of North Dakota State University, Fargo. These strains are described in Table 1. All strains of *C. m. sepedonicum* were grown in NBY broth (33) at 22 C on a gyrotory shaker at 175 rpm. Strains JM109 (38) and DH5 α (Bethesda Research Laboratories, Gaithersburg, MD) of *Escherichia coli* were used as hosts for cloning with M13 phage and pGEM4 plasmid vectors, respectively; they were grown in 2YT broth (22) at 37 C on a gyrotory shaker at 225 rpm.

DNA isolation and restriction digest preparation. Plasmid pCS1 of *C. m. sepedonicum*, spooled chromosomal DNA from strains of this organism, and plasmid or M13 phage cloning vectors were prepared as described previously (25).

Restriction digests (20 μ l total volume) contained chromosomal DNA (500 ng), restriction endonuclease, and the buffers and other components recommended by the suppliers of the restriction enzymes (New England Biolabs, Beverly, MA, and Pharmacia, Piscataway, NJ). Sufficient incubation times (1–2 hr) and quantities of enzyme (2–10 units) were used to assure that complete digests were obtained.

Probe construction and labeling. Standard methods (18,22) were used for cloning of DNA into plasmid and phage vectors. *Bam*HI restriction endonuclease was used to cleave replicative-form DNA from clone X3R-20R, a recombinant M13 phage that contains pCS1 fragment 8A (24). This *Sma*I fragment of the plasmid contains one copy of the highly repeated sequence from *C. m. sepedonicum*. The *Bam*HI fragments of clone X3R-20R replicative form were ligated into transcription vector pGEM4 (Promega, Madison, WI) that had been treated with calf intestine alkaline phosphatase after linearization with *Bam*HI. The recombinant inserts of pGEM4 clones were identified by sizing fragments released upon digestion with *Bam*HI endonuclease. Clone pBH12, which contains the 0.6-kb *Bam*HI fragment of pCS1 fragment 8A, was chosen as the probe for this study.

Radioisotope-labeled RNA probe (specific activity approximately 1.5×10^9 cpm/ μ g) was prepared by linearizing pBH12 with *Eco*RI and generating runoff transcripts. The protocol supplied by Promega was employed. Materials used in this procedure included T7 RNA polymerase (Pharmacia) and [α - 32 P]CTP (New England Nuclear, Boston, MA; specific activity 800 Ci/mmol). After the material was treated with RNase-free pancreatic DNase (Promega), oligodeoxyribonucleotides and unincorporated label were removed by passing the sample through a sterile Sephadex G-50 column equilibrated with TE buffer (18). The resulting preparation is termed pBH12 riboprobe.

Southern transfers and hybridization. DNA fragments in restriction digests were separated on horizontal gels containing 1.0% agarose. Standard electrophoretic separations were carried out at 3–5 V/cm in the Tris-borate buffer of Messing (22). The separated fragments were transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA) with 0.4 M NaOH by the method of Reed and Mann (30). Prehybridization and

hybridization conditions have been described previously (24). RNA probes were incubated at 95 C for 10 min in hybridization solution before use. High stringency washes and autoradiographic procedures have been described previously (24).

Analysis of autoradiograms. Autoradiograms were examined for variation in RFLP patterns among strains of *C. m. sepedonicum* by visual inspection and scanning densitometry. The basis for differentiation of RFLP pattern groups was addition or deletion of a distinct band or a marked change in band intensity. Minor differences in intensities of specific bands, such as those sometimes associated with the presence of variable small quantities of autonomous plasmid DNA in the chromosomal DNA preparations, were not used to define pattern groups.

Examination of RFLP pattern stability. RFLP pattern stability was evaluated by subculturing two distinct strains of *C. m. sepedonicum*: Cs3R and Cs106. Each strain was plated on NBY agar, and four typical colonies were inoculated into separate flasks containing 100 ml of NBY medium. Upon reaching stationary phase, a portion of each culture was streaked on NBY agar, and a single typical colony was selected for inoculation of another flask of the liquid medium. The process was repeated for a total of 11 cycles of growth on each medium. With each strain, chromosomal DNA was prepared from a portion of the original culture and each of the four separate serial subcultures. These DNA preparations were subjected to RFLP analysis.

Similarity coefficients and cluster analysis. Numerical analysis of RFLP data was performed by published methods (6,7,35). Restriction fragments that produced autoradiographic bands were assigned values of 1 or 0 for each strain, depending upon the presence or absence of the band under examination. With autoradiograms from *Bcl*II, *Bst*XI, and *Cla*I digests, only the readily resolved subset of bands below 6.1 kb was analyzed. Similarity coefficients were obtained by use of the equation of Nei and Li (26): $S = 2N_{xy} / (N_x + N_y)$. In this equation, N_x and N_y represent the total number of RFLP bands in autoradiograms of strains x and y , respectively, and N_{xy} represents the number of RFLP bands shared by the two strains. Cluster analysis was done with the NTSYS-pc software package (Exeter Publishing, Setauket, NY). The SAHN program from the software was used for this analysis. The quality of the resulting clusters was evaluated by determination of cophenetic correlation coefficients (32,35). These calculations involved the use of the COPH and MXCOMP programs on the software.

RESULTS

Probe construction. Recombinant M13 phage clone X3R-20R contains *Sma*I fragment 8A of pCS1. A single copy of the 1.3-kb repeated sequence is located near the middle of this 2.88-kb fragment (24). A detailed map of fragment 8A that indicates the sites for the restriction enzymes used in this work is shown

TABLE 1. Strains of *Clavibacter michiganense* subsp. *sepedonicum* subjected to restriction fragment length polymorphism analysis

Strain	Form of pCS1 ^a	Virulence ^b	Original host	Geographic origin ^c	Year of isolation ^c	Source ^d
Cs33113 ^e	A	+	Potato	British Columbia	pre-1979	ATCC
Cs3R	A	+	Potato	Alberta	1976	S. De Boer
Cs2531	A	++	Potato	Sweden	1956	PDDCC
CsME1	A	++	Potato	Maine	pre-1955	F. Manzer
CsIDNM-2	I	NT ^f	Potato	Idaho	1983	D. Clarke
CsND2	I	+	Potato	North Dakota	1984	NDSU
CsND7	I	++	Potato	North Dakota	1984	NDSU
CsNDSB3	I	+	Sugar beet	North Dakota	1984	NDSU
Cs106	I	++	Potato	West Virginia	pre-1945	S. De Boer
Cs20	I	++	Potato	British Columbia	1977	S. De Boer

^aA = autonomous; I = integrated.

^bVirulence evaluated by the method of Gudmestad et al (9): + = weakly or moderately virulent; ++ = highly virulent.

^cInformation supplied by source of strain.

^dATCC = American Type Culture Collection, Rockville, MD; PDDCC = Plant Disease Division Culture Collection, Auckland, New Zealand; NDSU = North Dakota State University, Fargo.

^eType strain.

^fNT = not tested.

in Figure 1. The 0.6-kb *Bam*HI fragment that is located almost entirely within the repeated sequence was subcloned and used to prepare the probe employed in this study. This probe, which selectively hybridizes to regions of the genomic DNA that contain the repeated sequence, does not yield the multiple extraneous bands observed in preliminary experiments with a nick-translated X3R-20R probe (23); these extraneous bands were found to result from hybridization of DNA of *C. m. sepedonicum* with the vector and with regions of pCS1 fragment 8A external to the repeated sequence (Oleson et al, unpublished results).

Selection of restriction enzymes for generating RFLPs. Most of the restriction enzymes used in this study recognize hexanucleotide or octanucleotide DNA sequences. All of the enzymes tested in detail produced complete digests of chromosomal DNA and generated a broad size range of DNA fragments. In each of the experiments described in Figures 2-4, fragments from 11

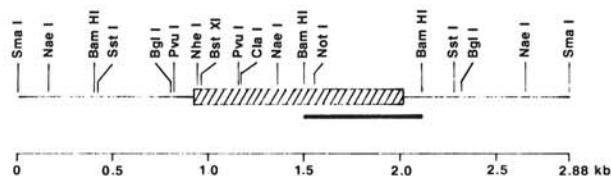


Fig. 1. Restriction map of *Sma*I fragment 8A of plasmid pCS1. No sites for *Bcl*I were present. The shaded rectangle denotes the repeated sequence. The underlined *Bam*HI fragment was subcloned into pGEM4 to yield pBH12.

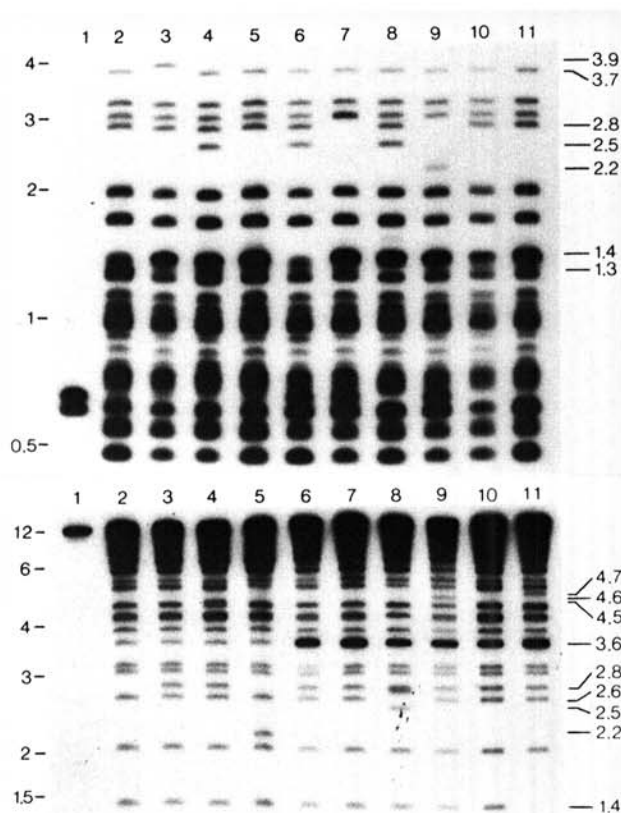


Fig. 2. Hybridization of Southern blots of restriction fragments of DNA from *Clavibacter michiganense* subsp. *sepedonicum* with the pBH12 riboprobe. Top panel: *Bam*HI-digested DNA; bottom panel: *Bcl*I-digested DNA. Lane 1, DNA from plasmid pCS1. Lanes 2-5, chromosomal DNA from plasmid-autonomous strains Cs33113, Cs3R, Cs2531, and CsME1, respectively. Lanes 6-11, chromosomal DNA from plasmid-integrated strains CsIDNM-2, CsNDSB3, CsND2, CsND7, Cs106, and Cs20, respectively. The numbers on the left indicate the sizes of fragments, based on a commercial 1-kilobase ladder, and those on the right indicate sizes of specific fragments used for strain differentiation. All sizes are expressed in kilobase pairs.

different DNA samples were electrophoresed, transferred to membranes, and hybridized with the pBH12 riboprobe. The DNA samples used in each experiment included purified pCS1 DNA, chromosomal DNA from four strains of *C. m. sepedonicum* that contain the autonomously replicating form of pCS1, and chromosomal DNA from six bacterial strains that contain the integrated form of the plasmid. Because of the high copy number of the repeated sequence in the genome of *C. m. sepedonicum*, the action of each of the restriction enzymes on chromosomal DNA from this organism resulted in the generation of more than 20 fragments that hybridized with the pBH12 riboprobe. The restriction enzymes produced RFLP patterns that varied in both resolution of the fragments that interacted with the probe and the number of unique, well-resolved bands useful for differentiation of the tested strains.

Polymorphisms produced by BamHI, BclI, BstXI, and SmaI. These four restriction enzymes produced RFLP patterns that were very useful for the differentiation of the tested strains (Figs. 2 and 3). The autoradiographic bands corresponded to restriction fragments with sizes ranging from 0.5 to approximately 15 kb. In general, the bands were well resolved, and several unique fragments were present. Although the patterns obtained with chromosomal DNA from the 10 strains are rather similar, they have easily recognizable differences. Seven fragments, with sizes ranging from 1.3 to 3.9 kb, permit differentiation of RFLP groups in *Bam*HI digests, whereas nine fragments of 1.4 to 4.7 kb vary in *Bcl*I digests of chromosomal DNA from the tested strains. Both *Bam*HI and *Bcl*I generated eight distinct RFLP patterns, of which seven were unique in the group of 10 tested strains

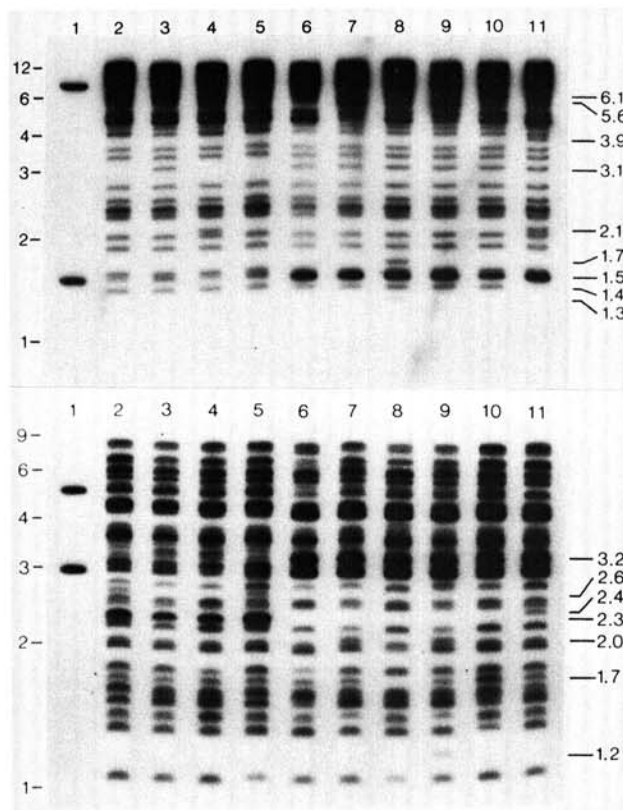


Fig. 3. Hybridization of Southern blots of restriction fragments of DNA from *Clavibacter michiganense* subsp. *sepedonicum* with the pBH12 riboprobe. Top panel: *Bst*XI-digested DNA; bottom panel: *Sma*I-digested DNA. Lane 1, DNA from plasmid pCS1. Lanes 2-5, chromosomal DNA from plasmid-autonomous strains Cs33113, Cs3R, Cs2531, and CsME1, respectively. Lanes 6-11, chromosomal DNA from plasmid-integrated strains CsIDNM-2, CsNDSB3, CsND2, CsND7, Cs106, and Cs20, respectively. The numbers on the left indicate the sizes of fragments, based on a commercial 1-kilobase ladder, and those on the right indicate sizes of specific fragments used for strain differentiation. All sizes are expressed in kilobase pairs.

(Fig. 2, Table 2). RFLP pattern differentiation of *Bst*XI digests used nine fragments with sizes ranging from 1.3 to 6.1 kb, whereas differentiation of RFLP groups in *Sma*I digests used seven fragments ranging in size from 1.2 to 3.2 kb. Action of *Bst*XI or *Sma*I on chromosomal DNA from the tested strains produced seven RFLP groups, of which four were unique (Fig. 3, Table 2). The combination of the results from the *Bam*HI patterns with those from either *Bcl*I, *Bst*XI, or *Sma*I permitted all of the tested strains to be differentiated (Table 2).

Polymorphisms produced by other restriction enzymes. The other restriction enzymes tested in detail were *Bgl*II, *Cl*aI, *Nae*I, *Nhe*I, *Not*I, *Pvu*I, and *Sst*I. They were less effective in producing fragments that permitted strains of *C. m. sepedonicum* to be distinguished. The number of RFLP patterns (not shown) obtained with the bacterial strains ranged from two to five for these restriction enzymes (Table 2). None of this group of enzymes was able to differentiate among strains carrying the free form of the plasmid. Fewer than half of the tested strains produced RFLP patterns that were unique with any of this group of enzymes. However, *Bgl*II and *Cl*aI were able to aid the differentiation of strains that could not be resolved by *Bam*HI or *Bcl*I. These enzymes may be useful with other strains of the pathogen that have not been tested. In addition to the enzymes listed here, less extensive studies have been performed with several other enzymes (*Acc*I, *Avr*II, *Bgl*II, *Bst*BI, *Dra*I, *Eag*I, *Eco*RI, *Eco*RV, *Fnu*DII, *Hae*III, *Hinc*II, *Hpa*I, *Kpn*I, *Nde*I, *Pvu*II, *Rsa*I, *Sau*3AI, *Sfi*I, *Sna*BI, *Spe*I, *Ssp*I, and *Xho*I). Many of these enzymes produced complete digests of DNA from *C. m. sepedonicum* only with

difficulty, and the presence of variable amounts of incompletely digested DNA caused the RFLP patterns to be of poor reproducibility. Other enzymes produced apparently complete digests that contained a series of large fragments of similar size that did not separate well under standard electrophoretic conditions (not shown).

Polymorphisms dependent upon the form of pCS1. Previous studies from this laboratory have shown that virtually all strains of *C. m. sepedonicum* possess plasmid pCS1 in either integrated or autonomous form; the site of integration has been localized to a 5.1-kb *Sma*I fragment of the plasmid, which also possesses a copy of the repeated sequence (24,25). The proximity of the repeated sequence to the site of integration permits the detection of plasmid-chromosome junction fragments uniquely present in integrated strains upon hybridization of Southern blots with the pBH12 riboprobe. The results obtained with *Nhe*I, shown in Figure 4, demonstrate this phenomenon. All integrated strains displayed bands of 4.5 and 6.0 kb, which were not present in the strains possessing the autonomous form of the plasmid. Both of the junction fragments also could be observed in RFLP patterns obtained with *Sst*I (not shown). One of the two junction fragments could be detected in RFLP patterns obtained with *Bcl*I (Fig. 2, lower panel, 3.6-kb band), *Bst*XI (Fig. 3, upper panel, 1.5-kb band), *Bgl*II, *Nae*I, *Pvu*I, and *Sma*I (Fig. 3, lower panel, 3.2-kb band).

Another aspect of the integrative process can be observed in the patterns obtained with *Nhe*I. A 2.8-kb fragment is present only in strains containing the autonomous form of pCS1 (Fig. 4). This fragment contains the site at which the plasmid integrates into the bacterial chromosome. In addition to the 10 strains examined in Figure 4, 19 other strains of *C. m. sepedonicum* have been tested and found to produce *Nhe*I RFLP patterns consonant with their plasmid status.

RFLP pattern stability. Genomic stability of the target sequences of the pBH12 riboprobe was tested by RFLP analysis of DNA isolated from selected strains before extensive subculture and after serial subculture that totaled 425 bacterial generations. Four separate samples of each strain were subjected to the extensive subculture. The results of this study with Cs3R, a strain containing the autonomous form of pCS1, are shown in Figure 5. It can be seen that there are no differences in the *Bst*XI or *Sma*I RFLP patterns obtained with the parental sample and any of the extensively subcultured samples. Analysis of patterns obtained with *Bam*HI and *Bcl*I also indicated no changes in the four parallel subcultures of strain Cs3R (data not shown). A parallel study of Cs106 revealed that this strain, which contains pCS1 in an integrated form, also possessed complete RFLP pattern stability over the time period examined.

Similarity coefficients and cluster analysis. Methods of numerical taxonomy were used to quantify the relationships among the tested strains. Because genetic differences related to the state of integration of pCS1 were presumed to be minor, integration-related bands on the autoradiograms were excluded from the calculations. Similarity coefficients were calculated for each pair of strains, using data from each of the restriction enzymes listed

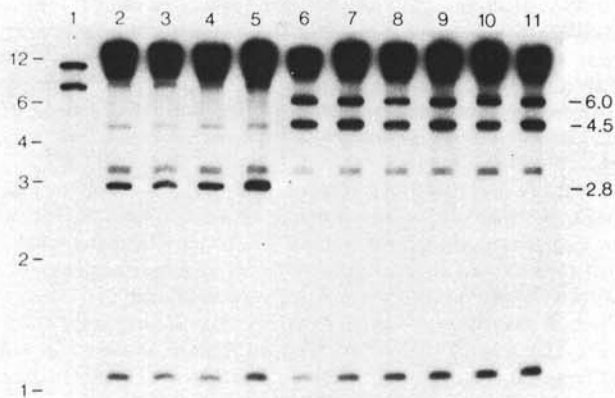


Fig. 4. Hybridization of a Southern blot of *Nhe*I-digested DNA from *Clavibacter michiganense* subsp. *sepedonicum* with the pBH12 riboprobe. Lane 1, DNA from plasmid pCS1. Lanes 2-5, chromosomal DNA from plasmid-autonomous strains Cs33113, Cs3R, Cs2531, and CsME1, respectively. Lanes 6-11, chromosomal DNA from plasmid-integrated strains CsIDNM-2, CsNDSB3, CsND2, CsND7, Cs106, and Cs20, respectively. The numbers on the left indicate the sizes of fragments, based on a commercial 1-kilobase ladder, and those on the right indicate sizes of specific fragments used for strain differentiation. All sizes are expressed in kilobase pairs.

TABLE 2. Summary of pattern groups produced by the restriction enzymes used for restriction fragment length polymorphism (RFLP) analysis of DNA from strains of *Clavibacter michiganense* subsp. *sepedonicum*

Isolate	RFLP pattern type										
	<i>Bam</i> HI	<i>Bcl</i> I	<i>Bgl</i> II	<i>Bst</i> XI	<i>Cl</i> aI	<i>Nae</i> I	<i>Nhe</i> I	<i>Not</i> I	<i>Pvu</i> I	<i>Sma</i> I	<i>Sst</i> I
Cs33113	Ba-1	Bc-1	Bg-1	Bs-1	Cl-1	Na-1	Nh-1	No-1	Pv-1	Sm-1	Ss-1
Cs3R	Ba-2	Bc-2	Bg-1	Bs-2	Cl-1	Na-1	Nh-1	No-1	Pv-1	Sm-2	Ss-1
Cs2531	Ba-3	Bc-3	Bg-1	Bs-3	Cl-1	Na-1	Nh-1	No-1	Pv-1	Sm-2	Ss-1
CsME1	Ba-4	Bc-4	Bg-1	Bs-1	Cl-1	Na-1	Nh-1	No-1	Pv-1	Sm-1	Ss-1
CsIDNM-2	Ba-5	Bc-5	Bg-2	Bs-4	Cl-2	Na-2	Nh-2	No-2	Pv-2	Sm-3	Ss-2
CsNDSB3	Ba-6	Bc-5	Bg-3	Bs-5	Cl-3	Na-3	Nh-2	No-3	Pv-2	Sm-4	Ss-3
CsND2	Ba-7	Bc-6	Bg-4	Bs-6	Cl-4	Na-4	Nh-2	No-4	Pv-2	Sm-5	Ss-4
CsND7	Ba-8	Bc-7	Bg-3	Bs-5	Cl-3	Na-2	Nh-2	No-3	Pv-2	Sm-6	Ss-3
Cs106	Ba-4	Bc-5	Bg-3	Bs-4	Cl-5	Na-2	Nh-2	No-2	Pv-2	Sm-3	Ss-3
Cs20	Ba-4	Bc-8	Bg-5	Bs-7	Cl-2	Na-2	Nh-2	No-2	Pv-2	Sm-7	Ss-3

in Table 2 except *NheI* and *PvuI*, and also using the combined data from these nine enzymes. Similarity coefficients for data from single RFLP patterns ranged from 0.857 to 1.000, whereas pairwise comparison of the combined RFLP data for the strains yielded values of 0.944 to 0.993. Cluster analysis of the similarity coefficients from the combined RFLP data, performed by the unweighted pair-group method with averages, yielded the phenogram shown in Figure 6. Virtually identical cluster patterns were obtained when clustering was done by the single-link or complete-link methods. The cluster data of Figure 6 also were analyzed to yield a cophenetic correlation coefficient. The resulting value, 0.923, indicates a very good fit of the similarity coefficient data to the cluster relationships shown in the figure.

DISCUSSION

RFLPs traditionally have been used as genetic markers to help localize specific genes (10) and in breeding programs to follow linked traits (1,29). RFLPs recently have been used in plant pathology as a taxonomic aid. For example, this approach has been applied to distinguish *Xanthomonas* pathovars (16) and to

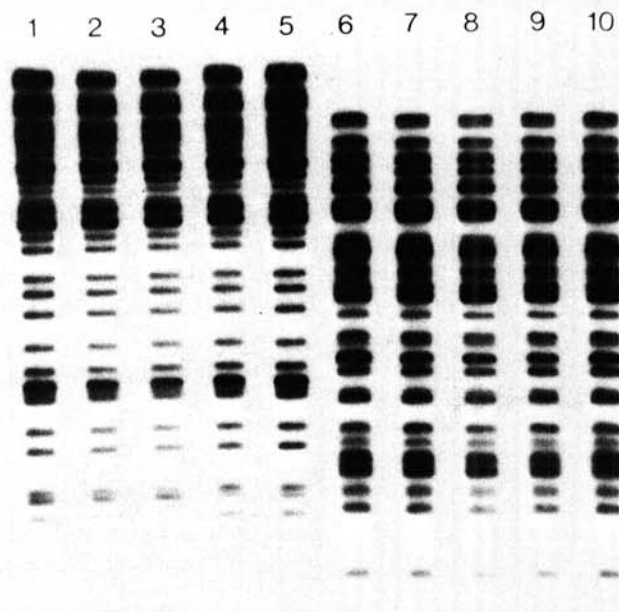


Fig. 5. Hybridization of a Southern blot of restricted DNA from *Clavibacter michiganense* subsp. *sepedonicum* with the pBH12 riboprobe. Lanes 1-5, DNA digested with *BstXI* endonuclease. Lanes 6-10, DNA digested with *SmaI* endonuclease. Lanes 1 and 6, chromosomal DNA from strain Cs3R before extensive subculturing. Lanes 2-5 and 7-10, chromosomal DNA from four separate clones of Cs3R after extensive subculturing.

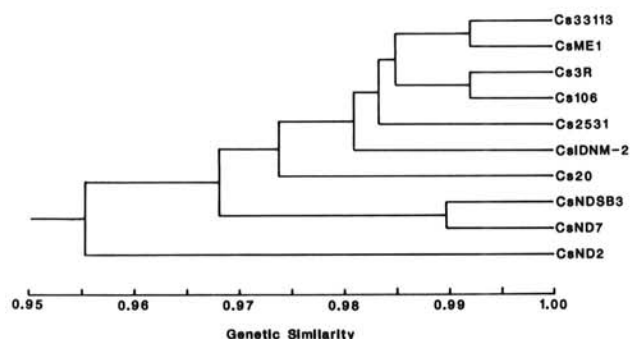


Fig. 6. Phenogram obtained by cluster analysis of restriction fragment length polymorphism similarity matrix data from 10 strains of *Clavibacter michiganense* subsp. *sepedonicum*.

help differentiate biotypes of *Fusarium* (19). The molecular probes used in these two studies were cloned anonymous DNA sequences. However, other studies with microbial systems have used cloned structural genes for ribosomal RNA (34) or specific polypeptides (37). Although most studies have used probes directed at single or low-copy-number sequences, probes directed at multiple-copy-number genomic targets, such as IS1 also have been employed (12,28). The results presented in this paper demonstrate the value of a probe directed at a high-copy-number target in the genome of *C. m. sepedonicum* for the production of RFLPs useful for strain identification in this species. The copy number of this repeated sequence in *C. m. sepedonicum* has been estimated to be greater than 50 (24). With virtually all of the enzymes tested, at least two dozen autoradiographic bands were observed in each chromosomal DNA digest. The large number of bands, caused by the high copy number of the target sequence, enhanced the ability of this single probe to permit differentiation between strains of the organism.

By the judicious selection of enzymes, it appears that one can separate virtually any pair of genetically distinct strains by RFLP analysis with the pBH12 riboprobe. The three pathogen strains isolated from a limited geographic region, CsND2, CsND7, and CsNDSB3, were resolved by five of the enzymes examined in detail. The two strains most difficult to resolve were Cs33113 (the type strain) and CsME1. However, two enzymes produced RFLP patterns that allowed these two strains to be differentiated.

There was substantial variation among the tested restriction enzymes in their ability to generate useful banding patterns. Of the enzymes used in this study, *BamHI* and *BclI* produced eight different RFLP patterns, whereas *PvuI* and *NheI* each produced only two different patterns. It also should be noted that with some enzymes (for example, *BclI*, *NheI*) a strong autoradiographic signal was obtained from unresolved high-molecular-weight fragments on the gel, and it is possible that polymorphisms exist in this region that could be revealed by recently developed pulsed field electrophoretic methods (2,8) that permit resolution of large fragments of similar size.

Some of the restriction fragments that hybridize with the pBH12 riboprobe involve the region of the chromosome of *C. m. sepedonicum* where integration of pCS1 occurs. Previous work has shown that integration occurs within a specific fragment of the plasmid (24), and the present study reveals that the integrative process is also specific for a single region of the bacterial chromosome. The fragment from pCS1-autonomous strains containing the target site for integration and the two related fragments containing the plasmid-chromosome junction region from pCS1-integrated strains were readily apparent in *NheI* RFLP patterns. One or two of these three integration-related fragments could be seen in RFLP patterns produced by several other enzymes. Detection of all three integration-related fragments in chromosomal DNA digests produced by all tested restriction enzymes may have been obscured by comigration of these fragments with other fragments that contain the repeated sequence or may have been prevented by cleavage of the integration site from the nearby repeated sequence responsible for the autoradiographic signal.

The genomic target of the molecular probe used in this study is a high-copy-number repeated sequence whose function remains to be determined. However, its size, approximately 1.3 kb, and copy number are similar to those of insertion elements that have been examined in other bacterial systems (14). The potential ability of the target sequence to function in insertional and transpositional events caused us to examine the stability of the RFLP patterns obtained with this probe. Analysis of DNA samples from plasmid-autonomous and plasmid-integrated strains of the organism subjected to extensive subculturing over a period of 10 mo revealed no change in RFLP patterns. These results indicate that intrachromosomal migrations of the repeated sequence in *C. m. sepedonicum* are not highly frequent events. This conclusion is supported by the high degree of similarity of the RFLP patterns from strains isolated over several decades from diverse locations. Studies on the sequence and function of the repeated sequence and examination of the stability of the RFLP patterns obtained

with the pBH12 riboprobe during culture of the pathogen in the natural plant host are in progress.

Recently, RFLP data have been used to probe the genetic diversity of strains of other plant pathogenic microbes (3,6,7). An examination of the 10 ring rot pathogen strains by the methods of numerical taxonomy revealed that they possess a high degree of similarity. The existence of closely related bacteria isolated from different geographical regions over a period of more than 40 years suggests a clonal population structure for this organism. *C. m. sepedonicum* occupies a relatively narrow ecological niche. It does not survive in soil (27), and growth in natural environments is thought to be limited primarily to vascular tissues of potato (5,20). The limited genetic base observed in this highly specialized pathogen is consonant with the recent study of McArthur and co-workers, in which they found that genetic diversity of natural populations of a soil bacterium, *Pseudomonas cepacia*, correlates directly with the degree of environmental heterogeneity (21).

Although the presence of unique bands can be demonstrated in some tested strains of *C. m. sepedonicum*, most of the bands obtained upon hybridization with the pBH12 riboprobe are shared by two or more strains of the pathogen. Many of the fragments were highly conserved and did not vary among any of the tested strains. Comparison of RFLP patterns from bacterial isolates with patterns produced by authentic strains of *C. m. sepedonicum* can serve as an adjunct to the currently used methods for identification of the ring rot pathogen, monoclonal antibody-based immunofluorescence, and pathogenicity testing in the greenhouse (5). Other potential applications of RFLP analysis of *C. m. sepedonicum* include aspects of epidemiological interest, such as studies on the source and spread of ring rot outbreaks and the evaluation of the relative competitiveness of mixed pathogen strains of differing virulence. Studies of this type currently cannot be done because of a lack of adequate morphological or physiological selective markers and the absence of differential hosts for different strains of the pathogen. Finally, it is expected that RFLP analysis will be of value in the disposition of litigation involving the sale of infected certified seed.

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