Distribution and Partial Characterization of pCS1, a Highly Conserved Plasmid Present in *Clavibacter michiganense* subsp. *sepedonicum*

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ABSTRACT

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Forty-nine strains of *Clavibacter michiganense* subsp. *sepedonicum*, (synonym *Corynebacterium sepedonicum*), the causal agent of potato bacterial ring rot, were screened for the presence of indigenous plasmids. Twenty-three of these strains contained a single plasmid with a molecular size of 50.6 ± 0.9 kb as determined by a combination of contour length measurements, comparison of electrophoretic mobility with intact plasmids of known molecular sizes, and summation of sizes of restriction fragments. Restriction digests and Southern hybridizations demonstrated that the plasmids from all positive strains were identical. The guanine plus cytosine content of this plasmid, designated pCS1, was determined to be 70.3%. Chromosomal DNA from the same *C. m. sepedonicum* strain had a guanine plus cytosine content of 72.4%. The plasmid copy number was found to be approximately 1.5 in strains that contain the autonomous form of pCS1. Southern hybridizations of chromosomal DNA from strains lacking the autonomous form of pCS1 revealed that all but one of the 26 tested strains contained the plasmid in integrated form. The extreme degree to which pCS1 has been conserved suggests that it encodes important, but unrecognized, metabolic functions.

Additional keywords: DNA probes, *Solanum tuberosum*, survey.

Potato bacterial ring rot, caused by *Clavibacter michiganense* subsp. *sepedonicum* (11) (synonym *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. & Burkh.), is one of the most serious diseases of potato (13,16,36,38). This is reflected by the fact that of all indigenous potato diseases monitored by state seed certification agencies, only ring rot has been assigned a zero tolerance level rating (36). Control of this disease is complicated by the presence of low, but pathologically significant, levels of *C. m. sepedonicum* in latently infected potato tubers and in tissues of other asymptomatic crop plant species (2,31). Detection of these small populations of the pathogen is essential to minimize the spread of bacterial ring rot and reduce economic losses to potato growers.

Traditional detection techniques for this pathogen have included symptom observation through field inspections, Gram staining, artificial host plant inoculations, and various immunodiagnostic procedures, including monoclonal antibody-based immunofluorescence (13,14). However, each of the above procedures has inherent drawbacks, such as a lack of sensitivity or specificity, or an inordinately long analysis time. These problems have contributed to the difficulty of detecting and quantifying low levels of the pathogen.

Recombinant DNA hybridization techniques may provide the basis of alternative or supplemental methods for the detection of *C. m. sepedonicum*. A key factor controlling the sensitivity and specificity of such methods is the nature of the molecular probe. We have screened a large number of geographically diverse strains of *C. m. sepedonicum* to ascertain the presence, distribution, and homology of common plasmids, which may contain DNA sequences that are potentially useful as probes for the pathogen. Three previous reports have documented the presence of plasmids in *C. m. sepedonicum* (5,18,28). However, each study was of a limited population and included no information on the relative virulence of the strains used.

In this communication we describe the results of this survey, the purification and properties of the single integrative plasmid found in all plasmid-positive bacterial strains, and a survey of strains that are apparently plasmid negative for the presence of chromosomally integrated plasmid sequences. A preliminary report has been published (27).

MATERIALS AND METHODS

Source and growth of bacterial strains. Many of the *C. m. sepedonicum* strains examined in this study were obtained from an ongoing ring rot screening program being carried out in the Department of Plant Pathology at North Dakota State University. Additional strains were obtained from the American Type Culture Collection (ATCC) and the Plant Disease Division Culture Collection (PDDCC) of New Zealand. Other strains were obtained from the sources listed in Table 1. All *C. m. sepedonicum* strains used in this study yielded a positive immunofluorescence reaction when tested with a *C. m. sepedonicum*-specific monoclonal antibody preparation provided by S. De Boer. Strains were tested for virulence with potato and eggplant by the procedure of Gudmestad et al (19). Nonpathogenic strains were spontaneous variants of pathogenic strains that had been maintained in the laboratory for several years.

Strains of *C. m. sepedonicum* were grown for plasmid extraction in NBY broth (35) at 22 C. The cultures were subjected to rotary shaking at 175 rpm until the A_{600} (Spectronic 20) reached 0.6–1.2.

Plasmid purification. Plasmids from *C. m. sepedonicum* strains were purified by a modification of previously published methods (3,10). All centrifugation steps were performed in a refrigerated centrifuge (4 C) at 8,000 *g* except where indicated. Cells from late log-phase cultures (250 ml) were sedimented by centrifugation and resuspended in 20 ml of TSB (0.05 M Tris-HCl buffer, pH 8.0, + 10% RNase-free sucrose). Five ml of lysozyme (5 mg/ml) in TSB was added, and the cells were incubated at 37 C for 20 min. The preparation was then treated with 25 ml of 0.05 M Tris-HCl buffer

(pH 8.0) and 50 ml of lysis solution (2% sodium dodecyl sulfate [SDS] + 0.05 M ethylenediaminetetraacetic acid [EDTA] in 0.05 M Tris-HCl buffer, pH 8.0) and gently mixed by inversion for 5 min. The lysate was treated with 4 ml of 3 M NaOH, mixed by gentle inversion for 10 min, neutralized by the addition of 8 ml of 2 M Tris-HCl buffer (pH 5.0), and mixed again for 10 min. Solid NaCl (23.4 mg/ml of neutralized solution) and 60 ml of salt-saturated phenol (redistilled phenol equilibrated with an equal volume of aqueous 3% NaCl) were added to the preparation. The resulting mixture was subjected to gentle inversion for 5 min, and the phases were separated by centrifugation for 45 min. The upper aqueous phase was collected and extracted with 100 ml of chloroform. The upper aqueous phase obtained after centrifugation for 15 min was removed and mixed with 2 volumes of absolute ethanol. After overnight storage at −20 C, the precipitated nucleic acids were sedimented by centrifugation at −10 C for 30 min and dried under vacuum. The dried pellet was allowed to resuspend overnight in sterile LTS (0.01 M Tris-HCl buffer, pH 7.5, + 0.01 M NaCl + 0.5 mM EDTA). The preparation was then subjected to 2 cycles of dye-buoyant-density centrifugation (32) using final concentrations of 49.5% (w/w) CsCl and 250 μg/ml of ethidium bromide (EtBr). The resulting plasmid solution was extracted with butanol (previously equilibrated with 49.5% CsCl) and dialyzed three times against 250 volumes of LTS. The concentration of plasmid DNA was generally high enough to obviate a concentrating step before restriction analysis.

Other DNA isolations. Chromosomal DNA from *C. m. sepedonicum* was purified by a published procedure (8) employing proteinase K digestion and phenol extraction, after lysis of the cells as described above. M13 phage replicative form I (RF) was purified according to the cleared-lysate method of Clewell and Helinski (7). Before use, all large-scale preparations of chromosomal and RF DNA were subjected to CsCl-EtBr gradient centrifugation followed by multiple butanol extractions and extensive dialysis against LTS. Small-scale preparations of plasmid DNA were isolated by the method of Ish-Horowicz and Burke (20).

Restriction digests and gel electrophoresis. Restriction digests (total volume 20 μl) contained 50–400 ng of plasmid DNA, 0.1 mg/ml of bovine serum albumin (nuclease-free), 1.0 mM dithiothreitol, 1–10 units of enzyme, and the restriction buffer recommended by the supplier. Low molecular weight (i.e., <12 kb) restriction fragments were separated on horizontal agarose gels (0.7–1.0%) run at 1–4 V/cm in TBE electrophoresis buffer (0.1 M Tris base + 0.1 M boric acid + 1.5 mM EDTA). High molecular weight restriction fragments were separated on horizontal agarose gels (0.5%) by using TNA electrophoresis buffer (0.04 M Tris base + 0.02 M Na acetate + 0.03 M acetic acid + 2 mM EDTA) run at 1 V/cm. Linear DNA standards (Bethesda Research Laboratories, Gaithersburg, MD) were used to determine the sizes of restriction fragments. Intact plasmids were electrophoresed on horizontal agarose gels (0.5%) at 5 V/cm in TA buffer (0.04 M Tris base + 0.02 M acetic acid + 2 mM EDTA) as described by Coplin et al (9). Indigenous plasmids from *Erwinia stewartii* SW-2 (9), purified by the standard procedure described above, were used as size standards for covalently closed circular (ccc) DNA molecules. Gels were stained in aqueous EtBr (0.5 μg/ml), destained in water, and photographed with Polaroid type 665 film with a 23A filter and a transilluminator emitting at 300 nm.

Southern transfer and hybridization. Southern hybridizations and high-stringency washes were performed as described by Maniatis et al (24) by using Zeta-Probe (BioRad Laboratories, Richmond, CA) membranes. Hybridization probes were prepared by nick translation (33) of pCS1 or cloned plasmid fragments (28), using [α-^{32}P]dCTP and a commercial nick translation kit (Bethesda Research Laboratories). Prehybridization was performed at 43 C in a solution composed of 50% (v/v) formamide, 4× SSCP (26), 0.5% BLOTTO (21), 500 μg/ml of denatured salmon sperm DNA, 10× Denhardt's solution (15), and 1% SDS. Following prehybridization, labeled probe DNA was added directly to the prehybridization solution, and hybridization was allowed to proceed at 43 C for 16 hr. The final washes of the membranes were performed at 50–68 C in 0.1× SSCP containing 1% SDS. Kodak X-Omat AR-5 sheet film was used for autoradiography. When required, exposures were enhanced by the use of a Dupont Cronex Hi-Plus intensifying screen.

Electron microscopy. Plasmid DNA was prepared for transmission electron microscopy according to the original procedure of Kleinschmidt as described by Davis et al (12), and shadowed with platinum-palladium. M13mp11 RF (7.224 kb) (26) was used as an internal size standard for contour length measurements. Open circular forms of the plasmid and the internal standard were generated by light-induced nicking of the DNA in the presence of EtBr (6).

Plasmid copy number. The copy number of the plasmid was measured by the nucleic acid hybridization method described by

TABLE 1. Plasmid status, degree of virulence, and geographic origin of 49 strains of *Clavibacter michiganense* subsp. *sepedonicum*

Strain	Plasmid presence[a]	Virulence[b]	Geographic origin	Source[c]
Cs9850	+	+	New York	ATCC
Cs33111	+	+	Canada	ATCC
Cs33113[d]	+	+	Canada	ATCC
Cs3M	+	−	Alberta	S. De Boer
Cs3R	+	+	Alberta	S. De Boer
Cs3NM	+	+	Alberta	S. De Boer
CsIDNM1	+	+	Idaho	D. Clarke
CsND1	+	++	N. Dakota	NDSU
CsME1	+	++	Maine	F. Manzer
CSca	+	+	California	A. Vidaver
CSmt	+	+	Montana	A. Vidaver
Cs43	+	++	Maine	S. Slack
Cs44	+	+	Maine	S. Slack
Cs2531	+	++	Sweden	PDDCC
Cs2532	+	++	Canada	PDDCC
Cs2534	+	+	New York	PDDCC
Cs2537	+	+	U.S.A.	PDDCC
CsFL1	+	+	Canada	R. Stall
CsBRR7	+	++	Brit. Columbia	S. De Boer
Cs16	+	++	New Brunswick	S. De Boer
Cs17	+	+	Maine	S. De Boer
CsRR1a	+	−	Alberta	S. De Boer
Cs5	+	++	New York	S. De Boer
CsND2	−	+	N. Dakota	NDSU
CsND3	−	++	N. Dakota	NDSU
CsND4	−	+	N. Dakota	NDSU
CsND5	−	++	N. Dakota	NDSU
CsND6	−	+	N. Dakota	NDSU
CsND7	−	++	N. Dakota	NDSU
CsNDSB1[e]	−	+	N. Dakota	NDSU
CsNDSB2	−	+	N. Dakota	NDSU
CsNDSB3	−	+	N. Dakota	NDSU
CsNDSB4	−	+	N. Dakota	NDSU
CsNDSB5	−	+	N. Dakota	NDSU
CsNDSB6	−	+	N. Dakota	NDSU
CsNDSB7	−	+	N. Dakota	NDSU
CsNDSB8	−	n.t.	N. Dakota	NDSU
CsSD1	−	+	S. Dakota	NDSU
CsR1	−	+	Brit. Columbia	S. De Boer
CsR2	−	−	Brit. Columbia	S. De Boer
CsR3	−	+	Brit. Columbia	S. De Boer
CsR4	−	+	Brit. Columbia	S. De Boer
CsR5	−	+	Brit. Columbia	S. De Boer
Cs14	−	−	Montana	S. Slack
Cs20	−	++	Wisconsin	S. Slack
Cs106	−	++	W. Virginia	S. De Boer
CsP45	−	+	Unknown	S. De Boer
Cs2535[d]	−	+	Canada	PDDCC
Cs2536	−	+	Maine	PDDCC

[a] + denotes presence of covalently closed circular plasmid DNA.
[b] −, Nonpathogenic; +, weakly or moderately virulent; ++, highly virulent; n.t., not tested.
[c] Strains obtained from ATCC or PDDCC retain their original numeric designations.
[d] Type strain.
[e] Strains CsNDSB1–CsNDSB8 were originally obtained as sugar beet endophytes (2).

Caro et al (4). Total bacterial DNA for this measurement was isolated by the method described above for highly polymerized chromosomal DNA, except that the lysed cell suspension, after treatment with proteinase K, was subjected to sonication at 0–10 C for 10 min. This process was performed to avoid preferential loss of either chromosomal DNA or plasmid DNA during subsequent fractionation steps. Agarose gel electrophoresis, followed by EtBr staining and Southern hybridization with a cloned, plasmid-specific probe, indicated that the size range of the sonic fragments was 0.3–2.0 kb for both total genomic DNA and plasmid pCS1. Dot hybridization, under the conditions for annealing and washing described above, was performed to measure the fraction of plasmid sequences in the sonic fragments of total genomic DNA from selected strains of the bacterium. Selected quantities (0.5–20 ng) of pCS1 fragment 8B, a *Sma*I fragment that has been shown to be pCS1-specific (28), were used as standards, and the same fragment was nick translated and used as the plasmid-specific probe. The areas of the hybridization membrane containing test DNA (1–10 μg) were excised, and the amount of ^{32}P-labeled probe bound to each membrane piece was determined by liquid scintillation spectrometry.

Base composition. The base composition of plasmid and chromosomal DNA was determined by reversed-phase high-pressure liquid chromatography (HPLC) essentially as described by Gehrke et al (17), using a Hibar-II 10 μm C18 reversed-phase column (EM Reagents) with a size of 4.6 mm ID × 250 mm. Conversion of the DNA to deoxynucleosides was performed by the three-enzyme method of Gehrke et al (17).

RESULTS

Plasmid isolation. Digestion with lysozyme at 37 C, followed by treatment with SDS, caused lysis of all *C. m. sepedonicum* strains tested during the course of this study. However, this organism was found to be refractory to the action of either lysozyme or mutanolysin at 0 C. Sarkosyl was less effective than SDS as a lytic agent for *C. m. sepedonicum* protoplasts. The major steps in the remainder of the procedure, selective alkaline denaturation of chromosomal DNA and density gradient centrifugation in the presence of ethidium bromide, are widely used for the enrichment of ccc DNA molecules (1,10,22,25,37).

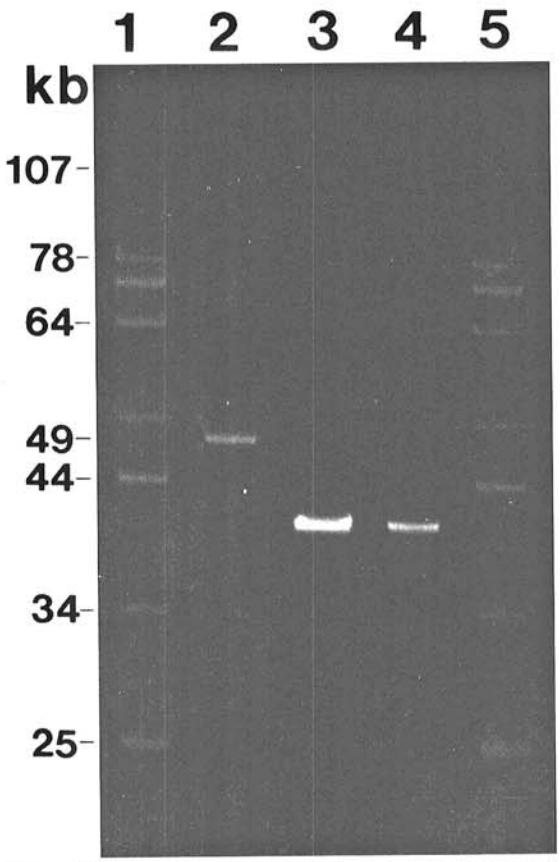

Fig. 1. Size estimation of the *Clavibacter michiganense* subsp. *sepedonicum* plasmid. Gel electrophoresis in the TA buffer system was performed as described in the text. Lanes 1 and 5, covalently closed circular plasmid size standards; lane 2, covalently closed circular plasmid preparation from strain Cs3R; lane 3, lambda phage DNA; and lane 4, plasmid from strain Cs3R linearized with *Xba*I restriction endonuclease.

In addition to our routine plasmid purification procedure, selected strains were also examined by two different methods for plasmid isolation. The methods used were the cleared-lysate/density-gradient method of Clewell and Helinski (7) and the alkaline quick-prep method of Ish-Horowicz and Burke (20). No differences in the number or sizes of plasmids recovered were observed. However, our standard isolation procedure resulted in the greatest yields of plasmid DNA from *C. m. sepedonicum*. Using this method, uniform yields of plasmid were obtained in replicate preparations from a given strain, although substantial variation in yield among different strains was observed. The quantity of purified plasmid obtained from strain Cs3R, a high-yielding strain, was 60 μg of plasmid DNA per 10^{12} cells. The standard purification procedure was used both for routine screening of *C. m. sepedonicum* strains for the presence of plasmids and for preparation of plasmid molecules for characterization studies.

Screening of strains for the presence of plasmids. Table 1 lists the bacterial strains used in this study, their geographic origins and virulence patterns, and the results of the plasmid screening survey. Twenty-three of 49 strains tested were plasmid positive, as indicated by the presence of a discernable ccc DNA band upon dye-buoyant-density centrifugation. No correlation of plasmid-positive cultures of *C. m. sepedonicum* with a specific geographic region or degree of virulence was noted. The plasmid from Cs3R, a strain yielding large quantities of the plasmid, was selected for molecular characterization.

Size of the *C. m. sepedonicum* plasmid. Intact plasmid DNA from all positive strains was subjected to electrophoresis in low percentage agarose gels. In all cases plasmid DNA migrated as a single heavy band with an apparent molecular size of approximately 50 kb (Fig. 1). Occasionally, a second band representing dimeric plasmid was observed in some plasmid preparations (data not shown). Plasmid DNA that had been cut with a restriction enzyme recognizing a single site on the plasmid resulted in a linear molecule that co-migrated with intact lambda DNA (Fig. 1) and abolished the dimeric band. Contaminating chromosomal DNA fragments migrated as a very light diffuse band slightly ahead of the linearized plasmid and were generally observed only in plasmid preparations that had been subjected to a single cycle of CsCl gradient centrifugation.

Additional verification of plasmid size was obtained by the summation of restriction digest fragments and by contour length measurements from electron micrographs of nicked plasmid DNA. Summation of restriction fragments obtained from various restriction digests (Fig. 2) resulted in size estimates ranging from 36.9 kb to 50.2 kb. The lower values may reflect the presence of incompletely resolved multiple bands with similar migrational rates, or selective loss of low molecular weight components by migration off the end of the gel. Contour length measurements of discrete, nonoverlapping plasmid molecules observed in electron micrographs (not shown) resulted in a size estimate of 50.6 ± 0.9 kb.

Plasmid homology of different strains. Plasmid DNA from selected strains was digested with a variety of restriction endonucleases to determine plasmid similarities. Electrophoresis patterns from all strains appeared identical (Fig. 3), which suggests that each positive strain harbors the same plasmid. This plasmid has been designated pCS1. Of the tested enzymes, only *Hin*dIII and *Eco*RI failed to cleave pCS1 (Fig. 2B, lanes 10 and 11). The failure of these two enzymes to cleave the plasmid was not due to the presence of an inhibitor, because digestion of an admixture of lambda DNA and pCS1 DNA yielded the expected lambda DNA restriction patterns (data not shown). Incubation of pCS1 with

other restriction enzymes resulted in patterns of varying complexity (Fig. 2). In general, restriction pattern complexities were directly related to the base composition of the enzyme recognition site. Restriction enzymes with AT-rich hexanucleotide recognition sites (*Asu*II, *Bcl*I, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Hin*dIII, *Sna*BI, *Spe*I, and *Xba*I) cleaved pCS1, a guanine plus cytosine (GC)-rich molecule (see below), at only a few locations. Most enzymes with GC-rich hexanucleotide recognition sites (*Bam*HI, *Bgl*I, *Kpn*I, *Pvu*I, *Sma*I, and *Xho*I) produced 1–2 dozen restriction fragments. Certain sequences seemed to be highly represented. For example, *Not*I, an enzyme with a GC-rich octanucleotide recognition sequence, cut pCS1 at approximately 18 different sites (Fig. 2A, lane 9).

Proof of plasmid homology was obtained by digesting plasmid DNA from all positive strains with *Sma*I and performing Southern hybridizations with radiolabeled pCS1. The results obtained with five representative plasmid-positive strains (Fig. 3) revealed that all plasmid fragments from each positive strain hybridized with the probe from strain Cs3R. Identical results were obtained with the other 18 plasmid-positive strains (data not shown). This clearly demonstrates that pCS1 is common to all plasmid-positive *C. m. sepedonicum* strains that have been surveyed to date.

Ubiquity of pCS1. A recent report from this laboratory has demonstrated the presence of pCS1, in integrated form, in the chromosomal DNA of several *C. m. sepedonicum* strains lacking the autonomous form of the plasmid (28). Because of the extremely wide distribution of *C. m. sepedonicum* strains containing ccc pCS1, it was of interest to determine the prevalence of integrated pCS1 sequences in strains that lack the autonomous form of the plasmid. This analysis employed Southern hybridization of digested chromosomal DNA from plasmid-negative strains with a radiolabeled probe consisting of a mixture of cloned, plasmid-specific pCS1 fragments. The study revealed that all but one of the 26 tested plasmid-negative strains of *C. m. sepedonicum* contained pCS1 in a stable, chromosomally integrated state. Only strain CsP45 was completely devoid of the plasmid sequences. The results with CsP45, Cs33113 (a representative strain that contains the episomal form of the plasmid), and Cs106 (a representative strain that contains the plasmid in an integrated state) are shown in Figure 4. The presence of some episomal plasmid DNA in the chromosomal DNA preparation from Cs33113 is responsible for the autoradiographic signal observed in lane 3. We have previously reported that some plasmid fragments produce weak hybridization signals with specific fragments of chromosomal DNA (28). One of the plasmid fragments in the probe is responsible for the faint band seen only in lanes 1, 3, and 4, which contain chromosomal DNA.

Plasmid copy number. Dot hybridization of total bacterial DNA with a plasmid-specific radiolabeled probe (*Sma*I fragment 8B of pCS1) (28) was used to determine the fraction of the total bacterial DNA represented by this unique plasmid fragment in four strains of *C. m. sepedonicum*. From these measurements and the sizes of pCS1 fragment 8B (2.88 kb) and the chromosome of a related coryneform bacterium (6,000 kb) (34), the copy number of pCS1 in each of the tested strains has been calculated. Strains Cs3R, Cs33113, and Cs9850, which all contain ccc pCS1, had plasmid copy numbers of 1.85, 1.16, and 1.48, respectively. The copy number of the plasmid in strain Cs106, which contains pCS1 in an integrated state, was found to be 1.26.

Base composition. Previously published information (39), in addition to our restriction digest results, suggested that DNA from *C. m. sepedonicum* has a very high GC content. This was confirmed by HPLC analysis of enzymatically digested DNA. Plasmid DNA from strain Cs3R was determined to have a GC content of 70.3%. Chromosomal DNA from the same strain was found to have a GC content of 72.4%. The HPLC analysis also revealed the presence of 1–2% RNA, perhaps present as a transcriptional intermediate or as a contaminant, in the plasmid DNA preparation.

DISCUSSION

Because this was the first extensive plasmid survey for this organism, it was important to use a purification procedure that resulted in acceptable recoveries and avoided selective loss of larger plasmids. In general, procedures involving an alkaline denaturation step permit the recovery and detection of plasmid

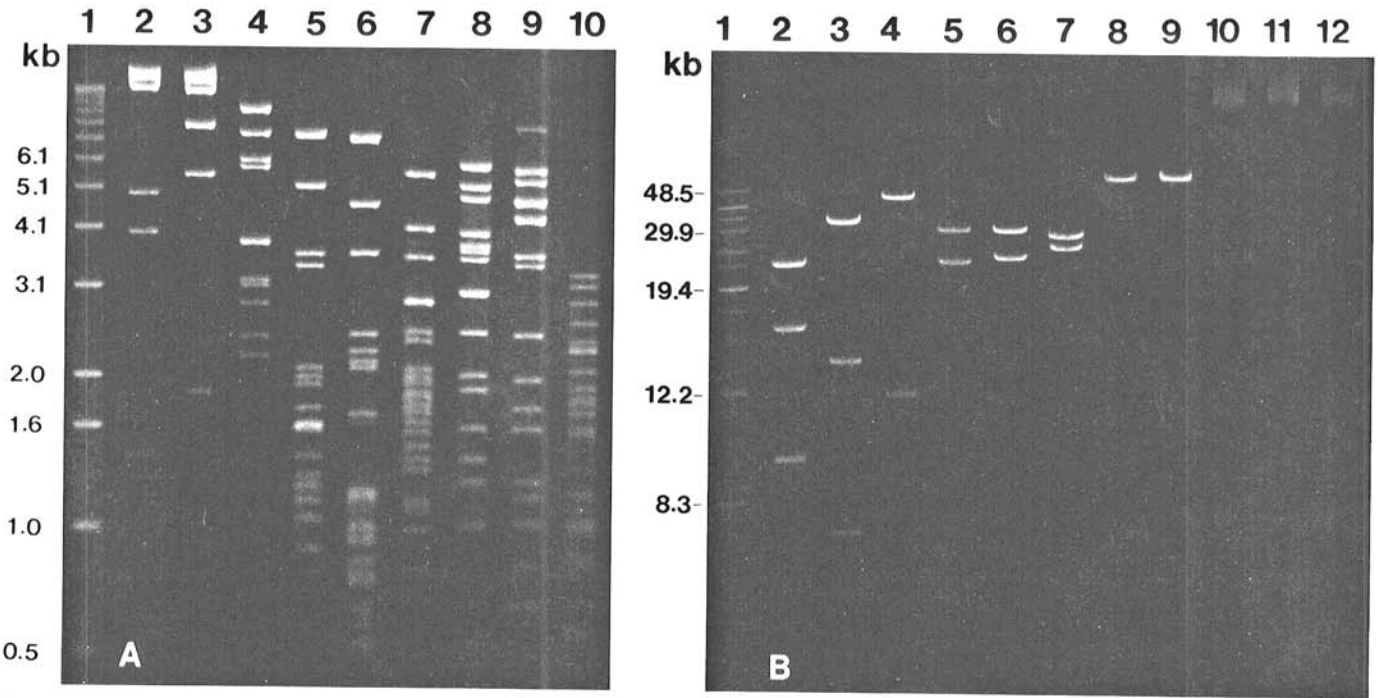

Fig. 2. Restriction fragment patterns of the *Clavibacter michiganense* subsp. *sepedonicum* plasmid. Plasmid DNA from strain Cs3R was digested with selected restriction endonucleases and subjected to agarose gel electrophoresis. Panel **A** (0.8% gel, TBE buffer system): Lane 1, 1-kb ladder; lane 2, *Cla*I; lane 3, *Nhe*I; lane 4, *Kpn*I; lane 5, *Pvu*I; lane 6, *Bam*HI; lane 7, *Xho*I; lane 8, *Sma*I; lane 9, *Not*I; lane 10, *Bgl*I. Panel **B** (0.5% gel, TNA buffer system): lane 1, high molecular weight ladder; lane 2, *Bgl*II; lane 3, *Sfi*I; lane 4, *Asu*II; lane 5, *Spe*I; lane 6, *Bcl*I; lane 7, *Sna*BI; lane 8, *Dra*I; lane 9, *Xba*I; lane 10, *Hin*dIII; lane 11, *Eco*RI; lane 12, intact pCS1. In the TNA buffer system covalently closed circular DNA molecules (panel **B**, lanes 10–12) migrate much more slowly than linear molecules of the same size (lanes 8, 9) and appear as diffuse bands.

molecules up to a size of approximately 150–200 kb (3,9, unpublished observations). The largest plasmid reported to be recovered consistently from *E. stewartii* SW-2, using the method of Coplin et al (9), is 107 kb. This plasmid is easily recovered by using the procedure developed for isolation of plasmid DNA from *C. m. sepedonicum*, which indicates that the method will permit the detection of all plasmids in this organism that have sizes up to at least 100 kb. The purification scheme described here worked well for all members of this species that were tested. The procedure has also been used to assess the presence of plasmids in *C. m.* subsp. *michiganense*, *C. m.* subsp. *insidiosum*, *Curtobacterium flaccumfaciens* pv. *betae*, *Rhodococcus fascians*, and *Corynebacterium glutamicum*. Viscous lysates were obtained in all cases and plasmid yields were reasonable. The percentage recovery of pCS1 from strain Cs3R is estimated at 35%, based on an observed yield of 60 μg per 10^{12} cells and the plasmid size and copy number determined in this study.

Gross et al (18), using a different plasmid purification procedure, reported the presence of a single 50 ± 4.5 kb plasmid in two of the three *C. m. sepedonicum* strains that were examined. The results of this study support their findings and provide a more precise value, 50.6 ± 0.9 kb, for the size of this conserved *Clavibacter* plasmid. During the course of this survey, we found that CSca, the single strain reported as being plasmid-negative by Gross et al (18), also harbors ccc pCS1. This minor difference in results probably reflects the variation in plasmid yield from different *C. m. sepedonicum* strains and the smaller scale of their isolation method.

Clark and Lawrence (5) recently reported the results from a plasmid survey of a limited number of *C. m. sepedonicum* strains. They identified a single common plasmid in 11 of 13 strains tested. Although they reported the size of the plasmid to be 46 kb, it is clear from the restriction patterns that the plasmid they examined is indistinguishable from pCS1. Clark and Lawrence estimated the copy number of the *C. m. sepedonicum* plasmid to be approximately 30 copies per cell. Our measurements provide a much lower average value of 1.5 copies per cell for three strains that contain the episomal form of the plasmid. This value agrees closely with the copy number of 1 to 2 originally proposed by Gross et al (18) for indigenous plasmids in three related *Clavibacter* species. Our study also yielded a copy number of 1.26 for a plasmid-integrated strain, Cs106, which is in good agreement with a copy number of one projected from the detection of only a single pair of plasmid-chromosome junction fragments in plasmid-integrated strains (28,29).

An interesting finding of this study was the presence of pCS1 in either free or integrated form in such a large percentage of the *C. m. sepedonicum* population. Bacterial strains in this survey were chosen to contain representative members originating from diverse geographic regions. An insight into plasmid stability was provided by including strains that had been isolated and subcultured over a period of at least 40 years. In addition, the strains surveyed in this study varied substantially in their virulence patterns and differed in the hosts from which they were originally isolated.

Previous attempts to assess the role of pCS1 in pathogenicity have been obscured by the widespread distribution of this plasmid and its ability to integrate. However, the identification of a plasmid-free pathogenic strain, CsP45, and detection of free or integrated forms of pCS1 in a number of nonpathogenic *C. m. sepedonicum* strains indicate that there is no direct correlation between pCS1 and pathogenicity. The availability of the plasmid-

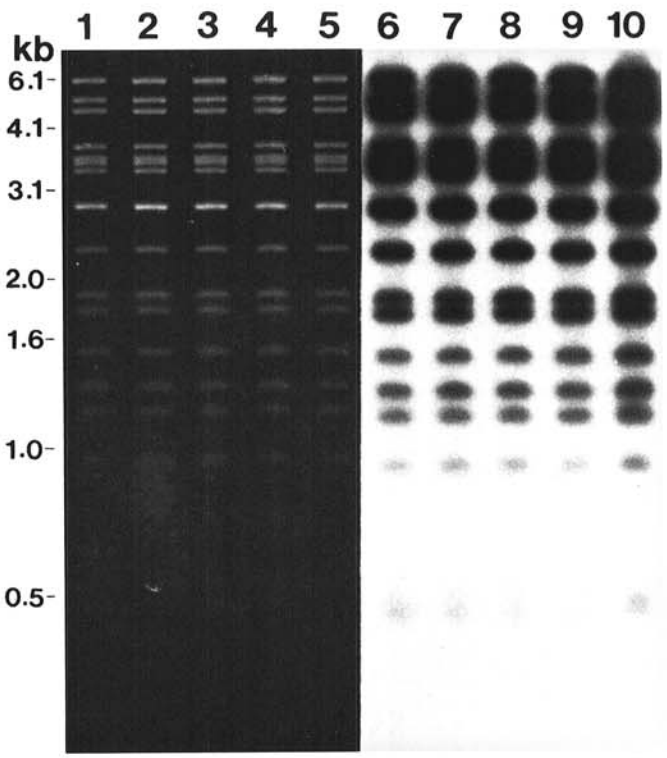

Fig. 3. Comparative *Sma*I restriction patterns of plasmid DNA from five representative strains of *Clavibacter michiganense* subsp. *sepedonicum*. Lanes 1–5, EtBr stain. Lanes 6–10, Southern hybridization with ^{32}P-labeled pCS1 from strain Cs3R. Lanes 1 and 6, strain Cs3R; lanes 2 and 7, strain CsIDNM1; lanes 3 and 8, strain Cs43; lanes 4 and 9, strain Cs33113; lanes 5 and 10, strain Cs2531.

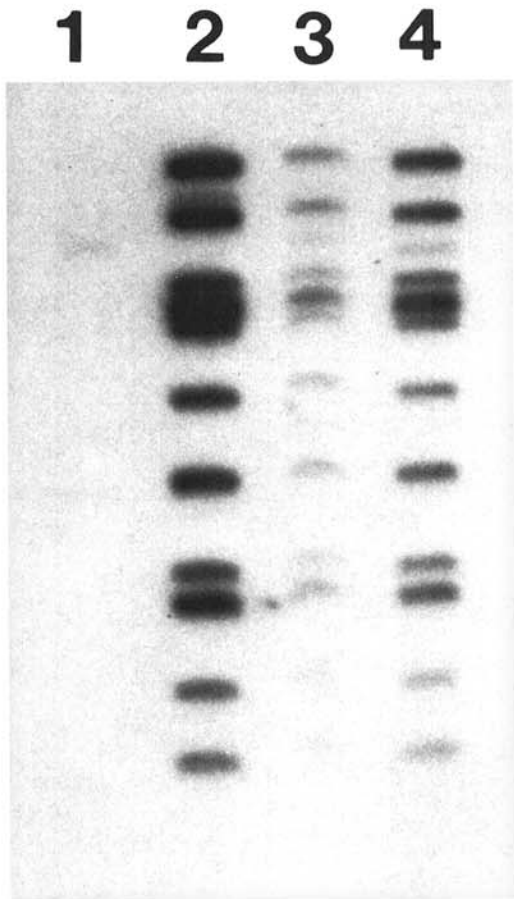

Fig. 4. Southern hybridization of pCS1 and chromosomal DNA from three strains of *Clavibacter michiganense* subsp. *sepedonicum* with a plasmid-specific probe. The DNA preparations were digested with *Sma*I restriction endonuclease and then subjected to electrophoresis and fragment transfer as described in the text. A mixture of cloned pCS1 fragments (28) was used to prepare the ^{32}P-labeled probe. Lane 1, chromosomal DNA from strain CsP45; lane 2, pCS1 DNA (purified from strain Cs3R); lane 3, chromosomal DNA from strain Cs33113 (a representative strain containing the autonomous form of pCS1); lane 4, chromosomal DNA from strain Cs106 (a representative strain containing the integrated form of pCS1).

free strain identified in this study will facilitate the development of a transformation system for this species and evaluation of the role of pCS1 in the physiology and ecology of this organism.

Other examples of apparently stable plasmids from phytopathogenic coryneforms have been reported. Murai et al (30) and Lawson et al (23) have described the presence of a single 117-kb plasmid in numerous *R. fascians* strains surveyed. Restriction analysis revealed a generally similar pattern for each of these *R. fascians* plasmids. Gross et al (18) found that five of 22 *C. m.* subsp. *nebraskense* strains contained a single plasmid with a molecular size of 51 kb. Unfortunately, Southern hybridizations were not performed in either case to confirm sequence homology among the plasmids identified. Although some phytopathogenic corynebacteria, e.g., *C. m. michiganense* (18), have a significant amount of plasmid diversity, the presence of single, highly stable plasmids appears to be a common occurrence in phytopathogenic coryneforms. These widely distributed plasmids may be useful sources of DNA sequences for pathogen detection procedures. Work currently in progress in this laboratory indicates that a cloned pCS1 fragment, which contains a repeated sequence also present in high copy number on the bacterial chromosome (28), is a promising candidate for use as a molecular probe in a sensitive hybridization-based method for detection of the ring rot pathogen.

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