Specific Identification of *Clavibacter michiganense* subsp. *sepedonicum* by DNA-Hybridization Probes

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ABSTRACT

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Three DNA sequences specific for the causal agent of bacterial ring rot of potatoes, *Clavibacter michiganense* subsp. *sepedonicum*, have been isolated from a library prepared from total DNA. One of the DNA probes is a *Pst*I fragment of 2.6 kilobases (kb), which contains a region that is repeated numerous times. By using a probe labeled with ^{32}P-dCTP, 0.5 ng of total DNA could be detected. The same detection limit was obtained with a biotinylated probe, whereas a sulphonated probe was only about one-third as sensitive. The presence of large amounts of nonhomologous DNA affected the signal considerably, especially when the homologous DNA constituted less than 5%.

Additional keywords: *Corynebacterium sepedonicum*, DNA probes, nonradioactive labeling, repeated sequences.

Clavibacter michiganense subsp. *sepedonicum* (Spieck. & Kotth.) Davis et al is the causal agent of bacterial ring rot of potatoes. The disease is transmitted mechanically and is spread by infected seed tubers. The bacteria can survive only in infected plant material and thus can be controlled effectively by the use of disease-free seed material. Specific, sensitive, and rapid diagnostic methods for *C. m. sepedonicum* are required for the control of the disease. Identification of the bacteria in seed material could prevent spread to elite stock programs and to noninfested geographical areas.

Serological methods are being increasingly accepted as sensitive methods for detection of bacterial infections in plant tissue (23). The sensitivity and specificity of these methods depend on the specificity of the antiserum used. *C. m. sepedonicum* has antigenic determinants in common with other bacteria associated with potato tubers in soil (3,5). Serological cross-reaction with contaminating or endophytic bacteria of potato tubers therefore may lead to misidentification. Diagnosis of the bacteria by immunofluorescent microscopy is relatively insensitive to serological cross-reaction because the bacteria can be characterized in addition by fluorescence intensity, shape, size, and layering. However, this method is not well suited for large-scale screening because it is labor intensive and requires a high level of expertise. Indirect diagnosis techniques, such as enzyme-linked immunosorbent assay (ELISA), dot immunobinding (DIB), and the agglutination tests, are well suited for handling large numbers of samples. Unfortunately, these methods are more sensitive to serological cross-reaction, which, for example, makes it impossible to distinguish between samples containing low concentrations of *C. m. sepedonicum* and those containing cross-reacting bacteria. To eliminate cross-reaction, De Boer and Wieczorek (6) produced monoclonal antibodies against whole bacterial cells of *C. m. sepedonicum*, and they isolated a number of clones specific to strains of *C. m. sepedonicum* from North America.

An alternative to serological methods of diagnosis is the use of DNA probes for identification of microorganisms. At the present state of the art, DNA dot blotting is somewhat more labor intensive than ELISA or DIB because each sample must undergo one or more extraction steps. However, DNA dot blotting is probably less labor intensive and more amenable to automation than the immunoflourescence microscopy method in which each individual sample must be examined carefully under the microscope. The DNA fragments used as probes have been found among randomly cloned fragments (22) or have harbored specific virulence factors (19). Here we present three DNA probes for identification and diagnosis of *C. m. sepedonicum*. There was only one report on the occurrence of plasmids in *C. m. sepedonicum* at the outset of this work. Gross et al (11) found a plasmid of 45 kilobases (kb) in two of three isolates but did not relate the plasmid to virulence. We therefore decided to look for specific fragments for *C. m. sepedonicum* among shotgun-cloned fragments of total DNA. While this work was in progress, Clark and Lawrence (4) found a plasmid of 45 kb in 11 of 13 isolates of *C. m. sepedonicum*, and Mogen and Oleson (18) showed that the sequences were integrated into the chromosome in isolates that did not carry this plasmid.

MATERIALS AND METHODS

Bacterial cultures. The clavibacter strains used in this study are listed in Table 1. In addition, 17 unidentified gram-positive and gram-negative bacterial strains were used. These have been isolated from healthy potato tubers, and they all react with antiserum raised against *C. m. sepedonicum* (Heide and Dinesen, *unpublished results*). The host organism for recombinant plasmids was *Escherichia coli DH5α* (Bethesda Research Laboratories, Gaithersburg, MD). Nutrient broth (Difco Laboratories, Detroit, MI) in a concentration of 0.8% was used for all cultures except for *E. coli,* which was grown in NY-medium (0.8% N-Z-Amine A [Sheffield Products, Lynnhurst, NJ], 0.4% Bacto yeast extract [Difco], 0.5% NaCl). *E. coli* was stored in 15% glycerol at −70 C; all other cultures were stored on nutrient broth stabilized with 2% agar at 5 C.

Isolation and purification of total DNA. Cultures were grown in 200 ml of 0.8% nutrient broth at room temperature on a rotary shaker. Cultures of *C. m. sepedonicum* were grown 5–7 days; all other cultures were grown 1–2 days. The cultures were harvested by centrifugation at 7,000 *g* for 5 min, washed in 30 ml of 50 mM Tris (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl and resuspended in 8 ml of 50 mM Tris (pH 8.0), containing 100 mM EDTA and lysozyme (5 mg/ml). After 2 hr of incubation, sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1% and 0.35 mg/ml, respectively, and the mixtures were incubated overnight. All incubations were at 37 C. After a second addition of SDS to a final concentration of 3.3%, the mixtures were extracted with phenol saturated with 50 mM Tris (pH 8.0), 1 mM EDTA (TE) for 2 hr by rolling the tubes on a Multimix Major (Luckham, Ltd., Sussex, England). Five milliliters of chloroform was added and rolling was continued for 5 min before the phases were separated by centrifugation at 20,000 *g* for 30 min. The

supernatants then were extracted with phenol/chloroform and three times with chloroform. Each phase separation was accomplished by a 5-min centrifugation at 2,500 *g*. After a subsequent 30-min incubation with RNase A at a final concentration of 0.1 mg/ml followed by a 30-min proteinase K treatment at a final concentration of 200 ng/ml, the DNA was precipitated with isopropanol, washed in 80% ethanol, dried, and redissolved in 9 ml of TE. Potassium iodide and ethidium bromide (EtBr) were added to final concentrations of 0.76 g/ml and 0.1 mg/ml, respectively. The solutions were centrifuged at 166,000 *g* for 40 hr in a fixed angle rotor. DNA bands were collected and EtBr was extracted with butanol saturated with KI and H_2O. The DNA finally was spooled and redissolved in 300 μl of TE.

DNA from *Solanum tuberosum* L. and *Bradyrhizobium japonicum* (Kirchner) Jordan was kindly provided by K. Gausing and J. S. Jensen, respectively, Århus University, Denmark. DNA from isolates not previously mentioned (see Fig. 3) was kindly provided by C. Christiansen, The Genetic Engineering Group, Denmark.

Cloning of *C. m. sepedonicum* DNA. Total DNA from *C. m. sepedonicum* strain NCPPB 2140 was digested with restriction enzyme *Pst*I (New England Biolabs, Beverly, MA) and ligated into the vector pGEM-3-blue (Promega Biotec, Madison, WI) as described by Pheiffer and Zimmerman (20). The ligation mixture was subsequently transformed into competent cells of *E. coli DH5α* (12). Recombinant plasmids were isolated and purified as decribed by Maniatis et al (15). The cloned fragments of *C. m. sepedonicum* were separated from agarose with Gene Clean Kit (Bio 101, San Diego, CA).

Blotting, nick-translation, and hybridization. Dot blots and Southern blots were made on Hybond N filters (Amersham International, Buckinghamshire, England) as recommended by the manufacturer. Unless otherwise stated, 25 ng of DNA was added to each dot in dot blots and 500 ng of DNA was added to each lane in Southern blots. Nick-translations were performed as described by Rigby et al (21) with 300 μCi [^{32}P]dCTP (specific activity of 3,000 mCi/mmol) (Amersham International) per microgram of DNA. Unless otherwise noted, 10 ng of ^{32}P-labeled DNA was added per 1 ml of hybridization solution. Prehybridization and hybridization were performed in 10× Denhardt solution (15), containing 50 mM sodium phosphate (pH 6.5), 0.1% pyrophosphate, 0.75 M NaCl, and 5 mM EDTA. Ten milliliters of solution was added per 120 cm^2 of filter. Filters were prehybridized for at least 1 hr at 65 C and hybridized overnight. After hybridization, filters were washed twice for 5 and 15 min in 2× standard saline citrate (SSC), containing 0.5% SDS at room temperature and twice for 2 hr and 30 min, respectively, in 0.2× SSC, containing 0.5% SDS. Temperatures for hybridization reactions and the final two washings were determined according to Anderson and Young (1) assuming a G+C percentage of 74.9 for *C. m. sepedonicum* (8). For perfectly matched hybrids, hybridizations were carried out at 88 C and washings at 73 C; for hybrids allowing 10% mismatch, hybridizations were carried out at 76 C and washings at 61 C; and for hybrids allowing 20% mismatch, hybridizations were carried out at 64 C and washings at 49 C.

Biotin labeling was accomplished by nick-translation as described by Rigby et al (21) with 1 μmole of biotin-11-dUTP (Bethesda Research Laboratories) per microgram of DNA. Bound biotin-labeled probes were visualized as described by Gebeyehu et al (9). Reagents were obtained from Dakopatts, Denmark. Sulfonation of DNA was performed with the Chemiprobe Kit (FMC BioProducts, Rockland, ME) as recommended by the manufacturer. Hybridization with biotinylated and sulfonated probes and posthybridization washings were carried out as described for ^{32}P-labeled probes with addition of 100 μg of denatured salmon sperm DNA per milliliter of the prehybridization and hybridization solutions. Probe concentrations during hybridizations were 100 ng/ml for biotinylated probes and 1 μg/ml for sulfonated probes.

TABLE 1. Strains of *Clavibacter*

Species and strain number	Strain designation	Source
Clavibacter michiganense subsp. *sependonicum*		
3	NCPPB 2140	
3A		I. Dinesen, Denmark
5		A. Alivizatos, Greece
6		A. Alivizatos, Greece
10	CS-PD-37	J. F. Chauveau, France
13		I. Dinesen, Denmark
31	2	L. Nielson, Sweden
32	3	L. Nielson, Sweden
33	4	L. Nielson, Sweden
34	6	L. Nielson, Sweden
35	77-2	A. Sletten, Norway
36	79-9	A. Sletten, Norway
40	CS-099	J. F. Chauveau, France
81	*13-California-A	S. Slack, United States
83	xx43 Manzer	S. Slack, United States
85	Maine A	S. Slack, United States
C. m. michiganese		
28	NCPPB 1064	U. Mazzucchi, Italy
29	1	P. Psallidas, Greece
30	18	P. Psallidas, Greece
91	36/3	S. Süle, Hungary
92	h-112	N. W. Schaad, United States
93	h-160 MOROCCO	N. W. Schaad, United States
C. m. insidiosum		
20		I. Dinesen, Denmark
C. m. nebraskense		
95	NCPPB 2580	
C. rathayi		
15		I. Dinesen, Denmark
C. tritici	NCPPB 1857	

RESULTS

Approximately 5,000 recombinant clones were recovered following cloning of *Pst*I-digested DNA of *C. m. sepedonicum*. Plasmids from 84 of these clones, designated pEJ1–pEJ84, were isolated. The fragment size ranges from 0.5 to 10 kb, and each of the plasmids harbors up to four *Pst*I fragments primarily because of the use of excess DNA of *C. m. sepedonicum* in the ligation reaction. The 84 recombinant plasmids were digested with either *Pst*I (fragments less than 5 kb) or *Bam*HI, *Hind*III, and *Pst*I (fragments larger than 5 kb) followed by agarose gel electrophoresis and transfer of the DNA to nylon membranes. The filters were hybridized with nick-translated total DNA from four of the cross-reacting bacteria and the closely related species *C. m. michiganense* (Smith) Davis et al, *C. m. insidiosum* (McCulloch) Davis et al., and *C. rathayi* (Smith) Davis et al. The hybridization was carried out under hybridization conditions allowing 20% mismatch with 7 ng of DNA from each bacterial species per milliliter of hybridization solution.

Nine fragments to which no or very weak hybridization occurred were selected for further screening. The selected fragments from the nine plasmids were isolated and hybridized to dot blots of total DNA from *C. m. michiganense*, *C. m. insidiosum*, *C. rathayi*, and 17 cross-reacting species.

Three fragments (the 1.5-kb *Pst*I fragment of pEJ64, the 2.6-kb *Pst*I fragment from pEJ79, and the 1.2-kb *Bam*HI-*Hind*III fragment of pEJ82) did not hybridize with DNA from any of those bacteria. In a final screening for specificity, each of the three selected fragments of *C. m. sepedonicum* was hybridized to Southern blots with total DNA from *C. m. michiganense*, *C. m. insidiosum*, *C. m. nebraskense* (Schuster et al) Davis et al., *C. rathayi*, *C. tritici* (Carlson & Vidaver) Davis et al, and 17 different serologically cross-reacting bacteria. They were hybridized additionally to dot blots of DNA from potato and total DNA from bacteria and lower eucaryotes covering a wide spectrum of microorganisms. DNA from strain NCPPB 2140 of *C. m. sepedonicum* was included as a positive control on each

blot. The results of probing with the 2.6-kb *Pst*I fragment of pEJ79 are shown in Figures 1–3. The autoradiograms were exposed for 30 min (Figs. 1 and 2) or 4 hr (Fig. 3), but even after extended exposure (up to 72 hr), only very faint signals appeared in addition to the signal of *C. m. sepedonicum* (data not shown). Similar results were obtained for the two other selected fragments of *C. m. sepedonicum* (data not shown).

To verify that the three selected fragments are common to isolates of *C. m. sepedonicum* of different origin, each fragment was hybridized to a Southern blot with DNA from 12 isolates of *C. m. sepedonicum* originating from Europe and North America. The autoradiogram in Figure 4 shows that the pEJ79 fragment hybridizes to all the isolates tested. In a dot-blot experiment, four additional isolates of *C. m. sepedonicum* were found to hybridize with pEJ79. Similar results were obtained for fragments of pEJ64 and pEJ82 of *C. m. sepedonicum* (data not shown). It can be seen from Figure 4 that hybridization of pEJ79 to genomic digests of *C. m. sepedonicum* gives rise to a multiple banding pattern. The patterns are seen even when hybridization and washes are carried out under highly stringent conditions allowing only 5% mismatch. This indicates that the fragment contains a region that is repeated several times in the genome. A multiple banding pattern also is seen when pEJ64 is used as probe. This pattern is different from that obtained when pEJ79 is used as probe: There is hybridization to fewer bands, and the signals are generally of lower intensity (data not shown).

The detection limits for ^{32}P-labeling, biotinylation, and sulfonation were compared by hybridizing probes to a dilution series of total DNA from *C. m. sepedonicum* NCPPB 2140 (Fig. 5). The isolated fragment from pEJ79 was used for ^{32}P-labeling, whereas the entire plasmid pEJ79 including the vector was labeled in biotinylation and sulfonation trials. The detection limit for ^{32}P and biotin labeling was 0.5 ng of total DNA, whereas the detection limit for the sulfonated probe was 1.5 ng.

To examine the degree of interference from nonhomologous DNA on the sensitivity of the probe, 25 ng of DNA from *C. m. sepedonicum* NCPPB 2140 was mixed with increasing amounts of DNA of *Pseudomonas syringae* van Hall before application to filters. Hybridization of ^{32}P-labeled DNA from pEJ79 demonstrates clearly that the presence of nonhomologous DNA affects the hybridization signal (Fig. 6). With 2.5 μg of DNA of *P. syringae*, the signal is reduced by a factor of approximately eight compared to that from a pure sample of *C. m. sepedonicum*.

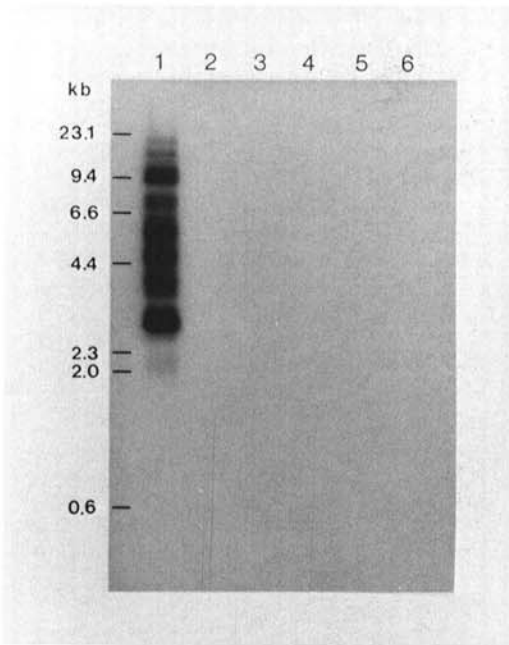

Fig. 1. Autoradiogram from hybridization of pEJ79 to a Southern blot of *Pst*I-digested total DNA from: lane 1, *Clavibacter michiganense* subsp. *sepedonicum* NCPPB 2140; lane 2, *C. m. michiganense*; lane 3, *C. m. insidiosum*; lane 4, *C. rathayi*; lane 5, *C. tritici*; and lane 6, *C. m. nebraskense*. Hybridization and washings were carried out to allow formation of hybrids with 20% mismatches. Exposure time was 30 min.

DISCUSSION

The strategy for selecting DNA fragments that can serve as probes for *C. m. sepedonicum* was based on the assumption that fragments specific to *C. m. sepedonicum* with respect to the related clavibacter species have a high probability of being generally specific. The overall DNA homology between *C. m. sepedonicum* and *C. m. michiganense*, *C. m. insidiosum*, and *C. m. nebraskense* is 56, 51, and 39%, respectively (8). In the hybridization with genomic DNA from the related and cross-reacting species to a Southern blot with DNA fragments from *C. m. sepedonicum*, there was no or very weak hybridization to nine fragments. The inserts of three clones (pEJ64, pEJ79, and pEJ82) were specific for *C. m. sepedonicum*. The inserts from pEJ64, pEJ79, and pEJ82 do not hybridize with DNA from the host organism, potato, or a number of microorganisms. These include closely related corynebacteria species, a number of bacteria isolates that react with polyclonal antibodies raised against *C. m. sepedonicum*, and 13 species covering a wide spectrum of genera. The three selected fragments all hybridize to the 15 isolates of *C. m. sepedonicum* tested. These isolates originate from different parts of Europe

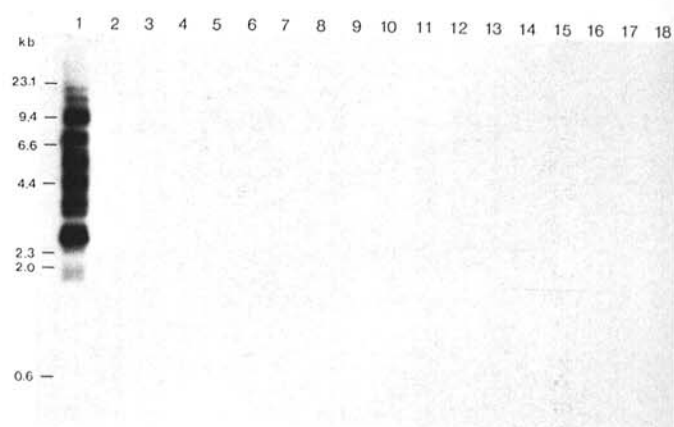

Fig. 2. Autoradiogram from hybridization of pEJ79 to a Southern blot of *Pst*I-digested total DNA from: lane 1, *Clavibacter michiganense* subsp. *sepedonicum* NCPPB 2140; and lanes 2–18, restricted DNA from 17 immunologically cross-reacting bacteria. Hybridization and washings were carried out to allow formation of hybrids with 20% mismatches. Exposure time was 30 min.

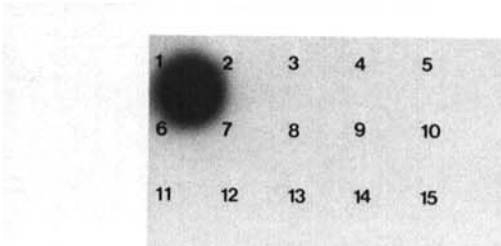

Fig. 3. Autoradiogram from hybridization of pEJ79 to a dot blot of total DNA from: **1,** *Clavibacter michiganense* subsp. *sepedonicum* NCPPB 2140; **2,** *Pseudomonas syringae*; **3,** *Xanthomonas campestris*; **4,** *Bradyrhizobium japonicum*; **5,** *Agrobacterium tumefaciens*; **6,** *Achromobacter avitratum*; **7,** *Escherichia coli*; **8,** *Erwinia carotovora*; **9,** *Actinobacillus equuli*; **10,** *Brevibacterium ammoniagenes*; **11,** *Bacillus subtilis*; **12,** *Streptomyces verticillatus*; **13,** Mycoplasma PG50; **14,** *Saccharomyces cerevisiae*; and **15,** potato (1 μg). Hybridization and washings allowed formation of hybrids with 20% mismatches. Exposure time was 4 hr with intensifier screen.

and the United States. The fragments therefore must be assumed to harbor sequences that are conserved among isolates of *C. m. sepedonicum*. The high degree of conservation combined with the high level of specificity indicate that the three fragments could serve as DNA probes for identification of *C. m. sepedonicum*.

Recently Verreault et al (25) published the identification of two clones with inserts from *C. m. sepedonicum* that hybridize strongly to DNA of *C. m. sepedonicum* and weakly to DNA from a number of other microorganisms including *C. m. michiganense* and *Erwinia carotovora* (Jones) et al. The inserts are relatively large (4 kb and 8 kb) and this may be the reason for the apparent lower degree of specificity than that found for the frements pEJ64, pEJ79, and pEJ82. Verreault et al (25) did not analyze to which degree the two DNA fragments are conserved among different isolates of *C. m. sepedonicum*. However, this is very important because Denny (7) found a specific fragment of *P. syringae* pv. *tomato* (Okabe) Young et al that hybridized to only some of the isolates of *P. s. tomato* tested.

The fragments of pEJ64 and pEJ79 each contain a sequence that is repeated in the genome of *C. m. sepedonicum*. The level of repetition is especially high for pEJ79 (Fig. 4). Recently Mogen and Oleson (18) reported the presence of a region of 1.3 ± 0.2 kb, which is present at high copy number in the chromosome of *C. m. sepedonicum*. In addition, they showed that there are two copies of this sequence on the plasmid pCS1, which is present

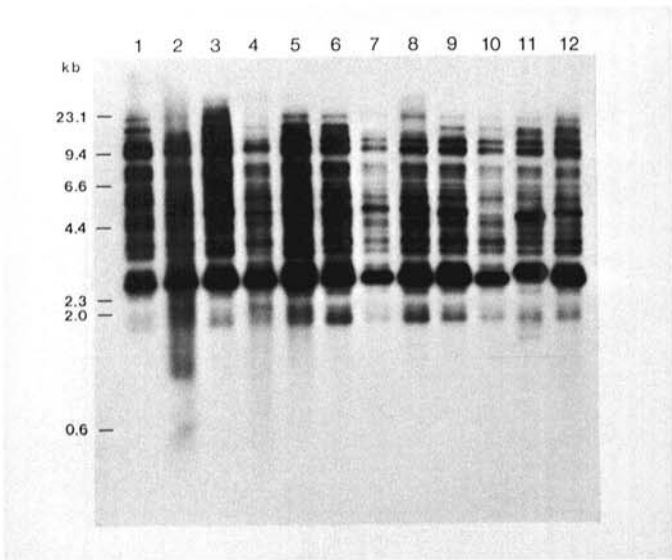

Fig. 4. Autoradiogram from hybridization of pEJ79 to a Southern blot of *Pst*I-digested total DNA from *Clavibacter michiganense* subsp. *sepedonicum* strains 3 (NCPPB 2140), 3A, 5, 6, 13, 32, 35, 36, 40, 81, 83, and 85 (lanes 1–12, respectively). Hybridization and washings were carried out to allow formation of hybrids with 20% mismatches. Exposure time was 30 min.

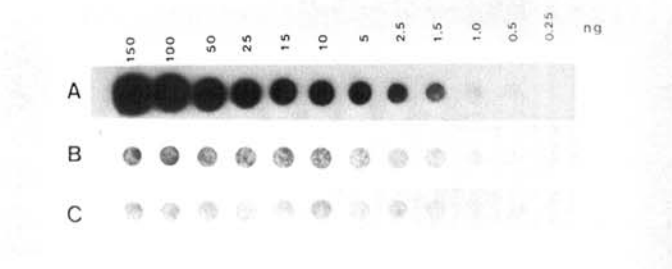

Fig. 5. Comparison of the detection limits for ^{32}P-labeled, biotinylated, and sulfonated probe pEJ79 on dot blots with dilution series of total DNA of *Clavibacter michiganense* subsp. *sepedonicum* NCPPB 2140. The filters were hybridized with: **A,** ^{32}P-labeled pEJ79 fragment (10 ng/ml); **B,** biotinylated pEJ79 (100 ng/ml); and **C,** sulfonated pEJ79 (1,000 ng/ml).

in most strains (18). The two plasmid copies are located on *Sma*I fragments of 2.9 and 5.1 kb, respectively. Mogen and Oleson evaluated the repeated sequence for use as a DNA probe (17) and concluded that it could not serve as a specific probe for *C. m. sepedonicum*. However, they gave no details concerning the strains tested against or the hybridization conditions used.

The repeated sequence of pEJ64 differs from the one found by Mogen and Oleson (18) because it does not hybridize to plasmid bands in DNA preparations from strain NCPPB 2140 of *C. m. sepedonicum*, which contains pCS1 (4) (data not shown). However, the fragment of pEJ79 does hybridize to a plasmid band in DNA preparations from strain NCPPB 2140 of *C. m. sepedonicum* (data not shown). We have compared the banding pattern obtained when the insert from pEJ79 is probed on a *Sma*I digest of total DNA to that obtained when the 2.9-kb *Sma*I fragment of pCS1 is used as a probe (18). Because of the extensive number of bands, a specific band-to-band comparison is not possible, but the overall banding pattern is very similar. Therefore we assume that the repeated sequence of pEJ79 is identical to that on the 2.9-kb *Sma*I fragment of pCS1. Also, by hybridizing chromosomal DNA to a *Sma*I digest of pCS1, Mogen and Oleson (18) showed that the only major repeated sequence present in both the chromosome and pCS1 is the repeated sequence of the 2.9-kb *Sma*I fragment. Because of the likely identity of the repeated sequences and the report that the repeated sequence hybridizes to DNA from species other than *C. m. sepedonicum* (17), the specificity of pEJ79 was further tested on a Southern blot with DNA from six different strains of *C. m. michiganense* (data not shown). This species has the highest DNA homology to *C. m. sepedonicum* (8). However, no hybridization occurred to the strains of *C. m. michiganense* under conditions allowing 20% mismatch. We therefore conclude that the fragment of pEJ79 is highly specific for *C. m. sepedonicum*. The observed differences between the specificity of the pEJ79 fragment and the repeated sequence from pCS1 can be explained in a number of ways: 1) the fragment of pEJ79 may not contain the entire repeated sequence; 2) different hybridization and washing conditions may have been used; 3) if Mogen et al (17) used a fragment that contains regions in addition to the repeated sequence, this would account for the observed hybridization to isolates not of *C. m. sepedonicum*; 4) Mogen et al (17) may have used species or strains not included in this study.

The use of DNA probes for diagnostic purposes depends to a large extent on the sensitivity that can be obtained. The detection limit for the probe pEJ79 was 0.5 ng for total DNA when ^{32}P was used for labeling and the autoradiogram was exposed for 16 hr. This amount of DNA corresponds to 2×10^5 bacteria assuming a genome size of 2,500 kb (2). The observed detection limit is lower than that usually obtained when the size of the probe is taken into consideration (10,13,14,25). This is also predictable because part of the probe is a repeated element. The detection limit of biotinylated pEJ79 was equal to that found with radioactive labeling, whereas the sulfonated probe was less sensitive. The result with the biotinylated probe is very promising

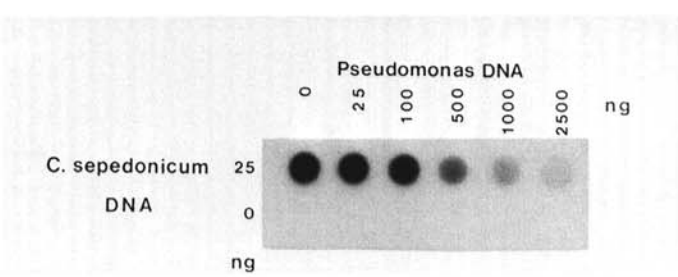

Fig. 6. Autoradiogram of dot blot with DNA of *Clavibacter michiganense* subsp. *sepedonicum* (25 ng) mixed with increasing amounts of DNA of *Pseudomonas syringae* (0, 25, 100, 500, 1,000, and 2,500 ng, dots 1–6) and DNA of pure *P. syringae* (0, 25, 100, 500, 1,000, and 2,500 ng). The filter was hybridized with the 2.6 kilobases fragment from pEJ79. Hybridization and washings were carried out to allow formation of hybrids with 10% mismatches. Exposure time was 60 min.

because nonradioactive labeling methods generally have been found to be less sensitive than labeling with ^{32}P (16,24). For the probes of *C. m. sepedonicum* identified by Verreault et al (25), the detection limit was 1.0–1.5 ng of total DNA with ^{32}P labeling. This is slightly higher than that obtained with pEJ79 despite the difference in size of the fragments. The reason is, however, that part of pEJ79 is repeated.

The presence of large amounts of nonhomologous DNA has a negative effect on the sensitivity of the probe. This effect is especially pronounced when DNA of *C. m. sepedonicum* constitutes 5% or less of the total DNA in the dot. Similar results were observed earlier (14). The weakening of the hybridization signal cannot be explained by low DNA binding capacity because nylon membranes with a binding capacity of 450 μg of DNA/cm^2 were used in this study. The observed effect might be explained partly by spacious hindrance preventing some of the bound DNA from being available for hybridization. This effect might clearly pose considerable problems when establishing a test system for *C. m. sepedonicum* from plant material based on a DNA probe because many cell constituents may have the same effect on the sensitivity.

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