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ABSTRACT. Bacteria isolated from greening-infected citrus, which have the typical ultrastructure and share some basic properties of the greening-causing agent, were compared with regard to their DNA properties. Both C-G content and DNA-DNA hybridisation revealed a high percentage of homology, consistent with the suggestion that the organisms are related. Pulsed field gel electrophoresis was employed to analyse the DNA of the isolates, after digestion with rare-cutter endonucleases. The electrophoretic patterns obtained further confirmed that the different isolates are the same bacterium. This technique may prove useful in identifying and separating greening isolates from other bacteria.

Greening is a severe disease of citrus, present in the countries of southern Africa, throughout Asia, and in the islands of the Indian Ocean (4). The disease is associated with a phloem-limited bacterium observed in infected material under the electron microscope (9). In 1984, a gram-negative rod was isolated from greening-infected citrus material bearing the same morphology under electron microscopy as the one previously described (3). Since then, a number of bacterial isolates from various countries have been obtained, all sharing the same ultrastructure and metabolic properties.

The most unique feature of DNA to have taxonomic importance is its mole percent cytosine plus guanine content (C-G content). In bacteria, this value ranges between 25 and 75% and the value is constant for a given organism (7).

Hybridisation between DNA of two bacteria depends on the linear sequence of nucleotides in their DNA's. Therefore, closely related bacteria share a large portion of their DNA, and thus result in a high homology value. This technique has been previously described for the comparative study of microorganisms (7).

Restriction endonuclease digestion, followed by agarose gel electrophoresis allows for a more precise analysis of DNA. Hence, pulsed field gel electrophoresis (PFGE) which provides a significant increase in resolution over unidirectional electrophoresis has been used to compare the resulting fragments of the restriction endonuclease digestion, or genomic fingerprints of bacterial isolates.

In this paper we compare the mole percent content and the genomic fingerprints of a number of greening isolates from various countries.

MATERIALS AND METHODS

Bacterial isolates. Eight isolates from greening-infected citrus material were used. Microorganisms from North-Eastern Transvaal, South Africa, include LC-1 from Letaba (3) and NC-1 from Nelspruit (14), both isolated from the columellae of infected oranges; isolates GC-1 and GL-1 were obtained from infected lemons from the Witwatersrand area, South Africa (14). Isolates O and M originate from Taiwan (14). Isolate 01 was isolated from an orange tree injected with LC-1 two years before (6). All these isolates shared the same ultrastructure and metabolic properties (14). Isolate LC was recovered from oranges from the same location as LC-1, but has a different colony morphology to the other isolates. Whereas LC-1 presented a small pin-point colony as described by Mochaba (14), LC showed a slightly larger, darker colony. Isolate DL-1 had the same morphology as isolate LC but was isolated from grapefruit infected with Australian citrus dieback (D. Böck, unpublished results). A culture of *Escherichia coli* K12 was obtained from the University of the Witwatersrand culture collection.

All greening and dieback isolates were grown in MIG, as described by Sibara (15), with vigorous aeration. *Escherichia coli* was grown on nutrient broth (Biolab, Johannesburg, South Africa).

DNA preparation. Liquid DNA samples were prepared according to the method of Marmur (11) with modifications (6). Cells were grown to an exponential growth phase, harvested and then lysed with lysozyme and SDS at a high pH to prevent the action of nucleases. Deproteinisation was accomplished with chloroform/isoamylalcohol and the nucleic acids recovered after ethanol precipitations (11). RNA was removed with the addition of RNase A.

Solid samples for PFGE were prepared according to the procedure of Promega Corporation (Madison, Wisconsin, USA). Briefly, cells from an overnight culture were mixed with molten low melting point agarose and poured into blocks. The blocks were incubated with lysozyme and sarkosyl overnight, and then with protein K and SDA. Blocks were washed in 50 mM EDTA and stored until used.

Thermal denaturation. The melting point (Tm) of the DNA of the putative greening organism was determined by thermal denaturation as described by Marmur and Dotty (12). The DNA sample dissolved in 1X SSC (0.15 M sodium chloride; 0.015 M sodium citrate; pH 7.0) at a concentration of 30 μg/ml was slowly heated (1 C/minute) from 25 C to complete denaturation, in a Varian 2200 spectophotometer (Varian, Australia) linked to a Julabo-programmer PRG microprocessor and a Julabo oil heater (Julabo, Germany). The absorbance was recorded as a function of the temperature, and the melting point was established as the midpoint of the absorbance increase. The C-G content was then determined using the equation: C-G = (Tm – 69.3) / 0.41.

DNA/DNA hybridisation. DNA/DNA hybridisation was performed on solid membranes (10). Nitrocellulose paper (Amersham, Amersham, UK)

was divided in (1cm^2) squares with soft pencil. Prior to spotting, the DNA samples were denatured with alkali solution (0.4 M NaOH) and were boiled for 10 min at 100 C before being quickly cooled on ice. Once cooled, 500 ng of DNA from each sample were spotted into each nitrocellulose square. The membrane was then baked in a vacuum oven for 2 hr at 80 C. Prehybridisation was carried out for 2 hr in a sealed bag at 68 C, and hybridisation continued at the same temperature for a further 18-20 hr. The hybridisation buffer and washes used were as described by Maniatis *et al.* (10). A total of 500 ng of DNA from isolate LC-1 was labeled with ^{32}P (Amersham, Amersham, UK) by the nick translation method (10) and was used as a probe. The nitrocellulose membrane was cut into the squares and the level of radioactivity for each of them was quantified on a Packard β particles counter.

Electrophoresis. Solid samples containing 2 μg of bacterial DNA were digested overnight at 37 C with 100 units of Not I endonuclease (Boehringer Mannheim, Mannheim, Germany) using the buffer recommended by the supplier. Digestion was stopped by the addition of 1 ml of 50 mM EDTA (pH 8.0) to the sample, before electrophoresis. Resulting fragments were separated on a CHEF II pulsed field electrophoresis system (Bio Rad, Richmond, California, USA) at 100 volts using a 1% agarose gel and 0.5X TBE buffer (8.9 mM Tris; 8.9 mM boric acid; 0.2 mM EDTA, pH 8.0). The buffer was recirculated and cooled during the electrophoresis to keep the gel at a constant temperature of 14 C. Samples were subjected to electrophoresis for 22 hr at a pulse time of 10 sec. As a reference, DNA from lambda bacteriophage digested with endonuclease Hind III (Boehringer Mannheim, Germany) was also run as molecular weight marker. After electrophoresis, the gel was stained in 0.5X TBE containing ethidium bromide (5 μg/ml), destained in distilled water and photographed with Polaroid type 665 film.

RESULTS

Mol percent C + G content. The purity of DNA samples was determined spectrophotometrically. Only samples showing an absorbance ratio at 260/280 nm between 1.8 and 1.9 were utilised. The denaturation pattern showed a sharp increase in absorbance, indicative of an RNA-free sample. The absorbance increased in the order of 39% during the denaturation process (data not shown) which reflects an intact DNA sample (1).

The C-G content of the DNA of selected isolates, as determined from the Tm values, was established as 59% for LC-1, 60% for NC-1 and 59.5% for isolate 01. Commercially available purified DNA from *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) was tested as a control for the thermal denaturation technique, and DNA from *Escherichia coli* K12 was purified as described in Materials and Methods and used as a control for the DNA purification technique. The experiments yielded C-G content values of 71.2% for *Micrococcus lysodeikticus*, and 50.4% for Escherichia coli.

When determining the Tm value of *Micrococcus lysodeikticus*, which was known to have a high C-G content (72%), the concentration of the solvent was changed to 0.01X SSC, thus allowing the DNA to denature at a lower temperature. The equation employed to estimate the C-G content was different, namely, C-G content = 2.64 X (Tm − 59.6) (15).

Homology among greening isolates. The homology between selected isolates was determined based upon the hybridisation values.

A total of 500 ng of sheared DNA from isolates LC-1, NC-1, GC-1 and isolate 01 was spotted onto nitrocellulose paper. For every experiment, 500 ng of DNA from isolate LC-1 was nick translated. After purification of the probe through a Sephadex G50 column (Pharmacia, Uppsala, Sweden) to eliminate the unincorporated nucleotides, the specific activity was established at 1.2 X 10^7 cpm/µg.

After hybridisation, the homology between isolate LC-1 and the other isolates were obtained. The value for the LC-1/LC-1 hybridisation was considered to represent 100% homology. All other homology values were established as fractions of the homologous hybridisation value. Isolate LC-1 was found to have a homology of 85% with NC-1, 86% with GC-1 and 86% with isolate 01.

Genomic fingerprinting. Endonuclease Not 1 was used to digest DNA from greening isolate NC-1, shown to have a similar C-G content as the other South African isolates GC-1 and 01, as well as O and M. In addition, DNA from isolates LC and DL-1 were also digested with Not 1 and included in the same gel.
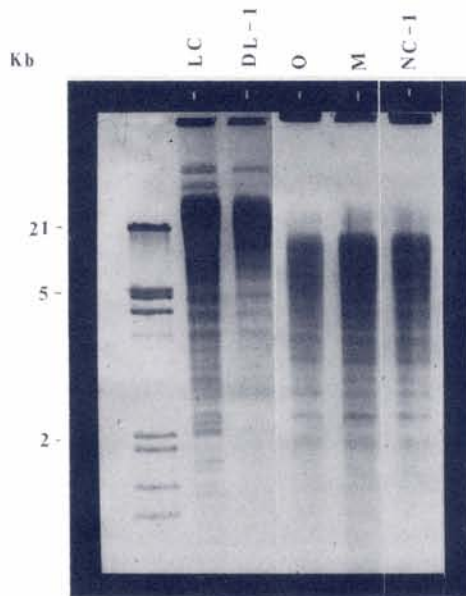

Fig. 1. Ethidium bromide stained PFGE showing the genomic fingerprint of various isolates digested with Not 1. Isolates DL-1 (from Australian citrus dieback) and LC (from South Africa) share a very similar profile. Isolates M and O (from Taiwan) and NC-1 (from South Africa) show identical fingerprints/although different from the previous pattern. A 1% gel was run at 100 v for 22 h with a pulse time of 10 sec. DNA from lambda phage digested with Hind III on lane 1 was run as a molecular weight marker. DNA sizes are in kilobases (Kb).

The result of the electrophoresis can be seen in Fig. 1. The digestion produced fragments of a molecular weight ranging from 1 to over 20 Kb. Fingerprints from isolates NC-1, O and M appeared identical, while the fingerprints from isolates LC and DL-1 were indistinguishable from each other, although different from the previous pattern.

DISCUSSION

The C-G content values obtained for the control microorganisms *Micrococcus lysodeikticus* and *Escherichia coli* fall well within the values previously being reported in the literature for these bacteria (12).

The C-G content value for greening isolates LC-1, NC-1, GC-1 and isolate 01 suggests similarities among them. The 60% value is a relatively high value, but is similar to that of known bacterial plant pathogens such as *Erwinia*, *Pseudomonas*, *Xanthomonas* or *Agrobacterium* species (2), where values of 55 to 65% are common.

Kemper *et al.* (8) have studied the C-G content of various fastidious xylem-limited bacteria, these having a homogeneous DNA composition of approximately 50.5% C-G content. Hence, these fastidious plant pathogens seem to differ from the greening isolates.

Mean DNA base composition are of particular taxonomic value for bacteria, since the range for the bacteria as a whole is so wide. However, although closely related bacteria have similar C-G content, two organisms with similar values are not necessarily closely related; this is because the C-G content does not take into account the linear arrangements of the nucleotides in the DNA, as with hybridisation techniques (7).

The results obtained from the hybridisations of isolate LC-1 DNA with other isolates suggests that there is a high degree of homology between the isolates LC-1, NC-1, GC-1 and isolate 01, and therefore a close taxonomic relationship between them. A homology of 80 - 90% is considered to be within a species (7). The homology of isolate LC-1 with the other greening isolates is greater or equal to 85%.

Genomic fingerprinting has been applied previously to strains of the citrus pathogen *Xanthomonas campestris* pv. *citri* (5), although PFGE was not used. The increased resolution that PFGE provides allows a more detailed comparison of fingerprints. It is possible to select the right electrophoresis conditions to resolve fragments of a desired molecular weight range.

The preferred restriction endonucleases for genomic fingerprinting are the so called rare-cutter enzymes, which recognise infrequent nucleotide sequences in DNA molecules and produce a small number of fragments, therefore resulting in a fingerprint useful for comparison. The enzyme used in this study Not I, recognizes the nucleotide sequence GC'GGCCGC and is one of the few known endonucleases with an eight-base recognition sequence, and thus it is expected to cut the bacterial genome less frequently than other endonucleases (13).

Fingerprints proved to be very reproducible. No variation of the fingerprints shown in Fig. 1 was detected after three repetitions of the gel. Although isolated from different countries, some isolates produce the same or indistinguishable pattern. In addition, LC, a bacterium isolated from the same source as LC-1 which has a different colony morphology to the putative greening organism, and a similar bacterium isolated from Australian citrus dieback (DL-1), revealed the same fingerprint. This similarity suggests a possible relationship between these two bacteria.

Data from thermal denaturation and DNA hybridisation is not available for isolates M and O, since they were only isolated after the assays were done. However, the fact that they both show an indistinguishable fingerprint to isolate NC-1 allows one to suggest that they also share a high degree of DNA homology and a C-G content very similar to that found for NC-1.

Fingerprinting may prove to be an important tool in identifying the greening isolates, and for comparison of isolates of different origin. The same technique could be applied to bacteria associated with other diseases, and so it may be useful in the study of the etiology of greening-like diseases like Australian citrus dieback.

The data shown in this study strongly suggests that the isolates which bear the typical morphology of the greening causing bacterium are in fact strains of the same bacterium, regardless of their country of origin, and have a C-G content well within the range of the known phytopathogenic bacteria.

ACKNOWLEDGEMENTS

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