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Genetic study of a bacterium isolated from "greening" infected citrus

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Centrifugation was carried out for 45 hours at a speed of 34000 rpm utilising a type 40 BECKMAN rotor (BECKMAN, USA) in a L8-55 BECKMAN ultracentrifuge.

The tubes were observed under ultraviolet light and a photograph taken when necessary with an EM NIKON camera loaded with 50 ASA AGFA film.

2.9 MOLECULAR WEIGHT ESTIMATION

An estimation of the molecular weight of pure DNA samples was made using agarose gel electrophoresis. The DNA preparations were run together with high molecular weight DNA markers, such as DNA of calf thymus (BDH), *Escherichia coli* k-12, *Bacillus subtilis*, *Agrobacterium tumefaciens*, and *Erwinia herbicola*, all of which were dissolved in 1 x SSC, adjusted to pH 7,0.

A volume of 200 ml of 1% agarose gel in TBE buffer (Appendix 4) was prepared and subsequently loaded with the samples. The electrophoresis was performed for 18 hours at 40 mA (constant current), using the above mentioned TBE buffer as running buffer. No running dye was included.

Bearing in mind that the logarithm of the molecular weight of the DNA is inversely correlated to the distance migrated by the DNA sample on the gel, a standard curve was plotted using the data from the reference DNAs.

2.10 DNA RESTRICTION

DNA was purified according to the modified method of Marmur (1961) described in 2.6. The DNA concentration was adjusted to 1 µg per ml in 1 x SSC, adjusted to pH 7,0.

2.10.1 RESTRICTION ENZYMES

DNA was digested with restriction enzymes Eco RI, Hind III and Bgl II (Boenringer Mannheim, West Germany) using different enzyme concentrations. The enzyme buffer was that recommended by the manufacturer (Appendix 5) and this was always 1/5 of the total reaction volume. The incubation temperature was as recommended by the supplier (37°C) and the samples were digested overnight.

The digestion was ended by heating the mixture at 65°C for 10 minutes before cooling on ice. Once cold, the reaction mixture was rapidly loaded onto the agarose gel (Maniatis et al, 1982; Boehringer Mannheim, 1986, West Germany).

2.10.2 AGAROSE GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis was used to separate the different fragments obtained after digestion of DNA (Maniatis et al, 1982). Agarose (1%) was dissolved in 250 ml of TBE buffer (Appendix 4) by boiling the

mixture. Ethidium bromide (10 µl of a stock solution of 10 mg per ml) was added, the mixture poured into the gel former and allow to harden. Once this was accomplished, the total digestion mixture for every restriction reaction was loaded into the wells. Fycoll (5 µg, see appendix 4) was loaded as running dye. For reference purposes, lambda DNA marker II (Boehringer Mannheim, West Germany) was run on the same gel.

The electrophoresis was performed for 5 hours at 40 mA (constant current) using TBE buffer (Appendix 4) as running buffer, after which the gel was observed under ultraviolet light (Transilluminator, Ultraviolet Products USA.) and a photograph taken when necessary with a POLAROID camera.

2.11 DNA HOMOLOGY

2.11.1 IMMOBILISATION OF DNA

First, 1 ml of DNA purified as described in 2,6 at a concentration of 0,1 µg per µl in 0,1 x SSC, adjusted to pH 7,0 was sheared twice in a sonicator for 30 seconds each time. An appropriate number of squares (1cm^2 each) were drawn on the nitrocellulose paper (Whatman, USA) with a pencil, and 0,5 µg of sheared DNA spotted onto each square. Each DNA sample was spotted in duplicate.

The nitrocellulose paper was gently located on top of the denaturing solution (Apperdix 6) for 5 minutes and then on the neutralising solution (Appendix 6) for 5 minutes. Both procedures were performed at room tem-

perature. The nitrocellulose blot was then dried in a vacuum oven at 80°C for 2 hours.

2.11.2 PREHYBRIDISATION

The nitrocellulose blot was washed with 100 ml of 3 x SSC, pH 7,0, for 10 minutes at room temperature. The blot was then placed inside a plastic bag and sealed on three sides.

Herring sperm DNA (Boehringer Mannheim, West Germany) in a volume of 300 µl from a stock solution of 0,15 mg per ml was boiled in an eppendorf tube for 10 minutes and quickly cooled on ice before adding it to the rest of the prehybridisation solution (Appendix 6). This solution was warmed in a waterbath at 37°C and the whole mixture added to the plastic bag and sealed. The plastic bag was incubated at 42°C for 3 hours in an orbital incubator (80 rpm), after which the bag was open and the prehybridisation solution discarded.

2.11.3 LABEL

The radioactive label used was deoxycytidine 5'-(α-P^{32}) triphosphate triethylammonium salt in stabilised aqueous solution at 10 mCi/ml, with a activity of 250 microcurie (Amersham, UK).

2.11.4 NICK TRANSLATION

The method used was derived from those of Maniatis et al (1982) and Rigby et al (1977). A volume of 2 µl of labelled CTP was used for the reaction mixture (Appendix 6), following the instructions of the supplier (Amersham, UK) and Maniatis et al (1982).

A volume of 1 µl from a DNA stock solution of 1 µg per µl in 1 x SSC, adjusted to pH 7,0 was mixed with the rest of the reaction mixture (Appendix 6).

The reaction mixture was incubated in a waterbath at 12°C for 4 hours and stopped with 2 µl of 0,5 M EDTA. It was then passed through a G-50 sephadex column (Pharmacia, Uppsala, Sweden) in TE buffer (Appendix 4), from which 4 drop fractions were collected in eppendorf tubes. These fractions were introduced in vials and counted in a scintillation counter (Packard 2230, USA) in order to determine which fractions contained the nick translated DNA and which contained the unincorporated dNTPs. The fractions containing the nick translated DNA (first peak of radioactivity) were pooled and used as a labelled probe.

Alternatively, a Geiger counter was used to establish the two different peaks of radioactivity. The activity in cpm per µg of DNA was established before the labelled DNA was used as a probe.

2.11.5 HYBRIDISATION

After the prehybridisation period, the total labelled probe was boiled for 10 minutes together with 1,5 ml of Herring sperm DNA from a stock solution of 5 mg per ml, and the mixture cooled on ice.

A volume of 20 ml of the hybridisation buffer (Appendix 6) was warmed in a waterbath at 37°C, and then mixed together with the prepared DNAs before pouring into the hybridisation plastic bag containing the nitrocellulose blot. The bag was sealed and incubated at 65°C for 20 hours in an orbital waterbath (80 rpm) inside a perspex box (Johnson, 1981).

The nitrocellulose blot was then removed from the bag and rinsed with 100 ml of 5 x SSC at room temperature to remove the dextran sulphate of the hybridisation solution.

2.11.6 POSTHYBRIDISATION

The nitrocellulose paper was washed twice with 50 ml of posthybridisation solution (Appendix 6) for 30 minutes at 42°C in an orbital waterbath (80 rpm) inside a plastic bag and a perspex box. Finally, it was washed with 100 ml of 5 x SSC, containing 0,1% SDS for 30 minutes at 55°C.

2.11.7 COUNTING

The nitrocellulose paper was cut into the 1 cm^2 squares mentioned in 2.11.1, and each paper was introduced into a separate vial to which 10 ml of scintillation liquid (Appendix 7) was added. Each vial was counted for β particles and the data recorded as cpm.

2.11.8 HOMOLOGY DETERMINATION

The number of counts recorded for LC-1 DNA utilising the LC-1 labelled probe was considered as 100% homology. The homology of the different microorganisms with LC-1 was establish through the equation:

Homology = counts for sample DNA / counts for LC-1 DNA x 100

RESULTS

3.1 ISOLATION OF MICROORGANISMS FROM CITRUS LEAVES

From each isolation experiment, 12 conical flasks (125 ml) with 30 ml of medium were inoculated with citrus leaf extract (table 1).

As control, three flasks containing each of the different media and no inoculum were also incubated. There was no evidence of contamination.

An isolation experiment was performed with both healthy and greening infected citrus leaves from each one of the material sources (see 2.3.1). Each experiment was done twice.

To monitor the growth, a gram stain was performed every 24 hours on each sample. The morphology of the microorganisms was studied under the light microscope and the cultures plated onto the same solid medium in order to obtain pure cultures. Once this was achieved, the catalase and oxidase tests were performed.

A total of 7 different microorganisms were isolated from the various citrus material, six of which were rods, and one being a coccus. These isolates were coded, according to the results of the colony morphology, the gram stain, and from phenotypic tests.

Some of the isolates were obtained from various material sources whereas others were only found in a particular area. Two (code 4 and code 05) were isolated from healthy leaves, the rest being isolated from greening infected leaves.

MEDIUM	NUMBER OF FLASKS	TEMPERATURE
523	2	37°C
523	2	25°C
MIG	2	37°C
MIG	2	25°C
Nutrient broth	2	37°C
Nutrient broth	2	25°C

Table 1 - Incubation procedure for the isolation experiments.

The microorganisms found were:

- code 01, isolated exclusively from citrus leaves from an orange tree from the Eastern Cape, inoculated two years previously by insertion of a disc of healthy leave tissue which had been soaked in LC-1, and maintained at the University of the Witwatersrand.

- code 05, isolated from both healthy and greening infected leaves from material collected from the Brits area.

- code 4, found in healthy leaves from the Letaba area (North Eastern Transvaal) and in greening infected material from the Brits area.

- code 7, isolated only from greening infected leaves from both the Brits and the Letaba area.

- code 9, found to be in greening infected leaves from Letaba and Nelspruit.

- code F-1, isolated from the greening infected material obtained from Brits.

- code F-2, isolated from the greening infected material collected from the Nelspruit area.

All microorganisms grew well in both 523 and MIG media (Appendix 1). No growth was obtained in the nutrient broth cultures. Although all isolates grew at both 25 and 37°C temperatures, better growth was observed at 37°C.

3.2 PHENOTYPIC CHARACTERISATION

The gram stain reaction and the colony morphology of the different isolates, as well as the results of the catalase and oxidase tests, are given in tables 2 and 3, respectively.

From the above phenotypic and morphological characteristics obtained strong similarities were observed between LC-1 and the microorganism isolated as code 01. They were both positive for the catalase and the oxidase tests, and possessed a closely related morphology.

The different isolates are shown in figures 2 to 8, together with LC-1 (Figure 1).

ORGANISM	GRAM STAIN	COLONY MORPHOLOGY
code 01	NEGATIVE	regular, whitish
code 05	NEGATIVE	irregular, whitish
code 4	POSITIVE	irregular, whitish
code 7	NEGATIVE	regular, yellowish
code 9	POSITIVE	regular, yellowish
code F-1	POSITIVE	regular, orange
code F-2	POSITIVE	regular, yellowish

Table 2 - Gram stain and colony morphology of the isolates from citrus leaves.

ORGANISM	CATALASE	OXIDASE
code 01	POSITIVE	POSITIVE
code 05	POSITIVE	POSITIVE
code 4	NEGATIVE	POSITIVE
code 7	NEGATIVE	NEGATIVE
code 9	POSITIVE	NEGATIVE
code F-1	NEGATIVE	NEGATIVE
code F-2	POSITIVE	NEGATIVE

Table 3 - Catalase and oxidase test results for the isolates from citrus leaves.

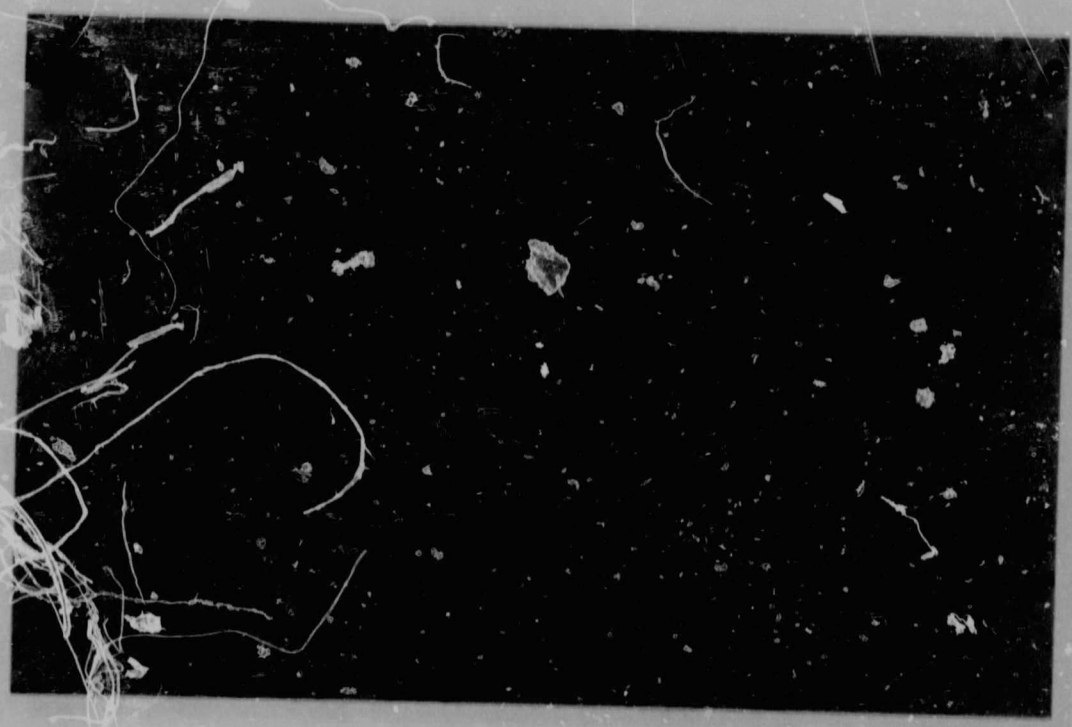


Figure 1 - Gram stain of LC-1.



Figure 2 - Gram stain of code 01.



Figure 3 - Gram stain of code 05.

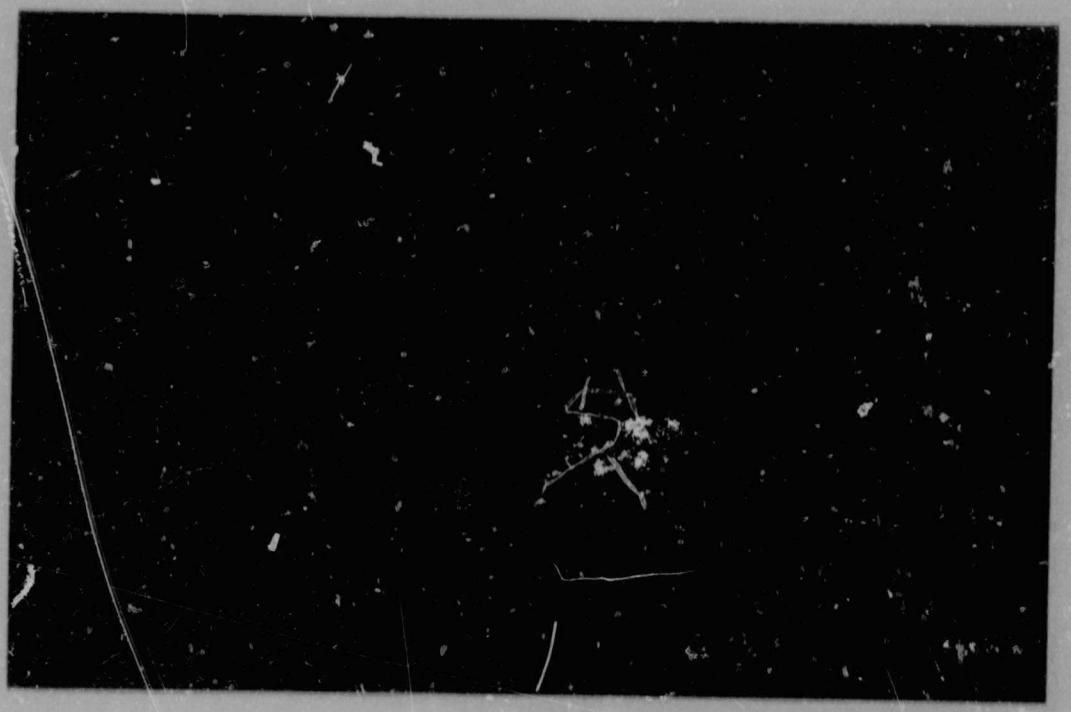


Figure 4 - Gram stain of code 4.



Figure 5 - Gram stain of code 7.



Figure 6 - Gram stain of code 9.

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