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

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The base composition of DNA has been determined also by correlating the mol %C+G content and the buoyant density of DNA in CsCl density gradient (Rolfe and Meselson, 1959; Sueoka et al, 1959; Schildkraut et al, 1962). However, this method needs large amounts of DNA and unusual bases in the DNA molecule may alter the resulting mol% C+G content (Marmur and Doty, 1962).

A simpler procedure to determine the mol% C+G content was proposed by Fredericq et al (1961), based on the ratio of the absorption of DNA at 260 and 280 nm after denaturation of the DNA molecule at pH 3. Ulitzur (1972), describes yet another method for determining base ratio of native DNA based on an empirically demonstrated correlation between ultraviolet absorption spectra of native DNA's and their base contents. According to this method, the ratio of the absorbance at any wavelength in the range from 240 to 255 nm and any wavelength in the range from 265 to 280 nm, increases linearly with increasing mol% C+G content. At absorbance ratios of 240/280, 245/270, and 240/275 (nm), the standard deviation of the calculated base ratio content was below ± 0,85. However, today the Tm analysis is considered to be more reliable for estimating the mol% C+G content.

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 mol% C+G content, two organisms with similar mol% C+G content are not necessarily closely related; this is because the mol% C+G content do not take into account the linear arrangements of the nucleotides in the DNA, as the hybridisation techniques do (Johnson, 1984).

If native DNA is subjected to thermal denaturation and slowly brought to a lower temperature, the simple strands anneal with one another, to form again double stranded molecules. The rate and efficiency of this process

depend on many factors, including the mean chain length of the DNA, the annealing temperature and the ionic strength of the medium (Wetmur and Davidson, 1968; Wetmur, 1976). Some random pairing always occurs, but since it is the complementary regions of the two strands that form the most stable duplexes (as a result of the high efficiency of their pairing) the reassociation is favoured. Shortly after this phenomenon had been discovered it was shown that when DNA preparations from two related strains of bacteria were mixed and treated in this manner, hybrid DNA molecules formed. When similar experiments were conducted with DNA preparations from two unrelated organisms, no hybridisation could be detected as upon annealing, duplexes are formed only by specific pairing between single strands with complementary sequences.

There are two general methods for homology studies. The first method, often referred to as the free-solution method, involves the reassociation of the single-stranded nucleic acids in solution. Reassociation of DNA may be measured optically using a spectrophotometer (Britten et al, 1974; De Ley et al, 1970), or it may be measured by incubating a small amount of denatured DNA having a high specific radioactivity with a large excess of unlabelled denatured DNA (Gillespie and Spiegelman, 1965). In the later instance, the ability of the labelled fragments to form duplexes with unlabelled DNA is assayed by absorption to hydroxyapatite (Bishop, 1970) or by resistance to hydrolysis by an enzyme known as S1 nuclease (Crosa et al, 1973).

In 1963, Nygaard and Hall found that native DNA, denatured DNA, and RNA/DNA hybrids would bind to nitrocellulose paper where RNA would not.

In 1966, Denhardt described a procedure for covering the DNA binding sites on nitrocellulose membranes. This made it possible to first immobilise a given amount of denatured DNA on the membrane and to treat it with a mixture that blocked all other sites which could potentially bind DNA.

Subsequent addition of a second DNA preparation thus allowed complementation to be assessed. Thus, the membrane procedure became readily available for DNA/DNA experiments in homology, replacing the agar method.

By the use of nitrocellulose membranes, DNA or RNA can be determined by either direct binding or by competitor experiments.

In the direct binding method, a given amount of denatured DNA (labelled) or RNA is incubated under standard conditions with various single-stranded DNA preparations that have been immobilised on nitrocellulose membranes. After the incubation the unbound labelled DNA is washed away and the radioactivity remaining on the membrane measured. The percent homology is expressed as the amount of heterologous binding divided by the amount of homologous binding x 100 (Johnson, 1984). The results obtained with this method are similar to those using the S-1 method. The advantage of the direct dot technique over the competitor method is that small amounts of DNA are required whereas large amounts of DNA are required in the latter procedure, where unlabelled denatured reference DNA is fixed onto nitrocellulose membranes. A direct binding reaction, used for a reference point, is performed between the homologous denatured labelled DNA in solution and the membrane-bound reference DNA. The competitive reaction has the same components as the direct binding reaction but additionally contain high concentration of unlabelled denatured DNA in solution (Johnson, 1984).

To be taxonomically useful, the data from experiments on nucleic acid hybridization must be expressible in quantitative terms. It is therefore necessary always to start from a particular reference strain.

The total amount of information concerning bacterial genetic homologies is still relatively small. Nevertheless, the technique is a powerful one.

Its great potential advantage is that it can be used to explore gross genetic homologies in the many bacterial groups where no mechanism of genetic transfer are known, and where, in consequence, biological hybridisation experiments are precluded (Stanier et al, 1972).

A further method of analysing DNA is by assessing the fragments resulting from restriction endonuclease digestion. Restriction endonucleases are endo-deoxyribonucleases that digest double-stranded DNA after recognising specific nucleotide sequences by cleaving two phosphodiester bonds, one within each strand of the duplex DNA.

Restriction endonucleases are categorised in classes I, II and III (Boehringer Mannheim, West Germany). Type I and III enzymes carry the ability to modify nucleic acid (methylations) and an ATP requiring restriction (cleavage) activity in the same protein. Both types of enzymes recognise unmethylated recognition sequences in substrate DNA, but type I enzymes cleave randomly whereas type III enzymes cut DNA at specific sites.

Type II restriction system consists of a separate restriction endonuclease and a modification methylase. A large number of type II restriction enzymes have been isolated (Roberts, 1982), many of which are useful in molecular cloning. These enzymes cut DNA within or near to their particular recognition sites (Sqaramella, 1972), which typically are four to six nucleotides in length with a two fold axis of symmetry (Maniatis et al, 1982).

Each restriction enzyme has a set of optimal reaction conditions which are given by the supplier. The major variables are the temperature of incubation and the composition of the buff. (Maniatis et al, 1982).

A number of strategies are used to construct maps of the sites at which restriction enzymes cleave DNA; it is usually necessary to employ more than one of them to obtain maps that are sufficiently accurate and detailed to be useful. The techniques most commonly used are:

- A simultaneous digestion with a combination of restriction enzymes (Lawn et al 1978).

- Sequential digestion of an isolated DNA fragment with a second restriction enzyme (Parker and Seed , 1980).

- Partial digestion (Smith and Birnstiel, 1976).

However, due to the size of a bacterial chromosome the map of a bacterial DNA cannot be made with simple techniques. In this case, the pattern of the different restricted DNAs are compared.

In addition to chromosomal DNA many bacteria possess extrachromosomal fragments of DNA.

In 1963, several authors demonstrated the existence of resistance to one or more drugs in bacteria (Akiba et al, 1960; Datta, 1962; Watanabe, 1963). Very soon an analogy was found between such resistance, the so-called R factor and the sex factor F of *Escherichia coli* K-12 (Mitsuhashi et al, 1960), which was found not to be part of the bacterial chromosome and, like R factors, could spread through bacterial populations (Lederberg, 1952). These extrachromosomal DNA molecules were termed plasmids. Numerous types of plasmids have been described since in many bacterial species. Bukhani et al (1977), gave a large list of different plasmids with a wide range of functions. They can code for drug resistance, tolerance to heavy metal ion, toxin production, and even tumour induction and pathogenicity in plants and animals.

Tooze (1973) described the pathogenicity of *Agrobacterium tumefaciens* which can induce tumours in plants as due to the Ti plasmid (Montoya et al, 1977).

In the last two decades, it has become clear that the genetic complement of a bacterial cell lies not only in the main chromosome but, in many cases, also in extrachromosomal elements such as plasmids which carry genetic material capable of phenotypic expression. What contribution such extrachromosomal entities make to a particular bacterial phenotype, either by direct expression or interaction with the chromosomal DNA of the cell, is just starting to be understood. Consequently, several of the newer taxonomic methods have been and are being directed towards the characterisation of the total genetic complement of bacteria (Broda, 1979; Harwood, 1980; Hardy, 1981), including plasmids.

In the early experiments, plasmid DNA was isolated as linear molecules. However, with the development of gentler methods of releasing DNA from cells, several plasmids were shown by electron microscopy to be circular (Freifelder, 1968) and this is the form in which most, if not all, plasmids are present in the cell.

For all the procedures described above, mol% C+G content, hybridisation and plasmid detection,the DNA has to be released from the bacterial cell and purified. In vivo, DNA is rarely found as a completely free molecule. In higher animals, it is protected by specialised proteins usually of a very basic nature, called histones, whereas in bacteria the proteins are replaced by oligoamines (Ayad, 1972; Hayes, 1968). The bacterial chromosome has been shown to have a single DNA molecule with a molecular weight of about $2,8 \times 10^9$ and to be circular (Cairns, 1963).

Numerous attempts have been made to isolate bacterial DNA with the conventional biochemical techniques and to establish its exact molecular

weight value. During these studies it became evident that chain-length varied with the procedure used, and that the apparent molecular weight of DNA could be significantly decreased by mechanical shearing. The molecular weight of DNA is usually determined by light scattering measurements or by measurements of sedimentation rates or intrinsic viscosity, or by a combination of measurements of sedimentation coefficient and intrinsic viscosity. Valuable information may also be obtained by direct observation of the thread-like DNA molecules in the electron microscope or by autoradiography (Davidson, 1969). A molecular weight of 2-2,5 x 10^8 daltons was the highest found with bulk isolation techniques (Kelly, 1967; Klesius and Schuhardt, 1968). However, unbroken DNA can be isolated from bacteria in a small quantity (Davern, 1966).

Although direct length measurement by electron microscopy are used, co-electrophoresis in agarose gels of DNA fragments of known length with the DNA to be analysed is among the most widely used methods for molecular weight estimation of DNA (Boehringer Mannheim, West Germany).

The methods employed in isolating DNA vary according to the nature of the biological material involved. Methods for animal, plant and bacterial sources have been fully described (Smith, 1967). For microorganisms in general, one of the most satisfactory procedures is that of Marmur (1961) which involves disruption of the cells, denaturation of cell debris and removal of RNA by ribonuclease followed by selective precipitation of DNA with isopropanol. Chelating agents such as sodium dodecyl sulphate are added to prevent bivalent metal ion contamination and deprivation by deoxyribonuclease, which requires bivalent metal ions for its hydrolytic action. Other methods for isolation of DNA have been described (Kirby, 1968). The most serious problem in any attempt to isolate DNA from natural sources are the avoidance of nuclease degradation and of shear degradation (Davidson, 1969). The long thin threads which constitute the DNA molecules

are very easily broken, even by very gentle lysis of the cells (Davern, 1966).

The aim of this investigation was to attempt to characterise the DNA of one putative greening organism, LC-1, with regard to molecular weight, mol%C+G content, and if possible plasmid content and endonuclease restriction patterns.

The DNA of this isolate was also tested for the degree of homology with other isolates of the putative greening organism, as well as representative bacteria from several phytopathogenic genera.

In order to determine if the putative greening isolates varied markedly from other bacteria associated with the leaves of healthy and greening infected citrus, various isolation media were used to grow citrus-associated organisms and their DNA homology to LC-1 assessed.

MATERIALS AND METHODS

2.1 MICROORGANISMS USED

Several isolates from greening infected citrus were used. LC-1, isolated by Duncan (1985) from the Letaba area, Eastern Transvaal; NC-1, isolated by Mochaba (pers. comm.) from the columellae of infected oranges from the Citrus and Subtropical Research Institute (CSFRI) at Nelspruit, Eastern Transvaal, and GC-1, isolated by Mochaba from the columellae of infected lemons from the Witwatersrand area.

Pseudomonas phaseolicola, *Erwinia carotovora*, *Erwinia herbicola*, *Corynebacterium michiganense* and *Corynebacterium insidiosum* were provided by the Plant Protection Research Institute, Pretoria.

Bacillus subtilis, *Azotobacter vinelandii*, *Escherichia coli*, and *Agrobacterium tumefaciens* were obtained from the University of the Witwatersrand culture collection.

2.2 CULTURE MEDIA

Various media were utilised. These include MIG medium (Appendix 1), 523 medium (Kado and Heskett, 1970, Appendix 1) and nutrient broth (Biolab, South Africa).

2.3 ISOLATION TECHNIQUE

2.3.1 SAMPLE PREPARATION

Healthy and greening infected citrus leaves were obtained from Brits, Letaba, The Citrus and Subtropical Fruit Research Institute (CSFRI) at Nelpruit, Johannesburg and the Eastern Cape. All control greening free material came from seedlings from the Eastern Cape.

2.3.2 INOCULATION TECHNIQUE

The surface of the leaves was washed with distilled water and cotton wool to remove all dirt. Then, the leaves were treated with formaldehyde (30%) for 20 minutes in a sterile beaker, after which the leaves were allow to air dry. Once dried, the intact leaves were treated with ethanol (70%) for 10 minutes and rinsed in sterile water twice before cutting the leaves into thin strips with sterile scissors and forceps. Sterile conditions were maintained throughout the procedure.

These strips were passed through a sterile press to collect the extract which was subsequently used as inoculum for the different culture media (Duncan, 1985), 2 ml being used as inoculum for 30 ml of medium. All isolations were performed in quadruplicate, duplicate flasks being incubated at 25°C and 35°C in an orbital shaker with vigorous aeration (150 rpm).

2.4 PHENOTYPIC CHARACTERISATION

2.4.1 GRAM STAIN

The technique described by Brock (1979) was followed with slight modifications (Duncan, 1985).

2.4.2 OXIDASE TEST

One to two drops of 1% aqueous tetramethyl-p-phenylene diamine dihydrochloride were spotted onto filter paper (Whatman, USA) previously placed on top of a slide where a sample of bacterial colony had been placed beforehand. A violet colour indicated the presence of oxidase within the bacteria. Late colour changes were not taken into account (Larpent and Larpent-Gourgaud, 1970).

2.4.3 CATALASE TEST

A turbid solution was obtained in a test tube by adding 1-2 ml of distilled water to a bacterial sample. Two drops of hydrogen peroxide (3%) were added and the presence of bubbles recorded as a positive test indicating the existence of catalase within the microorganism (Larpent and Larpent-Gourgaud, 1970).

2.5 STOCK CULTURES

In all cases, stock cultures of bacterial strains were stored on appropriate agar slants at 4°C, and all cultures were checked for purity before utilisation in any biochemical test.

2.6 PURIFICATION OF DEOXYRIBONUCLEIC ACID

2.6.1 LYSIS AND PURIFICATION PROCEDURE

Three different methods were tried to purify DNA, these being the methods of Meyer and Schleifer (1975), Brenner et al (1969) and Marmur (1961). The latter method was subsequently used in all sample preparations with the modifications described below:

Bacterial cells in the exponential phase of growth (18-24 hours) were harvested in the J2-21 BECKMAN centrifuge (BECKMAN, USA) with the JA-14 BECKMAN rotor at 5000 rpm for 10 minutes, and the pellet (2 to 3 g) washed once and resuspended in 25 ml of saline-EDTA, adjusted to pH 9 (Appendix 2).

Lysozyme (Boehringer Mannheim, West Germany) was added (500 µl) and the mixture incubated for 20 minutes at 37°C, after which sodium dodecyl sulphate was added to a concentration of 1%. The mixture was then incubated for a further 45 minutes at 37°C.

The mixture became very viscous and was not easy to handle. Sodium perchlorate to a final concentration of 1 M was added as well as 0,5 volumes of a chloroform-isoamylalcohol (24:1) mixture, before cooling the mixture on ice for 30 minutes with occasional mixing, in order to produce an emulsion.

This resulting mixture was then centrifuged in a J2-21 BECKMAN centrifuge with a JA-14 BECKMAN rotor for 10 minutes at 10000 rpm and maintained at a temperature of 0°C, after which three phases were visible. The top aqueous phase was gently pipetted, avoiding the white solid interphase consisting of the protein residue. The DNA in the top aqueous layer was then precipitated by gently pouring 2 volumes of ice-cold ethanol (100%) on top of the supernatant. The DNA precipitate was recovered by gently stirring a glass rod, dried and dissolved in 0,1 x sodium citrate at pH 7,0 (0,1 x SSC, see appendix 2) until dispersion occurred, after which time the solution was adjusted to 1 x SSC by adding the appropriate volume of 10 x SSC.

The deproteinization step using the chloroform-isoamylalcohol mixture was repeated several times, utilising the same volumes to resuspend the DNA, until little protein residue could be found in the interphase. After the first deproteinization, only 15 minutes treatment each time was necessary before the subsequent centrifugation.

The DNA was then precipitated once again with ethanol (100%) and dried. This step removes the ribonucleotides. To ensure the removal of RNA, the DNA sample, resuspended in 1 x SSC, adjusted to pH 7,0 was treated with ribonuclease (Boehringer Mannheim, West Germany) at a concentration of 40 µg per ml at 37°C for 30 minutes, after which treatment further deproteinization steps were required.

When no residual protein was observed in the interphase, the dried DNA was dissolved in 9 ml of 0,1 SSC, pH 7,0; 1 ml of acetate-EDTA, adjusted to pH 7,0 (Appendix 2) was added after the DNA was totally dissolved. While the DNA preparation was stirred, 0,54 volumes of isopropanol were added dropwise. The DNA was then precipitated again with two volumes of cold ethanol (100%) and recovered with a glass rod.

After the last precipitation with ethanol (100%), the DNA was dissolved in 0,1 x SSC, adjusted to pH 7,0 to a concentration of 1 µg per µl which corresponds to an absorbance of 20 at 260 nm in a 1 cm path quartz cuvette before storage at -20°C for subsequent use.

2.7 BASE COMPOSITION VALUE

Two techniques were followed to determine the mol% C+G content of the various DNA samples.

2.7.1 THERMAL DENATURATION TEMPERATURE

DNA was purified following the method described in 2.6 from microorganisms in the logarithmic phase of growth and dissolved in 1 x SSC, adjusted to pH 7,0 with a concentration of 30 µg per ml, which corresponds to an absorbance of 0,6 at a wavelength of 260 nm, in a 1 ml path quartz cuvette.

The experiment was carried out in a 2200 VARIAN spectrophotometer (VARIAN, Australia) liked to a JULABO-programmer PRG microprocessor and a JULABO oil heater (JULABO, Germany).

The starting temperature of 25°C was increased at a rate of 1°C every minute till there was complete denaturation of DNA. Once the maximum absorbance increase was determined, the melting point was establish as the temperature corresponding to half the total absorbance obtained. The base composition value (mol% C+G content) was calculated using the following formula (Marmur and Doty, 1962).

$$\text{mol\% C+G} = (Tm - 69,3) / 0,41$$

DNA from *Micrococcus lysodeikticus*(Sigma, USA), and DNA from *Escherichia coli* purified in the same manner were also included as controls.

2.7.2 ULTRAVIOLET SPECTROSCOPY

The concentration of DNA was maintained at about 40 µg per ml in 1 x SSC, at pH 7,0 which corresponds to an absorbance of 0,8 at 260 nm. The samples were placed in 1 cm path quartz cuvettes in a 2200 VARIAN spectrophotometer, and the absorbance recorded over the wavelength range from 240 to 280 nm, using 1 x SSC, adjusted to pH 7,0 as a blank. DNA of *Micrococcus lysodeikticus* (Sigma,MO. USA) was used as a reference, and DNA of *Escherichia coli* as control.

The ratio of the absorbance at the wavelength 240/280, 240/275, and 245/270 nm were established and related to the ratio obtained from *Micrococcus lysodeikticus* and its mol% C+G content (72%) through the equations (Ulitzur, 1972):

$$Mol\% = (72 \times 0{,}0076) + (240/280_{sample}) - (240/280_{ref.})/0{,}0076$$

$$Mol\% = (72 \times 0{,}00576) + (240/275_{sample}) - (240/275_{ref.})/0{,}00576$$

$$Mol\% = (72 \times 0{,}0047) + (245/270_{sample}) - (245/270_{ref.})/0{,}0047$$

the mean of this three values is considered to be the base composition value of the microorganism.

A computer programme was written in BASIC for all the calculations (Appendix 3).

2.8 PLASMID EXTRACTION

Two procedures for plasmid isolation were attempted from total DNA previously isolated following the method described in 2.6.

2.8.1 AGAROSE GEL ELECTROPHORESIS

Horizontal gel electrophoresis was used to separate the possible plasmids from the bacterial genome.

Agarose (0,8%) in 200 ml of TBE buffer (Appendix 4) was prepared and boiled. Once the agarose was dissolved, ethidium bromide (10 µg per ml) was added to a concentration of 0,5 µg per ml. The mixture was poured into the gel former and allow to harden (Maniatis et al, 1982).

A total of 5 µg of DNA dissolved in 10 µl of 1 x SSC were loaded into each well together with 5 µl of Fycoll running dye (Appendix 4). Lambda DNA marker II (Boehringer Mannheim, West Germany) was loaded as a reference.

The electrophoresis was performed for 4 hours at 30 mA (constant current) with TBE (Appendix 4) as running buffer. When the electrophoresis was ended, the gel was observed under ultraviolet light and a photograph taken with a POLAROID camera (Maniatis et al, 1982).

2.8.2 CAESIUM CHLORIDE DENSITY GRADIENT

DNA (5 ml) at a concentration of 1 µg per ml in 1 x SSC, adjusted to pH 7,0 was mixed with 5 g of CsCl (MERCK) and 0,4 ml of ethidium bromide (10 mg per ml) in a Quick-seal polyallomer BECKMAN tube. The remainder of the tube was filled with paraffin oil (Maniatis et al, 1982).

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