GENETIC STUDY OF A BACTERIUM ISOLATED FROM "GREENING" INFECTED CITRUS.

A thesis submitted by Gonzalo Hortelano to the Faculty of Science, University of the Witwatersrand in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, June 1986.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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24th day of July, 1986.

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ABSTRACT

A bacterium was isolated from greening infected citrus material (Duncan, 1985) suspected of being the actual causing agent, and coded LC-1. Beside the attempts to classify LC-1 through phenotypic features, the uniqueness of this gram-negative, spore forming, rod made its classification difficult.

The present work involved a genetic study of LC-1 with the aim of partial characterisation of its DNA. For this purposes, the mol% C+G content of the DNA and its molecular weight estimation were established, and the existence of plasmids and defined restriction patterns investigated.

Another microorganism was isolated from greening infected citrus leaves during the execution of this investigation, this being a gram-negative rod bearing the same morphology as LC-1, namely code 01. Its DNA, together with the DNA of other similar isolates (NC-1 and GC-1) was compared to the DNA of LC-1. The mol% C+G content of all organisms was established and the homology between them determined through DNA/DNA hybridisation.

In order to establish the relationship of LC-1 to other bacteria, the DNA homology between LC-1 and the phytopathogenic *Erwinia*, *Corynebacterium*, *Pseudomonas*, and *Xanthomonas*, as well as between *Azotobacter vinelandii*, and *Bacillus subtilis*, was established.

Futher the DNA of LC-1 was compared with the DNA from other organisms found associated with citrus plants.

High homology values were found between the different isolates from greened citrus considered on morphological and biochemical data to be the

putative greening organism and LC-1. The mol% C+G content of all the isolates were similar, thus suggesting a close relationship amongst them.

Low homology values were found between LC-1 and all other bacteria tested.The uses of these results for taxonomy purposes and to obtain a genetic detection technique for LC-1 are discussed.

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INTRODUCTION

Greening of citrus is a severe disease in Southern Africa, known for a long time. The disease was first observed in South Africa in 1928 (Oberholzer, 1965) at De Wild in the Western Transvaal and in the White River area of the Eastern Transvaal Lowveld.

This disease has had several severe outbreaks in the past. According to Moll (1974) the disease has "sustained epiphytotic proportions since 1957". Greening was considered the most serious single-problem facing citriculture at that time (Catling, 1970).

Today several areas in Swaziland, Natal and the Cape province are affected with greening, and the disease is of major economic importance, not only in South Africa but in the citrus industry throughout the world (Moll, 1979).

This disease and other graft-transmitted greening-like diseases of citrus have been reported in many citrus growing countries of the world: stubborn in Arizona, Florida (Childs,1968), the Mediterranean countries and Morocco, greening in Brazil (Rossetti et al, 1969), Reunion, Swaziland (Catling, 1970), and Thailand. Other similar diseases are known in China, Israel, India, Indonesia, Iran, The Philippines and Taiwan (Sibara, 1982).

As early as 1965, field trials indicated citrus phsylla, *Trioza erytrea* (del Guercio) as the vector of greening disease (McCleans and Oberholzer, 1965) but the mechanism of this transmission were elucidated only in 1973 (Moll and Martin, 1973), when a light and electron microscopic study was made of phsyllids which had fed on infected plant tissue.

Most varieties of citrus are susceptible to greening. Old established lines are just as equally affected as those of new origin. Affected plants either exhibit symptoms throughout the entire tree, or, as is often the case, only in some branches.

The symptoms of African greening show seasonal variations. Towards the end of summer and beginning of autumn the trees apparently improve though this is only a false impression of recovery. As soon as the cooler season appears the normal symptoms of greening are clearly visible again.

The symptoms of greening disease in most citrus species are similar: retarded growth in infected sectors and a blotchy mottling of older leaves. Infected sweet orange trees grow poorly, bear abnormal fruit and their leaves show various chlorotic patterns (Moll and Van Vuuren, 1977). The very comprehensive description of greening symptoms given by McClean and Schwarz (1970), can be summarised as follows,there being two main leaf symptoms:

- A yellowing of the main and secondary veins which progresses to a blotchy mottle on mature leaves, and is usually associated with those leaves formed on vigorous shoots of the late summer flush.

- The leaves on terminal twigs are small, upright and show a variety of chlorotic patterns suggestive of zinc and iron deficiency. The leaves become leathery with prominent veins as the season progresses, and tend to drop prematurely while stay green and never attain the orange colour. Inside, the affected fruits appear a dirty greenish brown colour. Occasionally, they may contain aborted seeds. Larger fruits may have fertile seeds which can raise normal trees that appear to be free of the greening disease.

Schreider (1968) explained the reason for the induced mineral deficiency and stimulated starvation symptoms when he found localised sieve tube necrosis in diseased leaves, and indeed the greening organism is located in the sieve elements of infected tissues (Bove et al, 1970; Lafleche and Bove, 1970).

The starvation conditions are considered to be due to defective translocation of food materials within the diseased tree. Occasionally, cambial cells also undergo hypertrophy and subsequently compress the necrotic tissue. The secondary symptoms observed, seem to be the result of phloem necrosis. The most prominent of these is the accumulation of starch in all these parenchyma cells of the leaves. The consequence of all these anatomic aberrations is the faulty translocation of food material within the entire tree or a section thereof. Fruits fail to develop normally because of carbohydrate deficiency and the apparent inability of starch to move out of the leaves (McClean and Schwarz, 1970).

The etiology of greening has had a checkered history (Garnett, 1985). Due to the chlorotic symptoms of the diseased plant, greening was generally believed to be a consequence of some sort of nutrient deficiency. However, when grafting was proved to transmit the disease (McClean and Oberholzer, 1965) and the inability of fertilisers to alleviate greening symptoms was demonstrated, the virus theory became popular. This theory was suggested by McClean and Oberholzer (1965) and went unchallenged until 1970 when Lafleche and Bove, on electron microscopic evidence, reported the presence of a microorganism in the phloem sieve elements of infected material obtained from South Africa (Moll and Van Vuuren, 1977). These organisms were initially described as bearing closer resemblances to MLO's (Lafleche and Bove, 1970; Moll, 1974). Similar organisms were later seen in the haemolymph of *Trioza erytreae* by Moll (1974).

Many plant diseases which were initially ascribed to viruses were re-examined during the sixties as a result of observations made which showed that mycoplasma-like organisms (MLO's) were responsible for some of the so-called yellow diseases (Igwegbe, 1970; Fudl-allah et al, 1972; Clark et al, 1978) and indeed most of the so-called yellow diseases have been shown to be caused by either MLO's or riketsia-like organisms (Maramosch, 1974; Hopkins, 1977).

However, Moll and Martin (1974) described a prokaryote as the causal agent of greening disease in South Africa. Electron microscopic studies revealed three layers in the agents cell wall. An electron dense inner layer (8 nm), an electron transparent region (5-15nm), and a darker, electron dense outer layer (8nm).The entire envelope comprises a thickness of between 20 and 30nm (Moll, 1974). This is thicker than the simpler unit membrane found in mycoplasmas (Saglio et al, 1971). Thus, the mycoplasma nature of the greening organism has since been questioned and it is now believed that the agent causing greening disease is of a bacterial nature and not mycoplasma-like (Bove et al, 1979). Furthermore, penicillin has been showed effective against greening (Bove et al, 1979) whereas it does not affect mycoplasmas and spiroplasmas. However, tetracycline is by far the most effective antibiotic for treating affected trees (Milne et al, 1978).

In order to control the disease, a number of principles are currently applied. While some of them are focused at the vector others are aimed at eradicating the greening causing agent itself from affected trees. In South Africa, several chemicals are used both in nurseries and in the field to fight the disease. Thus, dimethoate (Perfektion), and tetracycline hydrochloride (citomycin) (Van Vuuren, 1977) are commonly used today. However, only a combination of measures against both vector and causal agent will lead in the future to satisfactory results.

Many attempts have been made to isolate and culture the organism described by Moll and Martin (1974). Different media designed for mycoplasmas, cell and organ preparations, embryonic eggs and fastidious plant pathogens were used unsuccessfully.

Sibara (1982) devised a medium named MIG (Appendix 1), which isolated an unusual bacteria from greened citrus, with a cell wall structure similar to that described by Moll and Martin (1974) and sensitive to penicillin. The principal constituents of this medium were those described by Levitt (1974) as being major components of phloem exudates of many green plants, although not necessarily citrus plants. Some of the components were, however, based on insect tissue culture formulations (Jones et al, 1976) and on nutrients of media developed for the growth of excised roots and plant tissues.

Subsequently, this medium has been used to isolate bacteria from greened citrus material from various areas of South Africa. A gram negative rod, coded LC-1, was isolated by Duncan (1985) from the columellae of oranges collected from Letaba (Eastern Transvaal). The envelope structure was studied and its dimensions establish to be of approximately 3000 by 350 nm (Ariovich and Garnett, 1985).

A number of isolates have to date been obtained from various cultivars throughout the Transvaal. From metabolic features (Mochaba, pers. comm.) and electron microscopic studies (Ariovich, pers. comm.), the various isolates can be classified into two closely related groups.

Metabolic tests have to date been unable to assign the isolates to any taxonomic group of bacteria (Mochaba, pers. comm.). Hence, it seemed essential to compare the DNA of a well studied isolate (LC-1) to that of the other putative greening isolates and other bacterial plant pathogens, as well as bacteria commonly associated with citrus in order to asses if

indeed the isolates of the putative greening agent were closely related aswell as to establish their apparent relationship to other groups of bacteria.

Historically, classification of bacteria has been based on similarities in phenotypic characteristics. Although this method has been quite successful, it has not been precise enough for distinguishing superficially similar organisms or for determining phylogenetic relationship among the bacterial groups. Nucleic acid studies were first applied to such problems more than 20 years ago and have, since, become of major importance in bacterial classification. There are several advantages to be gained by basing classification on genetic relatedness:

- A more unifying concept of a bacterial species [illegible].

- Classification based on genetic relatedness tend not to be subject to frequent or radical changes.

- Reliable identification schemes can be prepared after organisms have been classified on the basis of genetic relatedness.

- Information can be obtained that is useful for understanding how the various bacterial groups have evolved and how they can be arranged according to their ancestral relationship (Johnson, 1984).

In recent years, the growth of molecular biology has opened a number of new approaches to the characterisation of organisms and these approaches have already had profound impact on the taxonomy of bacteria. Of particular value are two different kinds of analysis performed upon isolated nucleic acids which furnish specific information about the genotype. These are the analysis of the base composition of DNA, and the study of

chemical hybridisation between DNA and DNA (Marmur et al, 1963; Mandel, 1969; Stanier et al, 1972).

DNA is a polymer of nucleotides. A nucleotide has three components. A purine or pyrimidine base, linked through one of its nitrogens by a 5-carbon cyclic sugar (deoxyribose) and a hosphate esterified to carbon 5 of the sugar. In the deoxyribonucleic acid there are only four types of nucleotides, these being distinguishable by their bases (Adenine-A, Guanine-G, Cytosine-C and Thymine-T). Polynucleotide chains are long, unbranched polymers formed by bridges between the 5' phosphate of one the nucleotides and the 3' hydroxyl of the sugar of the next (Kornberg, 1974).

In 1950, Chargaff showed that, as a rule, the amount of 6-amino bases (T+G), equalled the amount of 6-keto bases, signifying a paired relationship between these two types of bases. Bearing this in mind, and after the X-ray studies of Wilkins (1956) and others, Watson and Crick (1953) proposed a "double helix" structure for DNA. The double helix is formed by hydrogen bonding between the bases. Such hydrogen bonds are weaker than the usual chemical bonds, but the fact that so many of them occur along the length of the DNA double helix, at least two for each base pair, gives the molecule a higher degree of stability and rigidity. In order words, the bases of double-stranded DNA should give a quantitative relationship in which A=T and G=C or A+G/T+C=1 (Strickberger, 1968).

Since the pyrimidines T and C are smaller than the purines A and G, the only way to make a symmetrical helix was to make bonds between a purine and a pyrimidine. It turns out that the A-T and G-C base pairs have not only the same size but the same shape. Three hydrogens bonds link a cytosine with a guanine, whereas two hydrogen bonds link an adenine to a thymine.

The most unique feature of DNA that was recognised as having taxonomic importance was its mole percent cytosine plus guanine content (mol% C+G). Among the bacteria, the mol% C+C content ranges from 25 to 75 and the value is constant for a given organism (Johnson, 1984). A substantial divergence between two organisms with respect to mean DNA base composition reflects a large number of individual differences between the specific base sequences of their respective DNA's. It is prima facia evidence for a major genetic divergence and hence for a wide evolutionary separation. The very broad span of values characteristic of the bacteria as a whole accordingly reveals the great evolutionary diversity of this particular biological group and also suggests their evolutionary antiquity (Stanier et al, 1972).

Although DNA base composition may be determined chemically after hydrolysis of a DNA sample and separation of the free bases, it can be determined more easily by physical methods, and these are the principally used. The "melting point" temperature of DNA (i.e. the temperature at which it becomes denatured, by breaking of the hydrogen bonds that hold together the two strands) is directly related to the mol% C+G content, because hydrogen bonding between C-G pairs is stronger than between the A-T pairs. Strand separation is accompanied by a marked increased in absorbance at 260 nm, the maximum absorption of DNA, and this can be easily measured by ultraviolet spectrophotometry (Stanier et al, 1972).

During the controlled heating of a preparation of double stranded DNA in an spectrophotometer, the absorption increases by about 40%. The temperature at the mid-point of the sharp rise in the curve obtained by plotting temperature versus absorption is called the "melting temperature", or Tm. The Tm is correlated in a linear manner with the mol% C+G content of the DNA (Marmur and Doty, 1962). The higher the Tm value, the higher the mol% C+G content of the DNA (Johnson, 1981).

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