3.9.2 MOLECULAR WEIGHT ESTIMATION

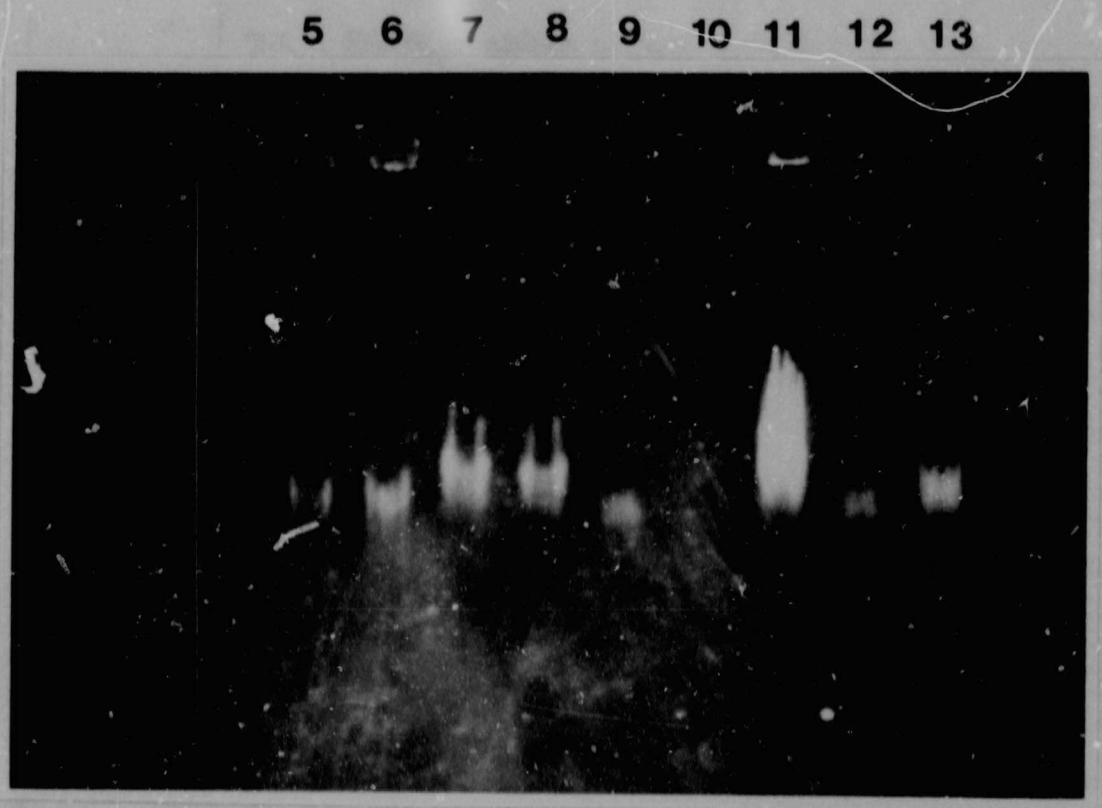
An estimation of the molecular weight of the DNA of LC-1 was made utilising agarose gel electrophoresis as described in 2.9. The result is shown in Figure 16.

Utilising the values obtained for the references (Appendix 9), a standard curve was plotted. Considering the molecular weight of the marker DNA's (Gillis et al, 1970) and the migration of LC-1 on the gel, a value of 2,8 x 10^9 daltons was estimated for LC-1 DNA.

3.9.3 DNA RESTRICTION

Digestion of DNA from LC-1 with various endonucleases, Eco RI (1,3 units per µl), Hind III (12 units per µl) and Bgl II (1,5 units per µl) was performed. In order to determine the optimal conditions for restriction, different enzyme concentrations were tested. The range of enzyme concentrations utilised being from 6 to 17 units per µg of DNA. The enzyme buffer was always kept as 1/5 of the total volume, distilled water being used to keep the whole digestion volume at 25 µl.

Three enzyme concentrations for each enzyme utilised were evaluated. The digestion solution being described in table 27. No clear restriction maps were obtained (figure 17).



Lanes 5,6 - LC-1.

Lanes 7,8 - *Agrobacterium*.

Lane 9 - *E.coli*.

Lane 11 - Calf thymus.

Lane 12 - *Erwinia*.

Lane 13 - *Bacillus*.

Figure 16 - Agarose gel run with high molecular weight DNA markers for the molecular weight estimation.

Lane 1 Lambda DNA marker II (2µg)					
	DNA µg	ENZYME µl	BUFFER µl	H_2O µl	TOTAL µl
(ECO RI)					
Lane 2	2	13	5	5	25
Lane 3	2	10	5	8	25
Lane 4	1	7	5	12	25
(HIND III)					
Lane 5	1	1	5	18	25
Lane 6	2	1	5	17	25
Lane 7	2	1,5	5	16,5	25
(BGL II)					
Lane 8	2	8	5	10	25
Lane 9	1	8	5	11	25
Lane 10	2	10	5	8	25

Table 27 - Different restriction conditions evaluated to digest DNA.

Figure 17 - Agarose gel run with the digested DNA samples.

DISCUSSION

Isolation of microorganisms from various citrus areas were performed using the isolation technique of Sibara (1982) as modified by Duncan (1985).

A variety of media exist for isolating plant pathogens (Agrios, 1978). Some are specific for a particular bacterial group whereas others are general and can be used to isolate a variety of microorganisms. Medium 523 (Kado and Hesket, 1970) is a general medium to isolate phytopathogenic bacteria and was chosen for culturing such organisms associated with citrus. MIC medium is routinely used for the isolation of putative greening organisms after a liquid enrichment technique where gram-negative organisms with appropriate dimensions are selected for further culture. This was also employed for culturing organisms on citrus leaves. Finally, nutrient broth was utilised as a common medium, suitable for the growth of a wide variety of microorganisms.

A variety of microorganisms were isolated from citrus material. From the results obtained (3.1), it follows that some of them were isolated exclusively from greening infected leaves (code 01, 7, 9, F-1 and F-2), while others were found in both healthy and infected leaves (code 05 and 4). Sometimes, the same bacteria was isolated from different sources. The literature suggest that many different organisms can be found in plant leaves (Agrios, 1978). Furthermore, the fact that different organisms can be found in healthy and infected leaves, can be explained due to the different nutritional conditions affecting both plants.

The isolates obtained were grouped together on gram stain, morphology, colony morphology as well as the reaction in the catalase and oxidase tests.

Seven different bacteria were isolated, six being rods and one a coccus. One organism had identical characteristics to LC-1, as described by Duncan (1985). This organism was isolated from leaves of a seedling inoculated with LC-1 two years earlier using a leaf patch technique.

The rest of the bacteria isolated from healthy and greened leaves bore no morphological nor biochemical similarities with LC-1. Although code 05 is also positive for the catalase and oxidase test, the morphology was different.

In order to compare the DNA of LC-1 with these isolates and other organisms, a procedure to produce pure DNA was essential.

Various procedures were utilised, including those of Brenner et al (1969), Meyer and Schleifer (1975), and Marmur (1961).

Considering the yield calculated from the 260/280 and 260/240 nm ratios, which are indicative of purity, the method of Marmur (1961) appeared to be the best (Tables 4 to 6). However, even this technique had to be adapted and modifications, particularly to the lysis procedure, introduced.

Thus, the amount of lysozyme utilised was increased to 20 µg per ml, and the volumes modified to make the DNA sample easier to handle. The number of cells harvested and the growth phase of utilised were found to be important, while small amounts of cells yielded a low amount of DNA which was denatured along the procedure, large amounts of DNA from large amounts of cells were impractical to handle and difficult to purify. Optimum numbers of cells was found to be about 2g of pellet (wet weight). If the cells were not in the logarithmic phase of growth, the yield of DNA was low.

According to Maniatis et al (1982), 260/280 and 260/240 nm ratios of 1,8 and 1,4, respectively indicate the purity of the DNA preparations. From the results obtained, the quality of the DNA samples is high, since only two DNA preparations of all those prepared gave a 260/280 nm ratio below 1,8.

In order to establish the mol% C+G content of the different DNA's, two techniques were utilised, the thermal denaturation temperature (Marmur and Doty, 1962), and the ultraviolet spectroscopy (Ulitzur, 1972). The reliability of both methods appears to be good (3.2). The Ulitzur method is advantageous since is faster and do not require expensive equipment. On the other hand, although the Tm technique takes a longer time due to the slow heating of the sample and requires a highly purified sample of DNA free of protein and RNA contamination, the results are generally considered to be superior (Johnson, 1984).

RNA absorbs at the same wavelength as DNA and displays hyperchromic shifts. DNA has a hyperchromic shift of about 40% while that for RNA is only 30%. Furthermore, the rising pattern, critical for calculating the Tm value, is much wider for RNA than it is for DNA, as RNA is a pool of different size molecules which denature at different temperatures (Lehninger, 1975). Hence, the purity of the sample can be determined using this procedure as well as the mol% C+G content.

An important point in this procedure is the ionic strength of the solvent buffer (1 x SSC, see appendix 2). In fact, the formula that relates the Tm value to the mol% C+G content varies with the buffer used (Marmur, 1961). From the thermal denaturation profiles which were smooth and characteristic, the DNA samples appeared to be pure in all cases (figures 10-14).

Purified DNA from both *Escherichia coli* and *Micrococcus lysodeikticus* were analysed with this procedure and yielded values published in the literature (Marmur and Doty, 1962). The mol% C+G content found for LC-1 was 59%, this being within the range of values found for known plant pathogens such as *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Azotobacter* (De Ley, 1967, table 24). *Bacillus* has a significantly lower value (43%, Marmur and Doty, 1962) than the DNA of LC-1. *Corynebacterium* are reported to have a higher mol% C+G content in some literature (Dopfer et al, 1982), although other authors (De Ley, 1967) found a much lower value for this genus. *Azotobacter* and *Bacillus*, were included in these experiments due to the fact that LC-1, a gram-negative bacterium, forms structures similar to spores or cysts.

The mol% C+G content of all greening putative organisms are very close (Table 9-12) and similar to the value found for LC-1 (59%). This was expected and verifies the morphological and biochemical studies performed on them which conclude that they are all very closely related.

A value of 59% is considered a relatively high mol% C+G content but many bacteria known to be plant pathogens have such a high mol% C+G content. Kamper et al (1985), have studied the homology between various fastidious xylem-limited bacteria as well as to members of the major phytopathogenic bacteria containing genera of gram-negative bacteria as well as their mol C+G%. All the xylem-limited bacterial strains had deoxyribonucleic acid composition of approximately 50,5 mol C+G% and a similar DNA molecular weight. Hence, these fastidious plant pathogens seem to differ from the putative greening organism.

Many methods have been described to establish the homology between DNA samples (Johnson, 1981). Competitive DNA hybridisation techniques are favoured by several workers, but these techniques require large amounts of DNA if the action of the homologous DNA is to be effectively swamped.

De Ley and Tutgat (1970) found that 700 µg of DNA was necessary to counteract the effect of 2 µg of labelled homologous DNA. In addition, competitive techniques exclude the direct comparison of two DNA preparations in the same solution. Therefore, a non-competitive filter method was adopted.

The direct binding method utilised in this work has been successfully used by various researchers with some variations.

Garvie (1976), followed the method of Denhardt (1966) and De Ley and Tutgat (1970) to immcbilise the DNA onto nitrocellulose paper. The hybridisation was carried out at 45°C in conical flasks with the hybridisation solution (dimethyl-H^3-sulfoxide) soaking the nitrocellulose paper. Their recorded radioactivity was 1000 to 2000 cpm for each homologous DNA-loaded nitrocellulose square, as compared with the 1796 cpm obtained in this work for LC-1 DNA-loaded nitrocellulose squares.

Seldin and Dubnau (1985), carried out taxonomic studies among different nitrogen fixing strains of *Bacillus*, using the method of Barinaga et al (1981) and Maniatis et al (1982) to immobilise the DNA. The amount of DNA immobilised was 0,5 µg. In this study similar amounts of DNA were used and similar labelling and hybridisation conditions as the hybridisation temperature, 65°C.

Stackebrandt and Kandler (1979), studied the taxonomy of the genus *Cellulomonas* with the proposal of seven new strains based on DNA-DNA hybridisation results. They followed the method of Denhardt (1966) to immobilise the DNA (7 to 12 µg / 0,5 diameter disks) and the method of McConaughy et al (1969) to hybridise (at 47°C) the H^3-labelled DNA to the DNA-loaded nitrocellulose paper filters. The scintillation cocktail employed by these researches was used for this work (PPO-POPOP mixture, see appendix 8).

The homology between DNAs as a source for bacterial taxonomy has been discussed by Johnson (1984). A major contribution of DNA homology studies has been to provide a more unifying concept of a bacterial species. Although the exact level of DNA homology above which one considers organisms as belonging to the same species is arbitrary, similar homology clusters have been found in all bacterial groups that have been investigated. Johnson (1973) also suggests what seemed to be reasonable cut-off points for delimitating species, and closely related species.

The results obtained from the hybridisation of LC-1 DNA with DNA from other putative greening isolates, suggest that there is a high degree of homology between these organisms, therefore suggesting a close taxonomic relationship between them. A homology of 80-90% is considered to be within a species (De Ley, 1967; Johnson, 1984). The homology of LC-1 to all these other isolates is similar (Table 15). When it is considered that the organism code 01 came from a plant infected with LC-1 at a very early subculture level it would appear that all isolates are almost identical. The DNA for the hybridisation reaction was produced from an LC-1 culture of 15 subcultures.

A major practical use of DNA homology data is for correlation with individual phenotypic tests. It is common to find that variability for a trait among strains within a DNA homology group as well as distinct DNA homology groups that differ from each other by only a few traits (Johnson and Ault, 1978; Johnson, 1980; Mays et al, 1982; Holderman et al, 1982). Correlating phenotypic test results with DNA homology groups enables investigators to select phenotypic tests that are required for the accurate identification of organisms belonging to these groups. In the current study, the DNA homology results match with the results of various phenotypic tests almost identical results for all isolates being obtained (Mochaba, pers. comm.).

The hybridisation between LC-1 and other microorganisms isolated from citrus leaves(Table 26) yielded lower homology values except for code 01 which is discussed above, suggesting marked differences between these isolates and LC-1. Code 9, isolated from greened material with a homology of 59% has reasonable homology although values under 70% are not considered to demonstrate a close relationship. The rest of the microorganisms isolated from citrus leaves had a lower homology value to LC-1 (Table 26).

Considering DNA homology together with morphological criteria, it appears that the isolates of the putative greening organism are unique and that other related bacterium are not found in healthy and infected citrus leaves from various sources.

However, the fact that one of the bacteria found in citrus have a reasonable DNA homology to the putative greening organism emphasises that any cloned fragment of LC-1 DNA considered as a potential diagnostic probe must be carefully checked for reaction to those bacteria.

The homology between LC-1 and the known phytopathogenic bacteria was low, none of them having a homology value greater than 30% (Table 25).

The homology with *Azotobacter*, a gram-negative, cyst former bacteria was also low and thus LC-1 appears to represent a unique bacterial group, whose classification is still to be achieved.

For this reason, further DNA characterisation of LC-1 was performed.

The properties of DNA which are important are the molecular weight, the presence or absence of plasmids and distinct restriction patterns.

The molecular weight of $2,8 \times 10^9$ daltons falls within the range of other gram-negative bacteria (Gillis et al, 1970) again supporting the criteria

that the organism can be considered to belong to this group despite its unique asymmetrical division process.

Although different restriction enzymes were used and different digestion solutions were utilised for each enzyme, a smear was obtained for all samples analysed on agarose gels. The DNA was obviously restricted but distinct patterns were not obtained. This suggests that LC-1 has many sites for the particular enzymes and that in this way there is no "unique" property of its DNA. Further studies to investigate the result using other restriction enzymes could be desirable.

The existence of plasmids was investigated using several methods, including those of Maniatis et al (1982).

Plasmids are known to import pathogenicity to some plant bacteria as well as unique biochemical properties. The case of the plant pathogen *Agrobacterium tumefaciens* is well studied. The bacterium carries a plasmid (Ti) which is transferred to the plant, inducing tumour production (Thomson, unpublisheddata). Further *Agrobacterium* sp. are known to contain other plasmids.

The techniques of Kado and Liu (1981) and Takahashi and Nagano (1984) being successfully used to release plasmids from *Agrobacterium* (Thomsom, unpub. data) were utilised by Babaya (1986) in an attempt to detect plasmids in LC-1. These studies yielded a negative result. Hence, in the current study other procedures were employed.

These methods are general procedures used to detect plasmids in a wide variety of bacteria. The fact that no plasmids were seen after DNA centrifugation on CsCl density gradient was used to detect unique plasmids, suggests that indeed plasmids were absent. However, the band

obtained on agarose gel electrophoresis (fig 15) suggested that DNA of a molecular weight of 1,5 x 10^6 daltons was present, which may be a large plasmid. Alternatively it could also be a form of the chromosomal DNA. Thus, although the existence of plasmids have not been firmly established they may be present. It is thus obvious that further plasmid analysis of these organisms needs to be undertaken. Furthermore, the presence of cryptic plasmids cannot be ruled out.

In conclusion, the experiments detailed herein describe procedures to prepare pure DNA from the putative greening organism. The mol% C+G content of the LC-1 isolate and other isolates is 59%, and the DNA homology between LC-1 and these isolates is high supporting the contention that these are all isolates of the pathogen.

This organism does not appear to be closely related to other phytopathogenic bacteria, although for one other bacteria isolated from infected citrus reasonable homology was apparent.

No confirmation of plasmids was obtained nor was a unique restriction pattern obtained using the three enzymes Eco R1, Hind III and Bgl II.

The properties of DNA detailed herein, together with other phenotypic tests should facilitate classification of the bacterium eventually.

Further, the results suggest that a DNA probe to facilitate detection of diseased material may be possibly but only if cognizance of the potential cross-reaction with other bacteria associated with citrus is taken.

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