MOLECULAR CHARACTERISATION AND DETECTION OF

Xanthomonas albilineans,

THE SUGARCANE LEAF SCALD PATHOGEN

by

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science to the University of Durban-Westville, Durban. It has not been submitted before for any degree or examination to any other University.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Xanthomonas albilineans, a xylem-invading bacterium, is the causative agent of leaf scald disease in sugarcane (*Saccharum* spp.). The disease is widespread and occurs in every major sugar-producing region of the world (Hughes, 1978; Ricaud and Ryan, 1989). Although the disease has been effectively controlled in most countries by cultivating sufficiently resistant sugarcane varieties, it still poses a serious threat to the sugarcane industry.

Severe losses were encountered in the noble canes (*Saccharum officinarum* L.) which were widely grown in the early years of this century (Ricaud and Ryan, 1989). The disease was brought under control by the replacement of the susceptible noble canes by resistant hybrid cultivars. This form of control is the only effective means of controlling the disease and has resulted in leaf scald disease losing its position as one of the most economically important sugarcane disease, in most countries, including South Africa. However, in these countries the disease continues to cause indirect losses such as: the discarding of promising but highly susceptible seedlings during selection trials; replacement of susceptible varieties which may be of considerable agricultural merit; and costs incurred in obtaining disease-free planting material and implementation of quarantine and other phytosanitary methods (Ricaud and Ryan, 1989).

Despite control by the use of resistant varieties, the serious threat posed by leaf scald disease is vindicated by the frequency of recent reports describing new outbreaks of the disease. In 1988, Akhtar *et al.* reported the first record of leaf scald in Pakistan and in 1993, Chen *et al.* reported the recurrence of the disease in Taiwan. In 1967, leaf scald disease was discovered in the United States Department of Agriculture World Collection of Sugarcane Clones, in Canal Point, Florida, which is an important international distribution centre (Hughes, 1978; Koike, 1992). Initially, the incidence was 5% to 6% of the total clones, but by 1990, the incidence of diseased clones had increased to 17%. Early in 1991, the disease was present in every commercial variety in Canal Point (Koike, 1992). In certain parts of Florida's sugarcane belt, the incidence of leaf scald had progressed from 0% in 1988 to 10% in 1991 (Koike, 1992). Davis *et al.* (1993) also reported that the incidence of leaf scald disease had increased in Florida, Guadeloupe and the Dominican Republic. The breakdown of resistance in some countries has been attributed to the introduction of new strains of the pathogen (Davis *et al.*, 1993; Egan, 1970). Various techniques have been used to demonstrate that variation in the leaf scald pathogen exists. However, genetic studies in *X. albilineans* are rare. In this study, molecular characterisation of geographical isolates of *X. albilineans* was performed in order to characterise these organisms further.

1.1 HISTORY AND DISTRIBUTION OF LEAF SCALD DISEASE

In the early 1920s, leaf scald disease was recognised as a bacterial vascular disease of sugarcane. This was due to investigations on the nature and cause of the disease by Wilbrink in Java, and North in Australia and Fiji (cited by Martin and Robinson, 1961; Ricaud and Ryan, 1989). The exact origin of the disease remains obscure. Early reports

on the occurrence of leaf scald disease suggest that the disease was present well before it was recognised as a separate disease. The earliest definite record of leaf scald was made in 1911 by North in Australia (Martin and Robinson, 1961). However, there is strong presumptive evidence that the disease occurred in New Guinea, Australia, Java and Fiji by 1900 (Martin and Robinson, 1961). In Java, the disease was known as "gomziekte" or "Java gum disease" for many years prior to 1920 when Wilbrink demonstrated that it was a separate disease to either sereh or gumming disease (Martin and Robinson, 1961). It was later discovered that the disease in Java was identical to Australian leaf scald disease following comparisons of the simultaneous studies of Wilbrink in Java and North in Australia and Fiji (North and Lee, 1924).

After being discovered in New Guinea, Australia, Java and Fiji prior to 1920, leaf scald disease spread to the Philippines in 1923 (Lee, 1923), Mauritius in 1928 and Hawaii in 1930 (Martin and Robinson, 1961). From these Indian Ocean and mid-Pacific Ocean areas, leaf scald disease spread to several South American countries (1940s - 1960s), to various Caribbean islands (1950s - 1960s), to North America (1960s), to most southern African countries and one west African country (1960s) and also in the 1960s to southern Asia (Egan, 1970). The spread of leaf scald into every major sugar-producing region in the world is illustrated by the fact that in 1950 leaf scald had been recorded in nine countries (Martin, 1950), by 1974 in 28 countries (International Society for Sugar-Cane Technologists [ISSCT] Standing Committee on Sugarcane Diseases, 1974) and by 1989 in 46 countries (Table 1.1) [Akhtar *et al.*, 1988; Davis *et al.*, 1993; Ricaud and Ryan, 1989]. The disease was first reported in South Africa in 1968 (Thomson, 1969) although it may have been present long before it was actually reported.

TABLE 1.1. Distribution of leaf scald disease in sugarcane-growing countries (Akhtar *et al.*, 1988; Davis *et al.*, 1993; Ricaud and Ryan, 1989)

Argentina	India	Réunion
Australia	Indochina	St. Lucia
Barbados	Indonesia	South Africa
Benin	Japan	Sri Lanka
Brazil	Kenya	Surinam
Burkina Faso	Malagasy R.	Swaziland
Burma	Malawi	Taiwan
Cameroon	Martinique	Tanzania
China	Mauritius	Thailand
Cuba	Morocco	Trinidad
Dominican R.	Mozambique	Uruguay
Fiji	Nigeria	U.S.A.
Ghana	Pakistan	Venezuela
Guadeloupe	Panama	Zimbabwe
Guyana	Philippines	
Hawaii	Peurto Rico	

1.2 LEAF SCALD SYMPTOMS

There are two distinct forms of leaf scald disease, namely the chronic phase and the acute

phase. Prior to the aetiology of leaf scald being established, the two phases were often regarded as separate diseases. The two phases sometimes occur independently, but more often changes from the chronic phase to the acute phase (Martin and Robinson, 1961). Infection can also result in a symptomless latent phase.

1.2.1 Chronic Phase

The symptom that is most characteristic of this phase is the presence on the leaf lamina of "white pencil-line" streaks along the length of the affected leaves (Fig. 1.1). These streaks are characteristic of the disease and are consequently used in disease diagnosis.

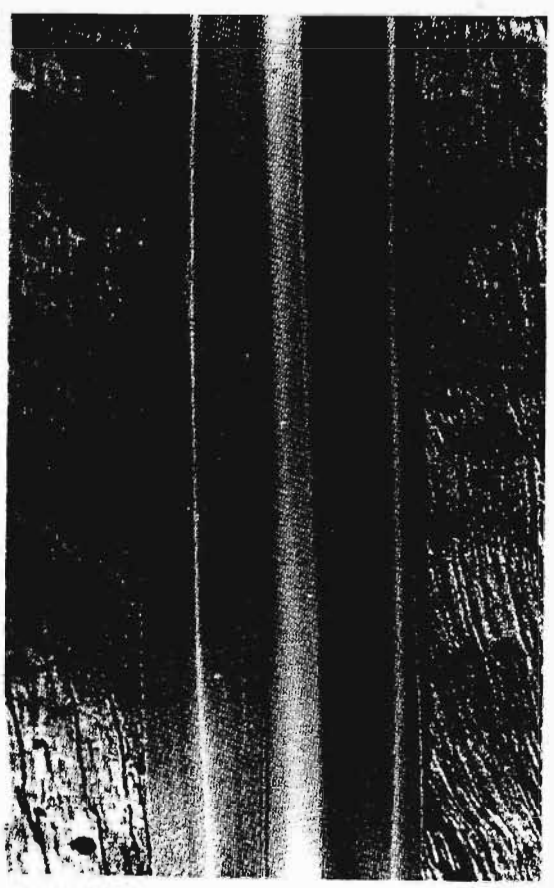

FIG. 1.1. Typical white pencil-line streaks along the leaf blade of infected sugarcane plants which is characteristic of leaf scald disease (Ricaud and Ryan, 1989).

The specific name of the pathogen is also derived from the appearance of these streaks (*albus* = white; *lineans* = lines). In young, newly attacked cane, the long narrow white stripes are the earliest symptom of infection. The white lines follow the direction of the main veins and often extend down into the leaf sheath where they may develop a purplish tinge. In resistant varieties, this is the only external symptom which may develop. As the infected leaves mature, the white lines tend to broaden and become more diffuse. This can lead to partial or complete chlorosis or whitening of the leaf blade. Broadening of the streak coincides with withering of the leaf tissue. The withering process commences at the tip of the leaf and proceeds along the line of the streak, giving a scalded appearance from which the name of the disease is derived. A prominent feature of the chronic form of leaf scald is the development of side shoots on mature infected stalks (Fig. 1.2). The

FIG. 1.2. Side shoot development on sugarcane stalks affected by leaf scald disease (left and right), compared to a healthy stalk (centre) [Martin and Robinson, 1961].

side shoots display all the symptoms which occur on the main shoots. The disease can also cause shoots to be stunted and wilted with the leaves becoming stiff and curling inwards at the tips (Fig. 1.3). Stalks affected in this way usually die and, in highly susceptible varieties, the whole stool may die. Another characteristic of leaf scald is the partial or complete aetiolation of leaves (Fig. 1.4).

FIG. 1.3. Infected sugarcane stalks showing wilted and inward curved leaves and side shoot development compared with a healthy stalk (extreme right) [Martin and Robinson, 1961].

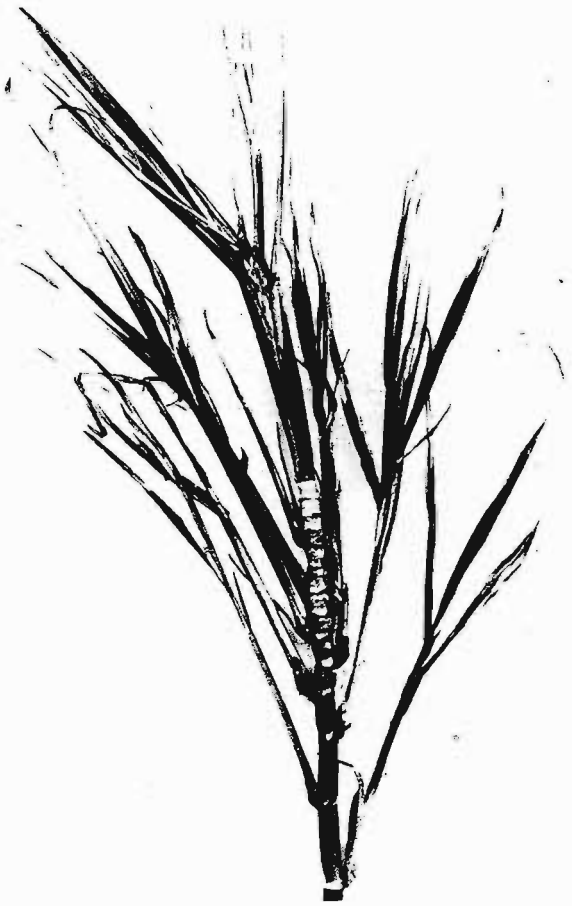

Fig. 1.4. Severely infected sugarcane stalk showing aetiolated leaves, premature drying and dying of leaf tips and leaves and side shoot development (Martin and Robinson, 1961).

Internal symptoms of chronically infected stalks are characterised by bright red streaks which are due to necrosis of the vascular bundles by the invading bacteria. The red streaks are more pronounced at the nodal regions, especially at the junction of the side shoots with the main stem. Badly diseased stalks may also develop lysigenous cavities.

1.2.2 **Acute Phase**

This phase is characterised by the sudden wilting and death of stalks often without

symptom expression. Entire shoots or one or two stalks within a stool may be affected. Whole fields may also be attacked in this manner, especially when prolonged dry weather is prevalent (Martin and Robinson, 1961). This phase is usually encountered when extremely susceptible varieties of sugarcane are cultivated.

1.2.3 **Latent Phase**

After ratooning or harvesting of infected setts, young shoots frequently exhibit chronic phase symptoms (Ricaud and Ryan, 1989). The affected shoots either die, continue to exhibit symptoms throughout the life of the plant or outgrow the disease and appear to recover. This "recovery" occurs more frequently in varieties that are tolerant to the disease and during favourable growth conditions (Ricaud and Ryan, 1989). However, pathogenic bacteria can be isolated from plants in which the disease is latent (Koike, 1992). Leaf scald symptoms may develop upon futher ratooning or at any stage of growth of plants originating from the infected setts. Both chronic and acute phase symptoms can result from a latent infection. The mechanism of latent infection is not yet fully understood (Ricaud and Ryan, 1989).

1.3 TRANSMISSION AND CONTROL

The disease is transmitted primarily by infected cuttings. The risk of spreading the disease between sugarcane-growing regions is greatest with tolerant varieties because they do not express symptoms if the disease is latent. Leaf scald can also be spread within a crop by

contaminated cane knives and mechanical harvesters (Taylor *et al.*, 1988). Other modes of transmission such as insects, rodents, wind-blown rain and through the soil have been proposed but strong evidence is lacking (Ricaud and Ryan, 1989).

The cultivation of varieties resistant to leaf scald disease is the most effective method of control. Resistant varieties of sugarcane are most often hybrids of *Saccharum officinarum* L. with *S. spontaneum* which has high resistance to leaf scald disease (Egan, 1971). An improvement of the thermotherapy technique developed by Steindl (1971) to obtain disease-free seed was also reported to be effective in controlling the disease (Ryan and Lopez, 1988). Other measures of control include disinfection of cane knives and blades of mechanical harvesters and strict quarantine procedures to avoid accidental or illegal introductions of the pathogen (Ricaud and Ryan, 1989).

1.4 ROLE OF ALBICIDIN

The factors involved in bacterial pathogenicity to plants are only partially understood. Pathogenicity determinants such as toxins, extracellular polysaccharides, pectolytic enzymes and plant growth substances have been implicated in the production of macroscopic disease symptoms, but this list is no doubt incomplete (Turner *et al.*, 1985). Other aspects of the disease process, such as the ability of the pathogen to evade recognition by the host, are still obscure. The phenomenon of specificity of the pathogen for a particular host or part of the host is still not understood.

Early light-microscope studies of infected tissue revealed that the pathogen, *X. albilineans* was present in leaf and stalk xylem elements, but not in chlorotic parenchyma cells adjacent to invaded vessels (Ricaud and Ryan, 1989). It was reported that chloroplasts were absent from the bundle sheath and surrounding parenchyma of the originally invaded bundle, whereas chloroplasts in the chlorotic zone did not disappear, but were reduced in size and disorganised. Orian (1942) postulated that the effect was due to a toxic metabolite of the pathogen acting on plastids before chlorophyll developed, and that development of a chlorotic zone around stripes on the leaf was due to slow disorganisation of plastids and consequent chlorophyll destruction.

In 1983, Birch and Patil examined infected tissue using electron microscopy and confirmed that *X. albilineans* was confined to xylem vessels during early disease development. The vessels often become blocked by the tightly packed bacteria. Chloroplasts were absent from cells surrounding invaded vessels in mature leaves, and from uninvaded white leaves emerging after invasion of sugarcane stalks by the pathogen. Proplastids, aetioplasts and vesicular forms but no mature or degenerating chloroplasts were present in white leaf areas. This evidence suggests that *X. albilineans* in invaded xylem may produce a diffusible phytotoxin which blocks chloroplast differentiation at the proplastid or aetioplast stages. Toxins produced by bacteria causing chlorosis in plants include: tabtoxin by *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *coronafaciens* (Engst and Shaw, 1992); coronatine by *P. syringae* pv. *tomato* and *P. syrinage* pv. *glycinea* (Ma *et al.*, 1991); phaseolotoxin by *P. syringae* pv. *phaseolicola* (Mitchell, 1976); tagetitoxin by *P. syringae* pv. *tagetis* (Jutte and Durbin, 1979); syringomycin by *P. syringae* pv. *syringae* (Surico and De Vay, 1982) and rhizobitoxine by *Rhizobium japonicum* (Johnson *et al.*, 1959).

Subsequent investigations (Birch and Patil, 1985; 1987a; 1987b) have verified the existence of a phytotoxin that is responsible for the symptoms of leaf scald disease in sugarcane. Chlorosis-inducing isolates of *X. albilineans* were found to produce a family of antimicrobial compounds *in vitro*. The major purified antimicrobial compound (albicidin) is bactericidal to *Escherichia coli*, causing a rapid and complete block to DNA synthesis, followed by partial inhibition of RNA and protein synthesis. Selective inhibition of DNA synthesis without evidence of DNA binding suggested a specific interaction of albicidin with an essential replication protein. Because chloroplasts employ prokaryote mechanisms of DNA replication, transcription and translation, it seemed possible that albicidin or a structural analogue produced in diseased plants might be involved in blocking chloroplast differentiation which characterises sugarcane leaf scald chlorosis. Partial characterisation of albicidin revealed that the compound is non-proteinaceous, has several aromatic rings and contains approximately thirty eight carbon atoms. Prior to these investigations, antimicrobial agents had not been reported for the genus *Xanthomonas* and the combined biological and chemical properties of albicidin do not appear to closely resemble those of any known antibiotic. The similar chromatographic behaviour of all components of the antimicrobial mixture produced in culture by *X. albilineans* suggested that these components, including albicidin, comprised a family of structurally related compounds as is commonly observed for secondary metabolites (Birch and Patil, 1985).

A study with mutants and revertants in albicidin production showed a close correlation between albicidin production and the ability to cause chlorosis (Birch and Patil, 1987a). Mutants which failed to inhibit *E. coli* in culture lacked the ability to induce leaf scald symptoms in inoculated sugarcane, in contrast to inhibitor-producing parent strains. In

order to further implicate albicidin in chlorosis induction, its effects on isolated plastids, tissue cultures and intact sugarcane plants were examined (Birch and Patil, 1987b). It was found that in isolated sugarcane proplastids, inhibition of DNA replication is a primary action of albicidin. Albicidin also caused permanent white chlorosis of shoots re-differentiating from treated callus but did not cause chlorosis when injected into the spindles of intact sugarcane plants. In addition, albicidin was isolated from white leaf stripes surrounding invaded xylem tissue in diseased plants. These results coupled with earlier ultrastructural and mutant studies strongly support the hypothesis that a member of the albicidin family of secondary metabolites produced by *X. albilineans* in invaded xylem causes chlorosis in sugarcane leaf scald disease by preferentially inhibiting plastid DNA replication, resulting in blocked chloroplast differentiation.

1.5 THE CAUSAL ORGANISM

Although Wilbrink and North (cited by Martin and Robinson, 1961) independently discovered that leaf scald disease was caused by a short, rod-shaped bacterium and published detailed descriptions of the organism, they did not name the organism because they could not stain the flagellum. Ashby (1929) found the organism to be actively motile by means of a single polar flagellum and proposed the name *Bacterium albilineans*, signifying a bacterium producing white lines which is a characteristic of the disease. The organism became known as *Phytomonas albilineans* when certain plant pathogens were placed in the genus *Phytomonas* (Martin and Robinson, 1961). After Dowson (1943) proposed the genus *Xanthomonas* for all yellow pigmented plant pathogenic bacteria with

a single polar flagellum, the leaf scald organism retained the name *X. albilineans*.

X. albilineans is a gram-negative rod, 0.25 to >0.3 μm in diameter by 0.6 to >1.5 μm in length. The yellow colour of the colonies are due to the presence of unique, brominated aryl-polyene pigments called xanthomonadins which are characteristic of all xanthomonads. Xanthomonadins have been shown to play a role in protecting *X. campestris* pv. *juglandis* against photobiological damage (Jenkins and Starr, 1982). *X. albilineans* is the only member of the genus *Xanthomonas* that has non-mucoid colonies. The bacterium grows optimally between 25 to 30°C, and at a maximum of 37°C. Diagnostic biochemical characteristics include: strict aerobic growth; hydrolysis of Tween 60 and esculin; growth in milk positive but proteolysis negative; no growth with ammonium salts, nitrates or asparagine as sources of nitrogen; nitrite not formed from nitrate; produces invertase, gelatinase and ß-glucosidase but not urease, and requires L-methionine and L-cysteine for growth (Ricaud and Ryan, 1989; Van den Mooter and Swings, 1990). The bacterium is best isolated and cultured on Wilbrink's agar (Ricaud and Ryan, 1989).

Apart from sugarcane, *X. albilineans* also naturally infects a few other members of the family Graminae. Typical leaf scald symptoms occur in these alternative hosts which includes *Brachiaria piligera* (F. Muell) Hughes, *Paspalum dilatatum* Poir., *P. conjugatum* Berg. (sour grass), *Imperata cylindrica* (L.) P. Beauv. var. *major* (Nees) C. E. Hubbard (blady grass) and *Zea mays* L. (maize). Leaf scald symptoms are also produced when *X. albilineans* is inoculated into artificial hosts e.g., *Coix lacryma-Jobi* L. (Job's tears), *Sorghum verticilliflorum* (Steud.) Stapf. (wild sorghum), *Bambusa vulgaris* Schrad. (tall

bamboo) and *Pennisetum purpureum* Schum. (elephant grass) [Martin and Robinson, 1961; Ricaud and Ryan, 1989].

1.6 VARIATION AMONG *X. albilineans* ISOLATES

Egan (1969; 1970) postulated that strains of the pathogen existed. Evidence for the existence of strains of *X. albilineans* is not concrete and is based largely on the differences observed in the severity of leaf scald and varietal reactions within and between countries. It is assumed that pathotypes of *X. albilineans* are responsible for different reactions against a particular variety in different geographic regions. Antoine and Pérombelon (1964) reported the occurrence of a new strain of the pathogen in Mauritius when the previously highly resistant varieties M147/44 and M202/46 became susceptible to leaf scald disease. Furthermore, the variety B34104 is resistant to both Mauritian strains but is highly susceptible in Guyana, while M147/44 is resistant in Guyana and to the old Mauritian strain. The variety Pindar was reported to be highly resistant in Australia, but moderately susceptible in Guyana and highly susceptible in Zimbabwe (Egan, 1969).

Baudin and Rott (1984 - cited by Ricaud and Ryan, 1989) studied 28 *X. albilineans* isolates from 11 countries using cultural, biochemical and physiological characteristics, bacteriophage typing and serological typing. They found little variation in cultural, biochemical and physiological characteristics among the isolates and could not justify their grouping into different biotypes. However, they separated the isolates into sero-groups and lysogenic-groups. Isolates from Mauritius and Réunion showed little variability and

belonged to a sero-group that was widely distributed. Isolates from tropical Africa (Cameroon, Burkina Faso and Kenya) also showed little variation, but they differed from other groups serologically and in lysogenic properties. However, there was no correlation between sero- and lyso-groups and virulence (Rott, 1984 - cited by Ricaud and Ryan, 1989). In 1984, it was concluded at an ISSCT workshop that while variation does exist in *X. albilineans*, there was no evidence for the existence of variety-specific races or pathovars (Ricaud and Ryan, 1989). A further study of the variability of 28 *X. albilineans* isolates by Rott *et al.* (1986) led to the grouping of the isolates into 3 serovars and 6 lysovars. A correlation between the serovars and lysovars was evident. Four isolates from tropical Africa formed a separate serovar and lysovar as did two isolates from the Caribbean zone. Isolates from different geographic origins belonging to 4 separate lysovar groups formed the third serovar. Two strains belonging to different serovars and lysovars were subsequently shown to react like pathotypes on *in vitro* sugarcane plantlets (Rott and Chagvardieff, 1987).

1.7 MOLECULAR CHARACTERISATION OF PLANT PATHOGENIC BACTERIA

Several molecular approaches have been used to compare and identify plant pathogenic bacteria. The most widely used techniques are serology, plasmid profile analysis, DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis, and DNA-DNA hybridisation. Other methods used to differentiate between plant pathogens include protein electrophoretic patterns (Van Zyl and Steyn, 1990; Vauterin *et al.*, 1991), gas

chromatography of fatty acids (Graham *et al.*, 1990; Stead *et al.*, 1992) and phage typing (Liew and Alvarez, 1981; Rott *et al.*, 1986).

1.7.1 Serology

The use of serology for identifying bacteria is almost as old as the science of plant pathology itself (Schaad, 1979). Serological techniques are currently the most widely used molecular biology tools for the identification of plant pathogens. The two most sensitive and accurate serological tests used are enzyme-linked immunosorbent assays (ELISA) [Alvarez and Lou, 1985; Civerolo and Fan, 1982; Nome *et al.*, 1980] and immunofluorescence tests (Brlansky *et al.*, 1982; Malin, *et al.*, 1983; Schaad, 1978). These tests were made more specific with the use of monoclonal antibodies instead of conventional (polyclonal) antibodies (Lin and Chen, 1986; Lin *et al.*, 1987; Permar and Gottwald, 1989).

The occurrence of a symptomless latent phase in sugarcane infected with *X. albilineans* has resulted in attempts to develop sensitive, specific and rapid techniques to detect the causal organism. In 1976, Léoville and Coleno (cited by Ricaud and Ryan, 1989; Ricaud *et al.*, 1978) used an immunofluorescent technique to detect *X. albilineans* in sugarcane stalks. The technique, which included an enrichment step, gave positive results with all diseased stalks showing symptoms. In addition, *X. albilineans* was detected in symptomless stalks, indicating that the technique could diagnose latent infections. Chatenet (1985 - cited by Ricaud and Ryan, 1989) used the technique on stalk juice without the

enrichment procedure to confirm the disease on stalks showing symptoms as well as on symptomless stalks. A simple and rapid micro-agglutination technique was developed by Ricaud *et al.* (1978) which proved reliable in plants showing doubtful symptoms. The technique was specific for *X. albilineans* and did not react with extracts from plants infected with gumming disease or chlorotic streak, both of which cause symptoms which can be confused with leaf scald disease. However, the technique was not sensitive enough to detect latent infection. In Australia, ELISA has been used successfully to detect *X. albilineans* in vascular extracts of leaves and stalks (Anon, 1980; 1981 - cited by Ricaud and Ryan, 1989). The sensitivities of various techniques have been reported using pure cultures of *X. albilineans*. Inderjit *et al.* (1991) compared indirect immunofluorescence, bacterial agglutination and microcapillary haemagglutination and found immunofluorescence to be the most sensitive technique. The highest sensitivity recorded was 10^4 cells/ml for the type strain of *X. albilineans*, using a polyclonal antiserum. Pillay *et al.* (1992) optimised an ELISA for *X. albilineans* and also reported a detection limit of 10^4 cells/ml for five isolates from different geographical regions.

Schaad (1978) compared direct and indirect immunofluorescence for the detection of *X. campestris* pathovars and found that indirect immunofluorescence was superior on the basis of reliability, simplicity and sensitivity. The sensitivity of the indirect immunofluorescence technique was illustrated by Malin *et al.* (1983) who reported the detection of 10^2 cells/ml of *X. campestris* pv. *phaseoli* in mixed populations containing 10^8 cells/ml of common bean seed bacteria. The sensitivity of ELISAs developed for plant pathogenic bacteria ranges from 10^3-10^6 cells/ml. Double-antibody sandwich ELISAs (Alvarez and Lou, 1985; Civerolo and Fan, 1982) and dot-ELISAs (Anderson and

Nameth, 1990; Lazarovits *et al.*, 1987) have been reported to increase sensitivity.

1.7.2 Plasmids in Plant Pathogenic Bacteria

The presence of one or more plasmids in plant pathogenic bacteria appears to be the rule rather than the exception (Panopoulos and Peet, 1985). Nearly all economically important plant pathogens have been reported to harbour plasmids (Table 1.2). Plant pathogens are not unusual in the size or number of plasmids they harbour, with a few exceptions. The vast majority of the plasmids in these bacterial species are still genetically cryptic, i. e., with no known function.

The discovery of virulence plasmids in *Agrobacterium tumefaciens* (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975) led to the search for virulence plasmids in a variety of pathogens. Early attempts to study plasmid genes involved either elimination of the plasmid or transfer to a suitable host. Non-specific mutations caused by curing agents, transfer of unrelated plasmids and the lack of selectable markers impeded this line of research. Current techniques used in the study of bacterial genes include cloning of DNA fragments and transposon mutagenesis. These techniques have shown that for many bacterial pathogens the genes coding for the synthesis of toxins, degradative enzymes, extracellular polysaccharides and those involved in pathogenicity and host range are typically located on the bacterial chromosome (Denny and Baek, 1991; Dow *et al.*, 1987; Salch and Shaw, 1988; Turner *et al.*, 1985). Nevertheless, a number of important

TABLE 1.2. Plant pathogenic bacteria that contain plasmids (Coplin, 1989; Mazarei and Kerr, 1991; Xu and Gonzalez, 1991)

Agrobacterium	*Pseudomonas*	*Xanthomonas*
radiobacter	*cepacea*	*campestris* pv.
rhizogenes	*glumae*	*campestris*
tumefaciens	*solanacearum*	*citri*
	syringae pv.	*cyamopsidis*
Clavibacter	*angulata*	*dieffenbachiae*
michiganense subsp.	*atropurpurea*	*glycines*
insidiosum	*coronafaciens*	*hederae*
michiganense	*glycinea*	*malvacearum*
nebraskense	*lachrymans*	*manihotis*
sepedonicum	*papulans*	*oryzae*
tritici	*phaseolicola*	*pelargonii*
rathayi	*pisi*	*phaseoli*
	savastanoi	*pruni*
Curtobacter	*striafaciens*	*vesicatoria*
flaccumfaciens pv.	*syringae*	*vignicola*
oorti	*tabaci*	*vitians*
poinsettiae	*tomato*	
Erwinia	*Rhodococcus*	
amylovora	*fascians*	
chrysanthemi pv. *zeae*		
carotovora subsp. *carotovora*	*Spiroplasma*	
herbicola	*citri*	
stewartii		
uredovora		

virulence plasmids have been discovered and it appears that plasmids contribute significantly to the ecological fitness of plant pathogenic bacteria.

Plasmids which contribute to the ecological fitness of plant pathogenic bacteria are especially important when the bacteria are not present inside their host plants. Plasmid-borne traits such as bacteriocin production (Gantotti *et al.*, 1981; Slota and Farrand, 1982) and nutritional capabilities (Boominathan and Mahadevan, 1987; Gallie *et al.*, 1984) can enhance their ability in colonisation of new niches and competition with other bacteria, whereas other plasmid-borne traits such as pigmentation (Tuveson *et al.*, 1988) and resistance to antibiotics (Chiou and Jones, 1991; Minsavage *et al.*, 1990) and heavy metals (Desomer *et al.*, 1988; Sundin *et al.*, 1989) should increase their chances of survival in adverse environments.

The tumour-inducing (Ti) plasmids of *A. tumefaciens* are the most characterised virulence plasmids present in plant pathogenic bacteria. Tumour induction results from the production of auxins, cytokinins and indoleacetic acid which are encoded by a segment of the Ti plasmid (T-DNA) that stably integrates into the plant chromosome. T-DNA transfer and integration is controlled by six plasmid-encoded *vir* genes. Additional functions attributed to Ti plasmids include determination of host range, production of certain periplasmic proteins and bacteriocin sensitivity (Coplin, 1989). Other virulence plasmids include pPT23A, a 101 kb plasmid present in *Pseudomonas syringae* pv. *tomato*, which is responsible for the production of the chlorosis-inducing phytotoxin coronatine (Bender *et al.*, 1989). Plasmids present in *P. syringae* pv. *pisi* were implicated in pathogenicity as pathogenic strains contained 2-4 plasmids compared to non-pathogenic

strains which contained none (Mazarei and Kerr, 1991). Characterisation of multiple cryptic plasmids in *Erwinia stewartii* revealed that avirulent strains tended to have fewer plasmids than virulent strains (Coplin *et al.*, 1981).

Plasmids are also responsible for host range and race/cultivar specificity of some bacterial pathogens. Since bacterial phytopathogens do not produce host-specific toxins, the reason for susceptibility of plants to some pathogens and resistance to others is not well understood. It is known however, that host range in pathogens and disease resistance in plants depend on the genotype of both the pathogen and the plant (Coplin, 1989). A plant will be resistant to a given pathogen if it carries a dominant resistance gene that interacts with a dominant "avirulence" (*avr*) gene in the pathogen. This type of relationship between plant and microbe is termed a "gene-for-gene" relationship (Coplin, 1989). Avirulence genes are probably involved in the recognition of pathogens by plants and only indirectly with pathogenicity (Coplin, 1989). Six avirulence genes have been reported to be clustered on a 90 kb plasmid in *X. campestris* pv. *malvacearum* (De Feyter and Gabriel, 1991). Avirulence loci are also located on plasmids in *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea* (Coplin, 1989) and on a 193 kb plasmid in *X. campestris* pv. *vesicatoria* (Stall *et al.*, 1986). Other genes however, appear to be required for both pathogenicity on susceptible hosts and induction of a hypersensitive response on resistant cultivars or non-hosts. These genes are necessary for the expression of an avirulence phenotype and have been termed *hrp* (*h*ost *r*ange and *p*athogenicity) genes. *Hrp* genes are normally chromosomal (Bauer and Beer, 1991; Daniels *et al.*, 1988) but are present on a megaplasmid in *P. solanacearum* (Boucher *et al.*, 1986).

The stability and ubiquity of plasmids in plant pathogenic bacteria has led to their use as an alternative method in the identification and differentiation of pathogens. Highly conserved plasmids or plasmid DNA sequences are frequently observed, possibly as a result of coevolution with their hosts to the point that they are stable and characteristic of a particular taxonomic group. An example of such a conserved plasmid is pCS1, a 50 kb plasmid present in *Clavibacter michiganense* subsp. *sepedonicum*. Clark and Lawrence (1986) studied 13 isolates from different countries and found the plasmid in 11 isolates. Mogen *et al.* (1988) extended this work to include 49 strains, 23 of which contained the plasmid. Plasmid DNA probes revealed that all but one of the remaining 26 strains harboured the plasmid integrated in the host chromosome. Another conserved plasmid, pEA28, was reported to be present in all strains of *Erwinia amylovora* isolated from various hosts and areas (Falkenstein *et al.*, 1989; Laurent *et al.*, 1989). Common plasmids also occur in a variety of other plant pathogens (Coplin *et al.*, 1981; Gross *et al.*, 1979; Lazo and Gabriel, 1987).

1.7.3 DNA Fingerprinting and RFLP Analysis

DNA fingerprinting and RFLP analysis using DNA probes have been successfully used to identify and differentiate between closely related strains of plant pathogenic bacteria. *X. campestris* comprises 125 pathovars based on pathogenicity to a particular host. Lazo *et al.* (1987) reported that pathovars of *X. campestris* can be distinguished by RFLP analysis. Hartung and Civerolo (1989) used statistical analysis of RFLP data to show that *X. campestris* isolates from Florida citrus nurseries formed a heterogenous group and were

not strains of *X. campestris* pv. *citri*. RFLP probes were also used to distinguish between more aggressive and less aggressive Florida citrus strains (Gottwald *et al.*, 1989; Hartung and Civerolo, 1991). Cook *et al.* (1989) used RFLP analysis involving DNA probes that specify virulence and the hypersensitive response, to study 62 strains of *P. solanacearum*, representing 3 races and 5 biovars. Statistical analysis revealed 2 major divisions which were not indicated by other means of classification. The technique was also used to predict accurately the race of 2 incorrectly classified strains. It was concluded that a revised classification of the species, based on RFLP analysis, was possible. Kuske *et al.* (1991) proposed that the taxonomy of mycoplasma-like organisms (MLO) be based on RFLP analysis, i.e., the pathogens' genotype, rather than phenotypic characteristics of the MLO-plant interaction. They argued that this would help to differentiate the hundreds of plant pathogenic MLO diseases described to date, and provide a basis for rapid identification of unknown MLO isolates.

DNA fingerprinting of plasmid DNA and bacterial genomes is a more direct approach of comparing bacteria than RFLP analysis using DNA probes. Lazo and Gabriel (1987) examined 117 different strains of *X. campestris*, representing 26 different pathovars, for plasmid content and plasmid DNA profiles and found that in most cases, strains of *X. campestris* that contained plasmids could be differentiated at the pathovar level on the basis of their characteristic plasmid profiles. DNA fingerprinting of the conserved plasmid pCS1, present in 48 out of the 49 strains of *C. michiganense* subsp. *sepedonicum* studied (Mogen *et al.*, 1988) revealed that the plasmid was identical in all strains, indicating that it could be used in identification of the bacterium, which was later confirmed (Mogen *et al.*, 1990). Fingerprinting of genomic DNA by pulsed field gel electrophoresis (PFGE)

has proved to be a powerful tool in comparing relatedness of plant pathogenic bacteria. Cooksey and Graham (1989) used the technique to show differences and relatedness among strains of *P. syringae* pv. *tomato* and differences among pathovars of *P. syringae*. Similar results were observed with strains of *X. campestris* pv. *vesicatoria* and pathovars of *X. campestris*. Grothues and Rudolph (1991) used the technique to differentiate between various species, pathovars and different strains within the same pathovar of phytopathogenic pseudomonads and concluded that genomic fingerprints are adequate means to identify strains and reveal information about relatedness of bacteria.

1.7.4 DNA-DNA Hybridisation

Nucleic acid hybridisation, which depends on the high degree of specificity inherent in the pairing of nucleotide bases, is a well established tool in molecular biology. As a result of this specificity, the technique is being applied for diagnostic purposes, including the detection of plant pathogens (Miller and Martin, 1988). An example of such an application is the plasmid DNA probe developed by Gilbertson *et al.* (1989) for the detection of the causal agents of common bacterial blight of bean, *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*. They reported rapid and specific detection of the pathogen in bean leaves. The detection limit of the technique was 10^3 colony-forming units. Paterson and Jones (1991) reported a detection limit of 2.2×10^4 colony-forming units/ml of *P. syringae* pv. *morsprunorum* using a DNA probe from *P. syringae* pv. *tomato*. However, the probe cross-reacted with other pathovars of *P. syringae* and was not able to detect the bacterium in leaf lesions. A DNA probe carrying

genes involved in phaseolotoxin production by *P. syringae* pv. *phaseolicola* was used to detect and identify the bacterium in pure and mixed cultures, seed-soak liquids and diseased plants (Schaad *et al.* 1989). This probe proved to be highly specific and was useful for the rapid identification of the pathogen. Other DNA probes have also been shown to be useful in the specific detection of plant pathogens (Bertaccini *et al.* 1990; Davis et al., 1992; Thompson *et al.* 1989).

1.8 SCOPE OF THE PRESENT STUDY

The occurrence of strains of *X. albilineans* was first suggested by Egan (1969; 1970) when previously highly resistant sugarcane varieties showed susceptibility to leaf scald disease. However, characterisation of *X. albilineans* isolates using traditional techniques, such as comparisons of morphological, biochemical and physiological characteristics, have shown that they form a homogenous group (Rott *et al.*, 1986). In this study, characterisation of *X. albilineans* isolates from South Africa, Mauritius, Australia and Fiji, using molecular techniques, was attempted in order to investigate genetic variation in this pathogen. Plasmid characterisation was attempted in order to determine its role in the South African isolate. The potential of a DNA probe, useful for the detection of the South African isolate, was also investigated. Genetic variation among *X. albilineans* isolates was examined using DNA fingerprinting techniques. It was hoped that such characterisation would provide further evidence for the existence of sub-groups of *X. albilineans*.

CHAPTER TWO

CHARACTERISATION OF A PLASMID FROM THE SOUTH AFRICAN ISOLATE OF *X. albilineans*

2.1 INTRODUCTION

Plasmids are widespread among all genera of plant pathogenic bacteria (Coplin, 1989). They are well known for playing a role in the ecological fitness of their hosts by conferring an advantageous trait in certain environments. Plasmids code for a variety of phenotypes in plant pathogenic bacteria which include : (a) virulence and pathogenicity (Chilton *et al.*, 1982; Gonzalez *et al.*, 1984; Mazarei and Kerr, 1991; Watson *et al.*, 1975); (b) avirulence and host range (De Feyter and Gabriel, 1991; Knauf *et al.*, 1982; Stall *et al.*, 1986); (c) production of phytotoxins (Bender *et al.*, 1989); (d) bacteriocin production (Ellis *et al.*, 1979); (e) production of plant growth hormones (Comai and Kosuge, 1980); (f) resistance to antibiotics and heavy metals (Chiou and Jones, 1991; Desomer *et al.*, 1988; Hendrick *et al.*, 1984; Minsavage *et al.*, 1990; Stall *et al.*, 1986; Sundin *et al.*, 1989); (g) metabolic traits (Boominathan and Mahadevan, 1987; Coplin, 1989; Laurent *et al.*, 1989) and (h) pigment production (Gantotti and Beer, 1982; Thiry, 1984). However, the majority of plasmids found in plant pathogens are still genetically cryptic, i.e., with no known function (Coplin, 1989).

The study of plasmids as accessory genetic elements in plant pathogenic bacteria has been

concentrated mainly in : (a) detection and characterisation of various strains, pathovars and species; (b) search of plasmid associated traits; (c) interstrain transmission and host range studies; (d) development of tools for *in vivo* and *in vitro* genetic analysis (conjugative transfer methods, cloning vectors and suicide plasmid systems) and (e) plant transformation systems. This chapter describes the study of a plasmid present in a South African isolate of *X. albilineans* and focuses on the first two of the afore-mentioned areas.

Apart from *X. albilineans* and the numerous pathovars of *X. campestris*, plasmids have not been reported from any of the other six species proposed for the genus *Xanthomonas* (Van den Mooter and Swings, 1990). Previous reports (Birch and Patil, 1987a; Lazo and Gabriel, 1987) failed to demonstrate the presence of plasmid DNA in *X. albilineans*. However, Singh and Pillay (1990) provided evidence for plasmids in five geographical isolates of *X. albilineans* using an alkaline lysis plasmid minipreparation method and agarose gel electrophoresis of isolated DNA. They observed a DNA band common to all five isolates and an additional band in a Mauritian and a South African isolate. Using a modification of the plasmid isolation procedure of Birnboim and Doly (1979), on the same five isolates of *X. albilineans*, Permaul *et al.* (1991) observed a common DNA band in all five isolates and one or two additional bands present only in the South African isolate. Restriction analysis revealed that only DNA from the South African isolate had restriction profiles that were characteristic of plasmid DNA. The size of the plasmid in the South African isolate was estimated to be 24.2 kb. This plasmid did not hybridise to DNA from the other four isolates. However, due to the similar electrophoretic pattern observed for uncleaved DNA from all five isolates, it was assumed that each isolate possessed a plasmid.

The initial objective of this chapter was to confirm whether four of the five geographical isolates used in this study viz., an Australian isolate, two Mauritian isolates and the type strain from Fiji, harbour plasmid DNA. The presence of stable plasmids provides a natural genetic marker that may have a taxonomic significance in differentiating these pathogens. The rest of this chapter is devoted to the physical and genetic characterisation of the plasmid present in the South African isolate of *X. albilineans*, designated pXA1. Physical characterisation of pXA1 involves identification of the two forms of the plasmid observed on agarose gels and construction of a restriction map depicting cleavage sites of various restriction endonucleases. Genetic characterisation involves the cloning of restriction fragments of the plasmid into *Escherichia coli* and assaying for gene expression in order to identify plasmid-borne genes.

2.2 MATERIALS AND METHODS

2.2.1 Growth and Maintenance of Bacterial Cultures

Bacterial isolates used in this study are listed in Table 2.1. *X. albilineans* isolates were positively identified previously (Seetal, 1989). They were grown at 28°C in modified Wilbrink's medium (20 g sucrose, 5 g peptone, 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$ and 0.05 g Na_2SO_3 per litre) for 1-2 days in broth and 3-4 days on agar plates. Cultures on agar plates were kept at 4°C and subcultured every 4-6 weeks. *E. coli* was grown at 37°C on nutrient agar (Biolab) and YT medium (8 g tryptone, 5 g yeast extract and 5 g NaCl per litre) and subcultured on YT agar plates at 4 week intervals. *E. coli* cells harbouring

TABLE 2.1. Bacterial isolates used in this study

Isolate	Geographic origin/ Host	Source[a]/ Reference
X. albilineans		
2901	Mauritius (Belle Rive)	MSIRI
2905	Mauritius (Medine)	MSIRI
LS155	Australia	BSES
PDDCC 196^T (= NCPPB 2969^T = LMG 494^T)[b]	Fiji	PDDCC
XA 86-1	South Africa	UDW
X. axonopodis	*Axonopus scoparius*	PPRI
X. campestris pv.		
malvacearum	*Gossypium* sp.	PPRI
pruni	*Prunus salicina*	PPRI
X. fragariae	*Fragaria ananassa*	PPRI
X. maltophilia[c]	Free-living	UDW
E. coli DH5αF'	-	BRL
E. coli HB101	-	Maniatis *et al.* (1982)

[a] BRL, Bethesda Research Laboratories, USA; BSES, Bureau of Sugar Experimental Stations, Queensland, Australia; LMG, Laboratorium Mikrobiologie, Ghent, Belgium; MSIRI, Mauritius Sugar Industry Research Institute; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; PDDCC, Plant Diseases Division Culture Collection, Auckland, New Zealand; PPRI, Plant Protection Research Institute, Stellenbosch, South Africa; UDW, Department of Microbiology, University of Durban-Westville, South Africa.

[b] T = type strain.

[c] Renamed *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993).

the cloning vector, pTZ19R and recombinant plasmids were grown on X-gal plates (YT medium supplemented with 50 μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside [X-gal], 1 mM isopropyl-ß-D-thiogalactopyranoside [IPTG] and 50 μg/ml ampicillin). Sucrose utilisation was tested on M9 minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl per litre, pH 7.4) containing 0.2% sucrose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$. *X. axonopodis, X. campestris* pv. *malvacearum, X. fragariae* and *X. maltophilia* were grown in peptone-glycerol medium (20 g peptone, 10 ml glycerol and 1.5 g K_2HPO_4 per litre) at 28°C and *X. campestris* pv. *pruni* was grown in BHI broth (Biolab) at 28°C. Long-term storage of bacterial cultures involved freezing of exponential phase broth cultures, supplemented with 15% glycerol, at -20°C and -70°C.

2.2.2 Screening for Plasmids in *X. albilineans*

The Qiagen Plasmid Kit was used to isolate plasmid DNA from the five isolates of *X. albilineans*. The procedure combines the alkaline lysis method of Birnboim and Doly (1979) with plasmid purification on a Qiagen anion exchange column.

Buffer P1: 100 μg/ml RNase A
50 mM Tris.HCl
10 mM EDTA
pH 8.0

Buffer P2: 200 mM NaOH
1% SDS

Buffer P3:	3.0 M potassium acetate, pH 5.5
Buffer QBT:	750 mM NaCl
	50 mM MOPS
	15% ethanol
	0.15% Triton X-100,
	pH 7.0
Buffer QC:	1.0 M NaCl
	50 mM MOPS
	15% ethanol
	pH 7.0
Buffer QF:	1.25 M NaCl
	50 mM Tris.HCl
	15% ethanol
	pH 8.5
TE:	10 mM Tris.HCl
	1 mM EDTA
	pH 8.0
TBE (5×):	45 mM Tris base
	45 mM boric acid
	1 mM EDTA
Gel loading buffer (6×):	40% sucrose
	0.25% bromophenol blue

The five isolates of *X. albilineans* were each inoculated into 10 ml of Wilbrink's broth and

grown to saturation at 28°C in an orbital shaker (Braun, Certomat U). 1.25 ml of culture of each isolate was pelleted twice in four 1.5 ml Eppendorf tubes for 1 min in an Eppendorf microcentrifuge at 15 800 × *g* and resuspended in 0.4 ml buffer P1. 0.4 ml of buffer P2 was added and the contents of the tubes were mixed gently by inversion and incubated at room temperature for 5 min. This step caused lysis of the bacterial cells with SDS, alkaline (NaOH) denaturation of plasmid and chromosomal DNA and degradation of RNA by RNase A. Denatured chromosomal DNA and cellular proteins were precipitated by addition of 0.4 ml buffer P3 and incubation on ice for 10 min. Precipitated material was pelleted at 15 800 × *g* for 45 min and the supernatant, containing plasmid DNA, promptly removed. Five Qiagen-tip 20 columns were equilibrated by applying 1 ml buffer QBT and allowing the columns to empty by gravity flow. Plasmid-containing supernatants were applied onto the columns and allowed to enter the silica gel resin by gravity flow. Each column was washed with 4 × 1 ml aliquots of buffer QC to remove contaminants. Bound DNA was eluted with 0.8 ml buffer QF and collected in Eppendorf tubes. DNA was precipitated with 0.7 volumes of isopropanol at room temperature and pelleted at 15 800 × *g* for 30 min. The supernatant was poured off and the pellets washed with 70% ethanol, vacuum dried (Speedvac Concentrator, Savant Instruments Inc.) for 10 min and dissolved in 20 μl of TE.

Horizontal agarose gel electrophoresis was used to analyse uncleaved plasmid DNA preparations and preparations cleaved with the restriction endonuclease *Eco*RI. Uncleaved DNA preparations in 1 × gel loading buffer were loaded in 0.7% agarose gels and electrophoresed at a constant voltage of 7.5 V/cm for 1.5 h in 0.5 × TBE. The plasmid preparation from each isolate was digested with 5 U of *Eco*RI for 1 h at 37°C. DNA

digests together with a DNA marker (DNA Molecular Weight Marker IV, Boehringer Mannheim) were loaded in 0.7% agarose gels and electrophoresed at 3 V/cm for 4 h in TBE buffer. Following electrophoresis, gels were stained with 2 μg/ml ethidium bromide for 15 min. The fluorescence of nucleic acid-containing bands was visualised with a UV transilluminator (UVP, Inc.). Gels were photographed, using Ilford FP4 Plus film, with a Minolta camera fitted with a UV and a Vivitar No. 25 (red) filter.

2.2.3 Isolation of Plasmid DNA from XA 86-1

Plasmid DNA from XA 86-1 (pXA1) was isolated using two methods i.e., a minipreparation and a large scale method, both based on the alkaline lysis procedure of Birnboim and Doly (1979).

2.2.3.1 Plasmid isolation on a miniprep scale

Solution A : 25 mM Tris.HCl

50 mM glucose

10 mM EDTA

pH 8.0

5 mg/ml lysozyme added just before use

Solution B : 0.2 N NaOH

1% SDS

Solution C : 3 M sodium acetate, pH 4.8

Phenol (equilibrated with 0.1 M Tris.HCl, pH 6.8)

The South African isolate of *X. albilineans* was inoculated into 10 ml of Wilbrink's broth and grown to saturation at 28°C in an orbital shaker. Bacteria from 3 ml of culture were harvested in 1.5 ml Eppendorf tubes by 2 × 1 min centrifugation steps in an Eppendorf microcentrifuge. Bacterial pellets were resuspended, by repeated pipetting, in 100 µl of solution A and incubated for 5 min at room temperature to allow cell wall degradation by lysozyme. The resulting protoplasts were lysed with 200 µl of solution B for 5 min on ice. Denatured chromosomal DNA and proteins were precipitated upon addition of 150 µl of cold solution C and a further 5 min incubation on ice. The precipitated material and cell debris were pelleted by centrifugation at 15 800 × *g* for 30 min at 4°C in a microcentrifuge. The plasmid-containing supernatant was extracted with an equal volume of phenol/chloroform by repeated inversion of the Eppendorf tubes and centrifugation at 15 800 × *g* for 3 min at 4°C to separate the aqueous and organic phases. The upper aqueous phase was collected and the phenol/chloroform extraction repeated until the protein layer at the interface was no longer visible. Following protein removal, the aqueous phase was finally extracted with an equal volume of chloroform (chloroform: isoamyl alcohol, 24:1) to remove all traces of phenol. RNA in the aqueous phase was degraded by addition of RNase (10 mg/ml) at a final concentration of 100 µg/ml and incubation at room temperature for 30 min. Phenol/chloroform and chloroform extractions were repeated to remove RNase and any residual proteins from the solution. Plasmid DNA was precipitated by the addition of 0.1 volume of cold solution C and 2 volumes of cold 100% ethanol for 1 h at -20°C or for 30 min at -70°C. Precipitated plasmid DNA

was pelleted at 15 800 × *g* for 15 min at 4°C, washed with 70% ethanol and dried briefly under a vacuum. Plasmid DNA pellets were resuspended in 20 μl of TE buffer (2.2.3.1) and stored at -20°C.

2.2.3.2 Plasmid DNA isolation on a large scale

TES : 10 mM Tris.HCl
1 mM EDTA
100 mM NaCl
pH 8.0

XA 86-1 was grown to saturation in 4 × 250 ml of Wilbrink's broth at 28°C. Each 250 ml culture was centrifuged separately at 9 500 × *g* for 10 min at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor). Each pellet was resuspended in 5 ml of solution A (2.2.3.1) and incubated for 10 min at room temperature. 10 ml of solution B (2.2.3.1) was added, mixed and incubated for 10 min on ice. After cell lysis, 7.5 ml of cold solution C (2.2.3.1) was added, mixed immediately and incubated for 10 min on ice. Each lysate was transferred to two polypropylene centrifuge tubes (JA-20.1 rotor) and centrifuged at 50 000 × *g* for 30 min at 4°C. The plasmid-containing supernatant was pooled and transferred equally into three polypropylene centrifuge tubes (JA-17 rotor) and the nucleic acids precipitated by the addition of 0.6 volumes of isopropanol to each tube and incubation for 15 min at room temperature. Precipitated material was subsequently pelleted by centrifugation at 15 000 × *g* for 30 min at room temperature. All three pellets were

resuspended in a total volume of 3.75 ml TES and added to a solution containing 7.3 g cesium chloride in 3.5 ml TES and the mixture transferred to a Quick-Seal (Beckman Instruments, Inc.) centrifuge tube. 0.25 ml of ethidium bromide (10 mg/ml) was added and the solution mixed. The Quick-Seal tube was filled with paraffin oil, sealed and centrifuged in a fixed angle rotor (Beckman Ti75) at 150 000 × *g* for 24 h at 10°C. After centrifugation, DNA bands were visualised with a UV lamp and the lower band, corresponding to covalently closed circular (CCC) plasmid DNA, collected by puncturing the side of the tube with a 21-G needle attached to a syringe. Ethidium bromide was removed from the DNA solution by extraction with *N*-butanol (saturated with TE buffer) and the extraction repeated until the DNA solution was colourless. CsCl was removed by dialysing 2 × 4 h against 2 l of TE at 4°C with stirring. Following dialysis, the concentration and purity of the DNA was estimated by measuring the absorbance of the DNA at 260 nm and 280 nm (Ultrospec II, LKB Biochrom Ltd.). DNA was stored in aliquots at -20°C.

2.2.4 **Identification of the Different Forms of pXA1**

The three isomers of plasmids are the CCC, open circular (OC) and linear (L) forms. The migratory pattern of these forms in agarose gels is usually constant, with CCC DNA migrating the fastest and OC DNA the slowest. However, the isomers of pXA1 do not appear to follow this pattern. To investigate this possibility, the structural nature of DNA bands observed after agarose gel electrophoresis was determined using three experimental procedures.

Firstly, 150 ng of pXA1 was heated in a boiling water bath for 5 min and rapidly cooled on ice to selectively remove OC and L forms (Civerolo, 1985; Coplin *et al.*, 1981). Secondly, 150 ng of pXA1 was adjusted to contain 20 μg/ml of ethidium bromide and irradiated under a shortwave (254 nm) UV lamp at a distance of 15 cm, in the dark, to selectively nick or partially relax CCC DNA to OC DNA (Civerolo, 1985; Coplin *et al.*, 1981). The third method involved cleavage of pXA1 with the restriction endonucleases *Bam*HI and *Xba*I, which cleave the plasmid at unique sites in each case to form L plasmid molecules. Plasmid samples subjected to these procedures were analysed together with untreated pXA1 by electrophoresis in 0.7% agarose gels (2.2.2).

2.2.5 Construction of a Restriction Map

A physical map showing the cleavage sites of various restriction endonucleases was constructed using the multiple endonuclease digestion method (Ausubel *et al.*, 1989). pXA1 was cleaved with a variety of restriction endonucleases (Table 2.2), either individually or in combinations and the resulting products separated by agarose gel electrophoresis (2.2.2). Gels were photographed and the migration distances of the DNA fragments measured from photographic prints. The sizes of the restriction fragments were calculated by linear regression analyses of $\log_{10}$ number of nucleotides versus relative mobility plots, using DNA Molecular Weight Markers II and IV (Boehringer Mannheim) and phage λ DNA cleaved with *Eco*RI and *Hin*dIII as DNA standards. The restriction map was deduced progressively from simple situations where enzymes cleaved the plasmid once or twice to more complex situations where cleavage occurred more frequently. A

TABLE 2.2. Restriction endonucleases used to digest pXA1 and their recognition sequences

Restriction endonuclease	Recognition sequence[a]
*Asn*I	AT/TAAT
*Ava*I	C/PyCGPuG
*Bam*HI	G/GATCC
*Bcl*I	T/GATCA
*Bfr*I	C/TTAAG
*Bgl*II	A/GATCT
*Cla*I	AT/CGAT
*Dra*I	TTT/AAA
*Eco*RI	G/AATTC
*Eco*RV	GAT/ATC
*Hae*III	GG/CC
*Hin*dII	GTPy/PuAC
*Hin*dIII	A/AGCTT
*Hpa*I	GTT/AAC
*Nco*I	C/CATGG
*Not*I	GC/GGCCGC
*Pvu*I	CGAT/CG
*Pst*I	CTGCA/G
*Sau*3A	/GATC
*Sfi*I	GGCCNNNN/NGGCC
*Spe*I	A/CTAGT
*Ssp*I	AAT/ATT
*Xba*I	T/CTAGA

[a] N = any nucleotide (A, C, G, T); Pu = either purine, G or A; Py = either pyrimidine, C or T; / = cleavage site

typical restriction reaction using a single enzyme comprised the following in an Eppendorf tube:

pXA1 DNA	10 μl	(190 ng)
deionised H_2O	3 μl	
restriction buffer (10×)	1.5 μl	
restriction endonuclease	0.5 μl	(5 U)
	15 μl total volume	

Restriction reactions were carried out according to the manufacturer's (Boehringer Mannheim) instructions. Double digests were carried out similarly when both enzymes had optimal activity in the same buffer. However, this procedure was modified when two enzymes cleaved optimally in different buffers. In this case, the restriction reaction time was increased to 2 h and the appropriate amount of NaCl and Tris.HCl added after 1 h to ensure optimal cleavage by the second enzyme.

2.2.6 Molecular Cloning of pXA1

In an attempt to determine the role of pXA1 in *X. albilineans*, restriction fragments of the plasmid were cloned into *E. coli*. The six *Eco*RI restriction fragments, designated EIa, EIb, EIc, EId, EIe and EIf were chosen. Fragments were ligated to pTZ19R, a 2.9 kb plasmid cloning vector, and the resultant recombinant DNA molecules transformed into competent *E. coli* DH5αF′ cells. Cloning of DNA fragments into the *Eco*RI site of pTZ19R resulted in insertional inactivation of the *lacZ* gene. Hence, *E. coli* cells carrying recombinant plasmids were easily differentiated from those harbouring the plasmid vector

only, on the basis of a colour reaction, after growth of transformants on media containing the chromogenic substrate X-gal and an inducer, IPTG.

2.2.6.1 Isolation of restriction fragments

TAE buffer (50×): 40 mM Tris base
20 mM glacial acetic acid
2 mM EDTA

Geneclean kit: 6 M NaI
Glassmilk
NEW wash

A total of about 1.5 μg pXA1 was cleaved with *Eco*RI for 90 min at 37°C and loaded into several wells in a 1% low gelling temperature agarose (type VII, Sigma) gel and electrophoresed in 1 × TAE until the bromophenol blue dye in the gel loading buffer reached the end of the gel. After staining in ethidium bromide to visualise the bands, slices of the gel containing the six *Eco*RI restriction fragments were excised and transferred separately into pre-weighed Eppendorf tubes. The weight of the gel slices was measured and their approximate volumes estimated (1 g = approximately 1 ml). DNA fragments present in the gel slices were isolated using the Geneclean kit (BIO 101, Inc.). 3 volumes of 6 M NaI were added to the tubes, mixed well and placed at 50°C until the agarose melted completely. 5 μl of Glassmilk suspension was added and the solutions mixed and placed on ice for 10 min to allow binding of the DNA fragments to the

insoluble silica matrix. The silica matrix with bound DNA was pelleted by spinning for approximately 5 s in a microcentrifuge and the supernatants discarded. The pellets were washed three times with ice cold NEW wash to remove contaminants. After the third wash, pellets were each resuspended in 12 μl of TE buffer and incubated for 3 min at 50°C to elute the bound DNA. The silica matrix was again pelleted and the supernatants containing the eluted DNA removed. A second elution was carried out using 8 μl of TE buffer to give a total of 20 μl of DNA solution for each of the six *Eco*RI fragments. An aliquot of the solutions was used to visually estimate their concentration on an agarose gel and the remainder stored at -20°C.

2.2.6.2 Restriction and dephosphorylation of the cloning vector

Prior to ligation to the pXA1 restriction fragments, the cloning vector, pTZ19R, was linearised with *Eco*RI. 10 μg of the vector was digested with an excess of *Eco*RI (25 U) for 1 h at 37°C. The restriction enzyme was then inactivated by heating at 70°C for 15 min. Aliquots of this reaction were used in the ligation reactions.

Dephosphorylation of the vector was necessary as cloning of EIa and EIb proved to be difficult. Removal of the 5'-phosphate groups from the vector by alkaline phosphatase prevented recircularisation of the vector, making more vector molecules available for ligation to the pXA1 fragments. In this procedure, *Eco*RI was not denatured by heating, but removed by extraction with an equal volume of phenol/chloroform. The aqueous

phase was collected and the DNA precipitated at -70°C for 15 min after adding 2 volumes of cold ethanol. Precipitated DNA was pelleted by centrifugation for 15 min and resuspended in 90 μl of 10 mM Tris.HCl, pH 8.3. 10 μl of 10 × dephosphorylation buffer and 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) were added and the reaction mixture incubated at 37°C for 30 min. After dephosphorylation of the vector, the enzyme was inactivated by the addition of 5 mM EDTA, 0.5% SDS and 100 μg/ml proteinase K (Boehringer Mannheim) and a further incubation step at 56°C for 30 min. The reaction mixture was extracted once with an equal volume of phenol and once with an equal volume of phenol/chloroform to remove the inactivated enzyme. The dephosphorylated DNA was precipitated at -70°C for 15 min after the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. DNA was pelleted by centrifugation for 15 min in a microcentrifuge, washed with 70% ethanol and resuspended in TE buffer at a concentration of 100 ng/μl.

2.2.6.3 Preparation of competent cells

<u>$CaCl_2$ solution</u>:	50 mM $CaCl_2$
	10 mM Tris base
<u>Glycerol solution</u>:	15% glycerol
	50 mM $CaCl_2$
	10 mM Tris base

E. coli cells were made competent, i. e., able to take up DNA, using $CaCl_2$. One ml of

an overnight culture of *E. coli* DH5αF' was diluted to 30 ml with YT broth. The culture was incubated at 37°C, in an orbital shaker, until the optical density of the culture reached 0.375 absorbance units at 590 nm. This step was critical as older cells reduced the efficiency of transformation. Cells were immediately placed on ice and centrifuged at 10 000 × *g* for 10 min at 4°C. The pellet was gently resuspended in 10 ml cold $CaCl_2$ solution and pelleted again. After washing, the cells were resuspended gently in 10 ml cold $CaCl_2$ solution and incubated for 20 min on ice. It was during this step that the cells became competent. The cells were then pelleted once more, resuspended gently in 2.5 ml glycerol solution and stored for 24 h at 4°C to increase competency (Ausubel *et al.*, 1989). Cells were subsequently stored, in aliquots of 100 μl, at -20°C.

2.2.6.4 Ligation of DNA fragments

DNA ligase catalysed covalent joining of the ends of the six *Eco*RI restriction fragments of pXA1 to compatible ends of the linearised cloning vector, pTZ19R. A typical ligation reaction consisted of equimolar amounts of the vector DNA and one of the six plasmid fragments, deionised water, ligation buffer and 1 U of T4 DNA ligase (Boehringer Mannheim). The reaction volume varied between 10 and 20 μl depending on the amounts of DNA solutions and water added. Reactants were heated at 45°C for 5 min before addition of the ligation buffer and DNA ligase to melt any cohesive termini that may have reannealed. Reaction mixtures were incubated for 20 h at 25°C. Reactions were stopped by the addition of 0.5 M EDTA, pH 8.0 to a final concentration of 25 mM and the reaction mixtures stored at 4°C.

2.2.6.5 Transformation

Recombinant plasmids present in the six ligation reaction mixtures were introduced into competent *E. coli* DH5αF' cells by transformation. 100 μl of frozen competent cells were allowed to thaw slowly on ice. 1 μl of the ligation reaction mixtures was added to the cells and the contents of the tubes mixed gently. Cells were incubated for 30 min on ice to allow the DNA to bind to the competent cells. Cells were subsequently heat-shocked for 2 min in a water bath set at 42°C, to allow DNA to enter the cells, and immediately placed on ice for 15 min. Cells were then transferred to test tubes containing 900 μl of YT broth and incubated for 1 h at 37°C in a shaking incubator to allow for the replication of recombinant plasmids before a selective medium was used. 100 μl of the cell suspension was then spread onto X-gal agar plates which were incubated overnight at 37°C.

2.2.6.6 Screening of transformed cells

White colonies on X-gal plates were isolated and sub-cultured on master plates and tested for the presence of foreign pXA1 DNA fragments by plasmid isolation and restriction analysis of plasmid DNA. Plasmid DNA was isolated from *E. coli* cells by further modifications of the procedure of Birnboim and Doly (1979), in order to process the large numbers of colonies that possibly possessed recombinant plasmids. Modifications to the procedure described in section 2.2.3.1 are as follows:

- RNase (100 μg/ml) was included in solution A.
- The 5 min incubation on ice after the addition of solution B was changed to a 5

min incubation at room temperature, to allow degradation of cellular RNA by RNase.

- Chromosomal DNA and cell debris were pelleted for only 10 min in a microcentrifuge, after the addition of solution C.
- Only one phenol/chloroform and one chloroform extraction was carried out.
- Plasmid DNA was precipitated at -70°C for 15 min.

Uncleaved plasmid DNA isolated from *E. coli* cells was compared with uncleaved pTZ19R on agarose gels. Plasmid DNA migrating slower than the cloning vector was suspected of containing pXA1 fragments. This was verified by cleaving the DNA with *Eco*RI and analysing the products on agarose gels.

2.2.7 Assaying for Expression of Cloned DNA Fragments

Four assays were used to determine whether cloned pXA1 fragments were being expressed in *E. coli*. Proteins present in *E. coli* cells carrying a pXA1 fragment were electrophoresed on SDS-polyacrylamide gels and compared with the proteins present in *E. coli* cells harbouring the cloning vector only. This procedure assayed for general expression of pXA1 DNA. Specific assays consisted of screening for the production of and resistance to albicidin, resistance to various antibiotics and growth on a sucrose minimal medium.

2.2.7.1 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out using the Havana Screening Electrophoresis System (Desaga) in a discontinuous buffer system according to the procedure of Laemmli (1970).

Acrylamide/bisacrylamide: 30 g acrylamide
0.8 g *N,N'*-methylenebisacrylamide
H_2O to 100 ml

Separation buffer (5×): 1.875 M Tris.HCl
0.5% SDS
pH 8.8

Stacking buffer (10×): 1.25 M Tris.HCl
1% SDS
pH 6.8

Electrode buffer (10×): 0.25 M Tris.HCl
1.92 M glycine
1% SDS
pH 8.3

SDS gel loading buffer: 50 mM Tris.HCl, pH 6.8
(Sambrook *et al.*, 1989) 2% SDS
0.1% bromophenol blue
10% glycerol
100 mM dithiothreitol added just before use

1% *N,N,N',N'*-tetramethylethylenediamine (TEMED)

8% $(NH_4)_2S_2O_8$

Staining solution: 0.25% Coomassie Brilliant Blue

4.5 volumes methanol

4.5 volumes H_2O

1 volume glacial acetic acid

Glass plates (11 cm × 11 cm) were assembled according to the manufacturer's instructions. A 1.5 mm thick 10% acrylamide separating gel was prepared by first mixing together 3.5 ml 5 × separating buffer, 5.8 ml acrylamide solution, 0.88 ml TEMED and 7 ml H_2O. The mixture was degassed and 0.25 ml $(NH_4)_2S_2O_8$ added. The mixture was rapidly swirled and immediately transferred to the gel mould. Sufficient space was left for a 1 cm stacking gel. The acrylamide solution in the mould was carefully overlaid with 20% ethanol to prevent oxygen from diffusing into the gel and inhibiting polymerisation. The gel was allowed to stand at room temperature for 1 h. Following polymerisation, the ethanol overlay was poured off and the top of the gel washed several times with deionised water to remove any unpolymerised acrylamide. The gel was drained properly and water removed from the inside of the glass plates with absorbent paper. A 4% stacking gel was prepared by mixing together 0.5 ml 10 × stacking buffer, 0.65 ml acrylamide solution, 0.25 ml TEMED, 3.53 ml H_2O, degassing and finally adding 75 μl $(NH_4)_2S_2O_8$. The stacking gel was poured directly onto the surface of the polymerised separating gel. A comb was inserted into the stacking gel solution and the stacking gel allowed to polymerise for 60 min at room temperature. After polymerisation was complete, the comb was removed, the wells washed with deionised H_2O to remove any unpolymerised acrylamide

and the gel mounted in the electrophoresis apparatus.

Protein samples were prepared from overnight cultures of *E. coli* cells carrying pXA1 fragments by first pelleting 1.5 ml of culture in a microcentrifuge for 1 min. Cells were resuspended in 100 μl of SDS gel loading buffer and placed in a boiling water bath for 5 min to denature the proteins. Chromosomal DNA was sheared by repeated pipetting with a micropippete and pelleted by spinning at 15 800 $\times$ g for 10 min. The supernatant containing SDS-polypeptide complexes was transferred to a fresh tube and stored at -20°C. Protein samples, together with a high molecular weight protein marker (Electrophoresis Calibration Kit, Pharmacia), were loaded into the wells of the gel with a Hamilton microlitre syringe. Samples were electrophoresed at 75 mA overnight until the dye front reached the bottom of the gel. Polypeptides in the gel were simultaneously fixed with methanol/glacial acetic acid and stained with Coomassie Brilliant Blue R250 by immersing the gel in staining solution for a minimum of 4 h at room temperature on a slowly rotating platform. The gel was destained for a prolonged period in methanol/glacial acetic acid solution without the dye. The destaining solution was changed at least three times and the gel stored in water containing 20% glycerol until a permanent record was made by photography using Ilford Pan F film.

2.2.7.2 Albicidin production and resistance

E. coli recombinant cells were screened for their ability to produce the phytotoxin albicidin and for resistance to albicidin. Cells were tested for albicidin production by

firstly spotting them onto YT/ampicillin agar plates and incubating the plates at 37°C overnight. Plates were overlaid with 2 ml Müeller Hinton (Biolab) top agar containing 0.2 ml of an overnight culture of *E. coli* HB101. Plates were reincubated at 37°C and examined for zones of clearing. As a control, XA 86-1 was also overlaid with *E. coli* indicator cells. Recombinant cells were tested for resistance to albicidin using the same technique. In this case, XA 86-1 was overlaid with each of the cells carrying a pXA1 DNA fragment.

2.2.7.3 Biochemical assays

Biochemical assays for the expression of pXA1 in *E. coli* consisted of antibiotic resistance tests and growth on sucrose as a sole source of carbon. Antibiotic resistance was tested on nutrient agar plates using antibiotic discs impregnated with the following antibiotics: ampicillin (10 μg), bacitracin (10 μg), chloramphenicol (30 μg), clindamycin (2 μg), colistin sulphate (10 μg), erythromycin (5 μg), fusidic acid (10 μg), gentamycin (10 μg), kanamycin (30 μg), lincomycin (30 μg), neomycin (30 μg), novobiocin (30 μ), penicillin G (1 U), sulfamethoxazole (25 μg), tetracycline (10 μg), and trimethoprin (1.25 μg). *E. coli* cells were first grown in YT broth supplemented with ampicillin. 0.1 ml of these cultures were spread on nutrient agar plates and the antibiotic discs applied when the plates dried. Plates were incubated overnight at 37°C. *E. coli* DH5αF′ and *E. coli* pTZ19R served as controls. Growth of the recombinant *E. coli* cells on sucrose as a sole carbon source was tested on M9 medium supplemented with ampicillin. Growth on this medium would indicate that pXA1 encodes a sucrase as *E. coli* DH5αF′ is incapable of growing on sucrose as a sole source of carbon.

2.3 RESULTS

2.3.1 Screening for Plasmids in *X. albilineans*

Electrophoresis of DNA isolated from the five geographical isolates of *X. albilineans* using the Qiagen Plasmid Kit and digestion of the DNA with *Eco*RI indicated that only the South African isolate harboured a plasmid. DNA isolation using the kit was rapid (± 2 h) and straightforward. Electrophoresis of uncleaved DNA (Fig. 2.1) showed that only the South African isolate possessed multiple bands, characteristic of plasmid DNA. DNA from the other four isolates migrated as a diffuse band, indicating that different sized fragments were present. The migration distance of these diffuse bands also differed for individual

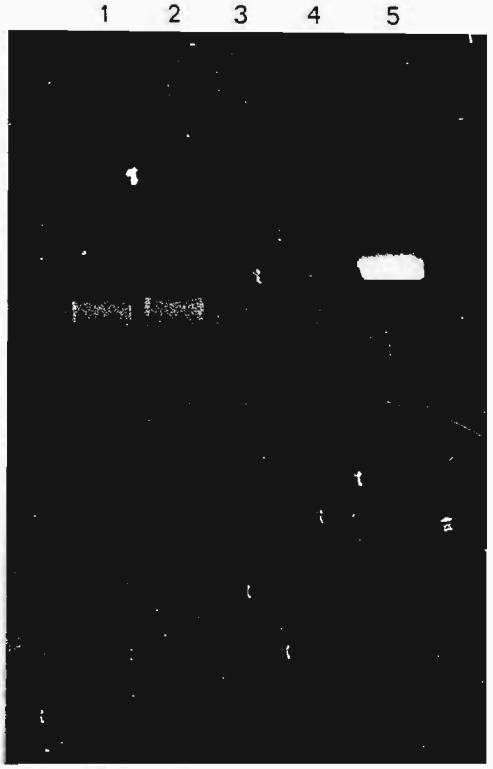

FIG. 2.1. Screening for plasmids in five geographical isolates of *X. albilineans* using the Qiagen Plasmid Kit. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; and lane 5: XA 86-1.

isolates. These observations suggested that the bands were composed of sheared fragments of chromosomal DNA. The DNA band of XA 86-1 having a similar migration distance was not diffuse, indicating that it was composed of molecules of the same size.

Restriction of the DNA isolated using the Qiagen Plasmid Kit, with *Eco*RI, and subsequent electrophoretic analysis (Fig. 2.2) again demonstrated that only the South African isolate harboured a plasmid. Electrophoresis of cleaved DNA from XA 86-1 showed discrete fragments. This is characteristic of plasmid DNA. In contrast, restriction and electrophoresis of DNA from the other isolates resulted in a "streak", consisting of unresolved DNA fragments. This "ladder" of DNA fragments is typical of endonuclease cleaved chromosomal DNA as endonucleases cleave chromosomal DNA at a much higher frequency, due to their larger size, compared to plasmid DNA.

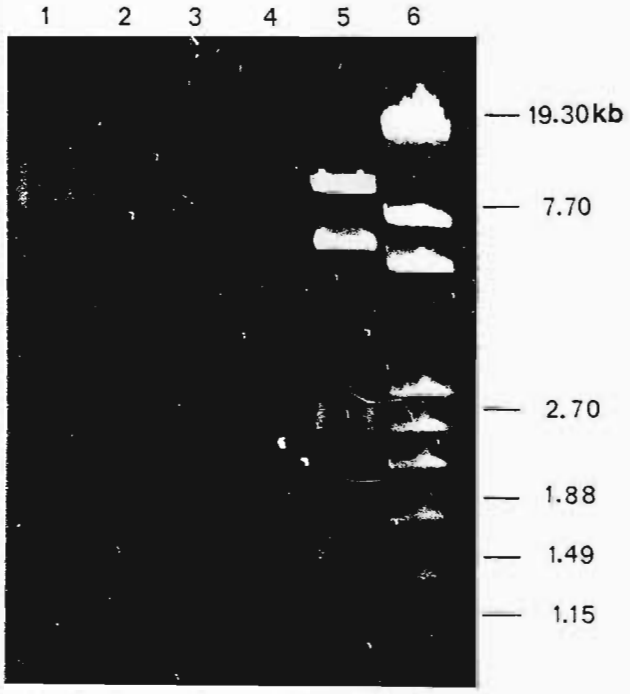

FIG. 2.2. Agarose gel showing products of *Eco*RI digestion of DNA isolated using the Qiagen Plasmid Kit. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker IV.

2.3.2 Isolation of Plasmid DNA

The minipreparation and large scale isolation procedures both reproducibly yielded plasmid DNA from XA 86-1. The minipreparation method yielded, in under 3 h, small quantities of plasmid DNA that was suitable as a substrate for *Eco*RI digestion. The large scale method yielded large quantities of DNA in a highly purified form. The optical density of the DNA solution obtained by this procedure was 0.38 at 260 nm. This corresponded to a DNA concentration of 19 ng/μl (OD_{260} of 1 = 50 μg/ml of double stranded DNA). The ratio of the DNA concentration to the protein concentration (OD_{260} :OD_{280}) of the solution was 1.7 which was an indication of pure DNA.

2.3.3 Identification of the Different Forms of pXA1

Two plasmid bands were usually observed after agarose gel electrophoresis of pXA1, with the predominant band migrating slower than the low-intensity-staining band. Of the three procedures used to determine the structural nature of these bands, two provided conclusive evidence of their identity (Fig. 2.3). The third isomer of pXA1 was also observed when a lane was overloaded with pXA1. Lane 3 of Fig. 2.3 shows the three forms of the plasmid. Nicking of pXA1 with ethidium bromide eliminates the middle band and increases the amount of DNA in the slowest migrating band (lane 2). This proves that the middle band consists of CCC plasmid DNA as ethidium bromide-induced nicking converts CCC DNA to the OC form. Heat treatment of pXA1 and subsequent electrophoresis did not produce the expected results (lane 1, Fig. 2.3). Athough L DNA was eliminated,

CCC DNA was also eliminated and an additional band was observed which migrated faster than CCC DNA but slower than the L form of the plasmid. The structural nature of this DNA band is unknown.

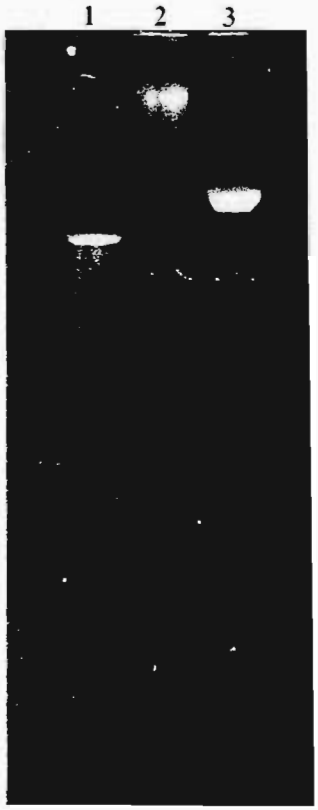

FIG. 2.3. Effects of heat treatment and nicking with ethidium bromide on the structural nature of pXA1. Lane 1: heat treated pXA1; lane 2: pXA1 nicked with ethidium bromide; and lane 3: untreated pXA1.

Identity of the plasmid bands was confirmed by restriction of pXA1 with enzymes that cleave the plasmid at a single site only (Fig. 2.4). Cleavage by *Bam*HI and *Xba*I converted CCC plasmid to L plasmid molecules. These L molecules migrated together with the fastest migrating pXA1 band. These two procedures proved that the fastest migrating pXA1 band consists of the L form of the plasmid and that the second band consists of CCC DNA. The OC form, which migrates the slowest was only observed in overloaded lanes.

FIG. 2.4. Identification of the linear form of pXA1 by digestion with restriction enzymes that cleave at a single site. Lane 1: pXA1 cleaved with *Bam*HI; lane 2: pXA1 cleaved with *Xba*I; and lane 3: uncleaved pXA1.

2.3.4 Restriction Mapping of pXA1

Of the twenty three restriction endonucleases tested, four enzymes viz., *Bcl*I, *Pst*I, *Sfi*I and *Spe*I did not cleave the plasmid. All the other enzymes cleaved pXA1 to completion with the exception of *Bgl*II which cleaved pXA1 to produce two DNA fragments. Eight restriction enzymes cleaved pXA1 at unique sites to form L plasmid molecules (Fig. 2.5). Most of the restriction enzymes used have recognition sequences that are 6 bp long. The two enzymes that have 4 bp recognition sequences (*Hae*III and *Sau*3A) digested pXA1 to such an extent that the fragments could not be resolved on agarose gels. Two enzymes

that have octameric recognition sequences were also tested. *Not*I had a single restriction site whereas the target sequence of *Sfi*I was not present in pXA1. The plasmid was cleaved by enzymes having both A-T rich and G-C rich target sequences. Profiles of some of the enzymes that have multiple restriction sites and two that do not cleave the plasmid are shown in Fig. 2.6. The number of restriction fragments produced by each enzyme tested, is summarized in Table 2.3.

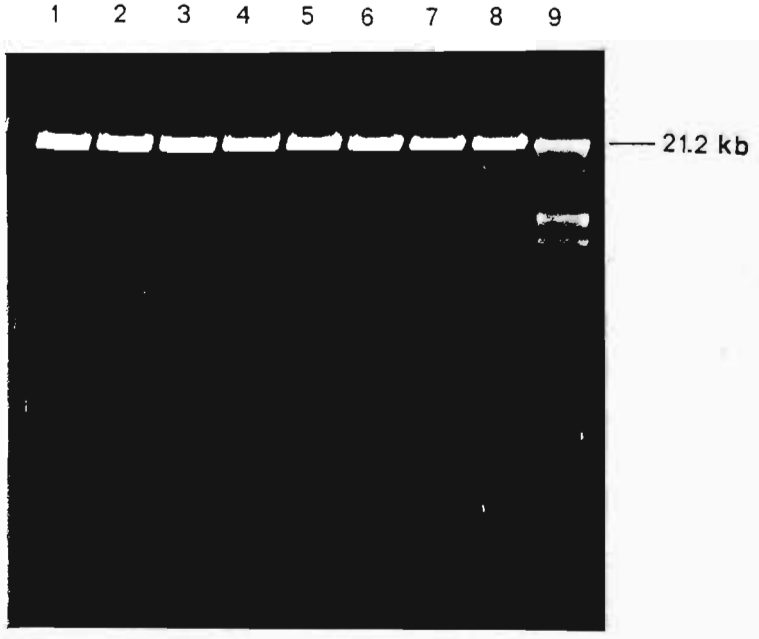

FIG. 2.5. Restriction digests with enzymes that cleave pXA1 at unique sites. pXA1 was linearised with eight enzymes. Lane 1: *Asn*I; lane 2: *Bam*HI; lane 3: *Bfr*I; lane 4: *Hpa*I; lane 5: *Not*I; lane 6: *Pvu*I; lane 7: *Ssp*I; lane 8, *Xba*I; and lane 9: phage λ DNA cleaved with *Eco*RI and *Hin*dIII.

TABLE 2.3. Number of pXA1 fragments produced for each restriction endonuclease

Restriction enzyme	Number of fragments[a]
*Asn*I	1
*Ava*I	7
*Bam*HI	1
*Bcl*I	-
*Bfr*I	1
*Bgl*II	2
*Cla*I	7
*Dra*I	2
*Eco*RI	6
*Eco*RV	3
*Hae*III	> >
*Hin*dII	> 12
*Hin*dIII	2
*Hpa*I	1
*Nco*I	3
*Not*I	1
*Pvu*I	1
*Pst*I	-
*Sau*3A	> >
*Sfi*I	-
*Spe*I	-
*Ssp*I	1
*Xba*I	1

[a] - = no restriction sites; > > = restriction fragments could not be resolved on agarose gels

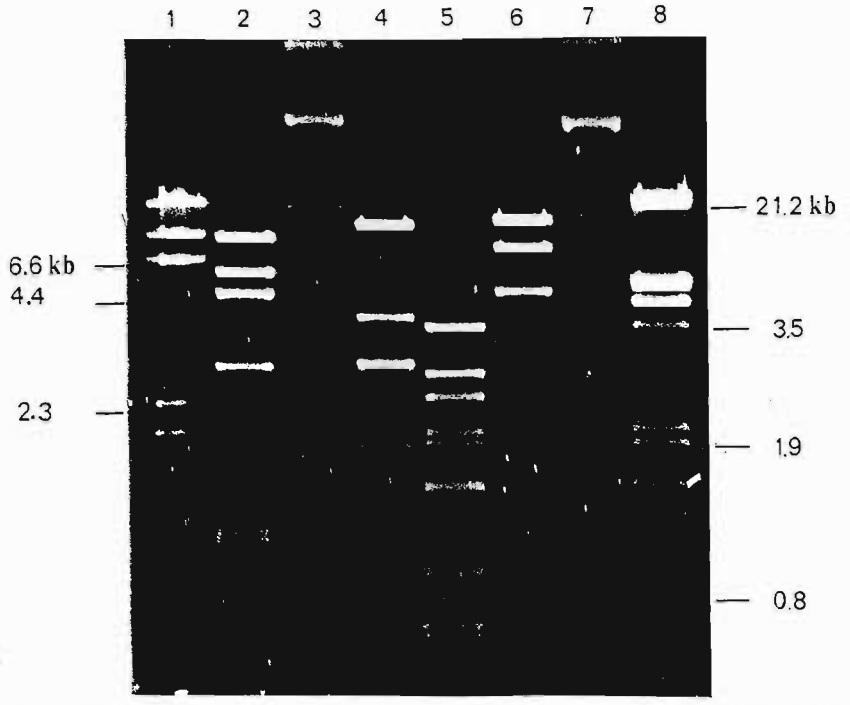

FIG. 2.6. Restriction profiles of pXA1 cleaved with various restriction endonucleases. Lane 1: DNA molecular weight marker II; lane 2: *Ava*I; lane 3: *Bcl*I; lane 4: *Cla*I; lane 5: *Hin*dIII; lane 6: *Not*I; lane 7: *Spe*I; and lane 8: phage λ DNA cleaved with *Eco*RI and *Hin*dIII.

The size of pXA1 calculated by addition of the sizes of restriction fragments of single and double digests, was approximately 24.9 kb. Mapping of DNA fragments with some of the combinations of restriction enzymes used, are shown in Figs. 2.7 - 2.9. A restriction map consisting of the cleavage sites of ten restriction endonucleases was constructed (Fig. 2.10). After the *Bam*HI restriction site was arbitrarily chosen as position 0 on the restriction map, digests containing combinations of enzymes that cleave at single sites were used to map their respective restriction sites.

For example, mapping of the *Bfr*I and *Xba*I sites was accomplished by performing *Bam*HI/*Bfr*I (lane 2, Fig. 2.7), *Bam*HI/*Xba*I (not shown) and *Bfr*I/*Xba*I (lane 3, Fig. 2.7) digests of pXA1. The distance between the *Bam*HI and *Bfr*I sites and the *Bam*HI and *Xba*I

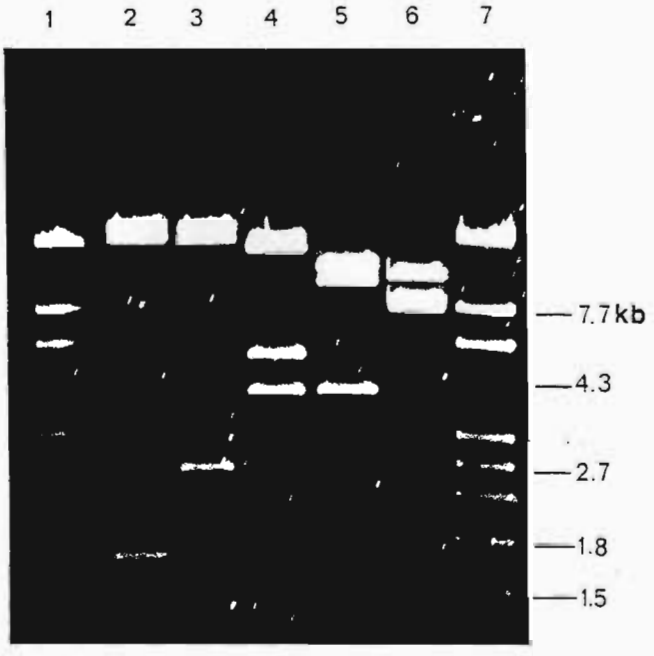

FIG. 2.7. Restriction profiles of double digests of pXA1 with combinations of restriction endonucleases, *Bam*HI, *Bfr*I, *Xba*I, *Asn*I, *Hin*dIII and *Dra*I. Lanes 1 and 7: DNA molecular weight marker IV; lane 2: *Bam*HI/*Bfr*I; lane 3: *Xba*I/*Bfr*I; lane 4: *Asn*I/*Hin*dIII; lane 5: *Bam*HI/*Hin*dIII; and lane 6: *Asn*I/*Dra*I.

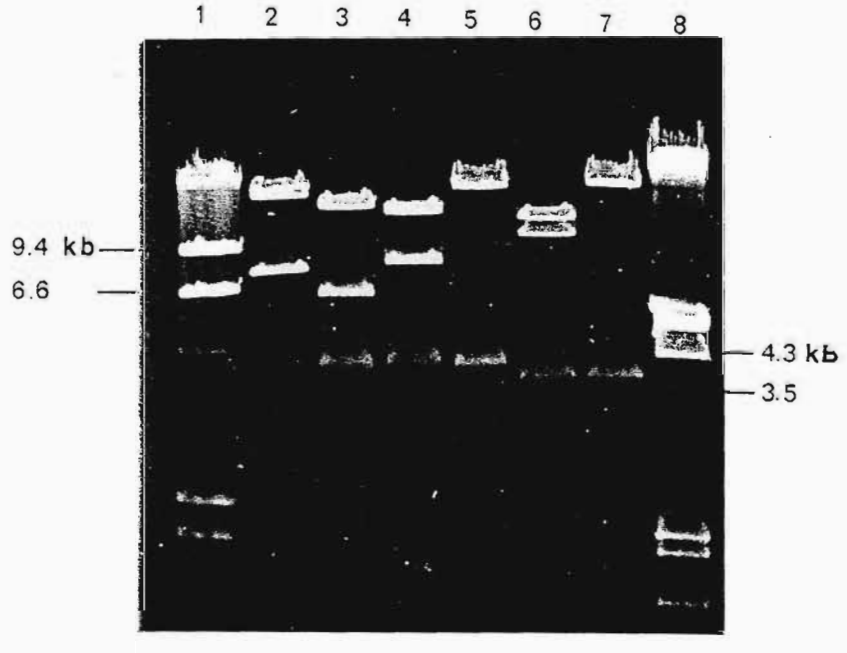

FIG. 2.8. Restriction profiles of double digests of pXA1 with restriction endonucleases, *Asn*I, *Bam*HI, *Eco*RV and *Hin*dIII. Lane 1: DNA molecular weight marker II; lane 2: *Asn*I/*Bam*HI; lane 3: *Asn*I/*Eco*RV; lane 4: *Bam*HI/*Eco*RV; lane 5: *Eco*RV; lane 6: *Bam*HI/*Hin*dIII; lane 7: *Hin*dIII; and lane 8: phage λ DNA cleaved with *Eco*RI and *Hin*dIII.

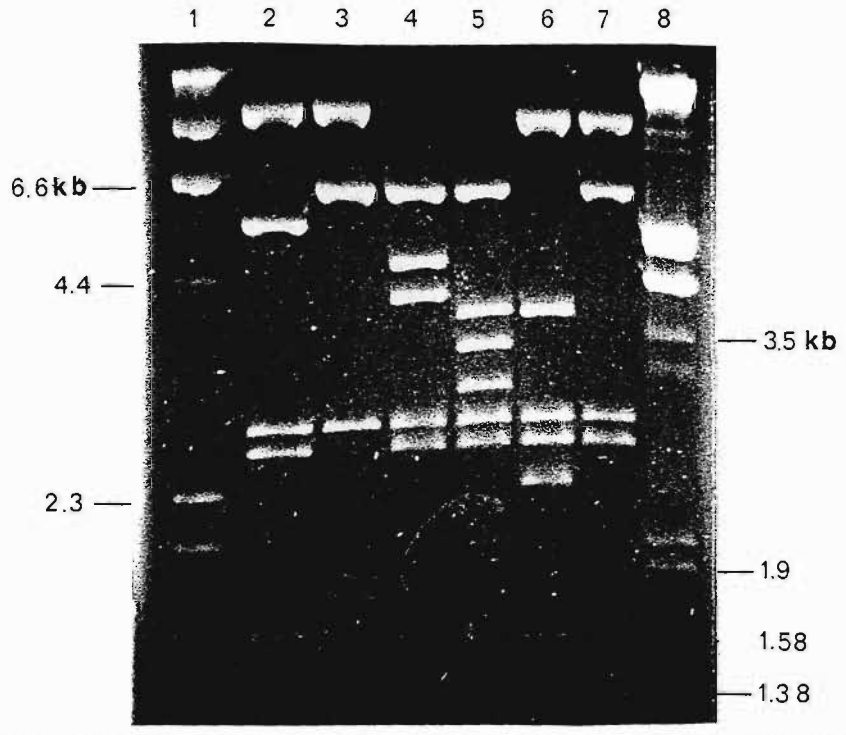

FIG. 2.9. Restriction profiles of double digests of pXA1 with *Eco*RI and other restriction endonucleases. Lane 1 : DNA molecular weight marker II; lane 2: *Bam*HI/*Eco*RI; lane 3: *Bfr*I/*Eco*RI; lane 4: *Eco*RV/*Eco*RI; lane 5: *Hin*dIII/*Eco*RI; lane 6: *Not*I/*Eco*RI; lane 7: *Eco*RI; and lane 8: phage λ DNA cleaved with *Eco*RI and *Hin*dIII.

sites were calculated to be 1.7 kb and 0.9 kb, respectively. These two sites were positioned on opposite sides of the *Bam*HI site as the distance between them was calculated to be 2.6 kb. Similarly, the *Asn*I site was mapped by performing *Asn*I/*Bfr*I (lane 1, Fig. 2.8) and *Asn*I/*Bam*HI (not shown) digests of pXA1. Since the smaller fragments of both these digests measured 6.0 kb and 7.7 kb, respectively, the *Asn*I site was positioned 6.0 kb from the *Bfr*I site and 7.7 kb from the *Bam*HI site. The positions of the six *Eco*RI restriction fragments were determined by double digests containing *Eco*RI together with other enzymes that had already been mapped. Profiles of these digests were compared with an *Eco*RI digest of pXA1 (lane 7, Fig. 2.9) to determine which *Eco*RI fragment had been cleaved by the second enzyme in the double digest.

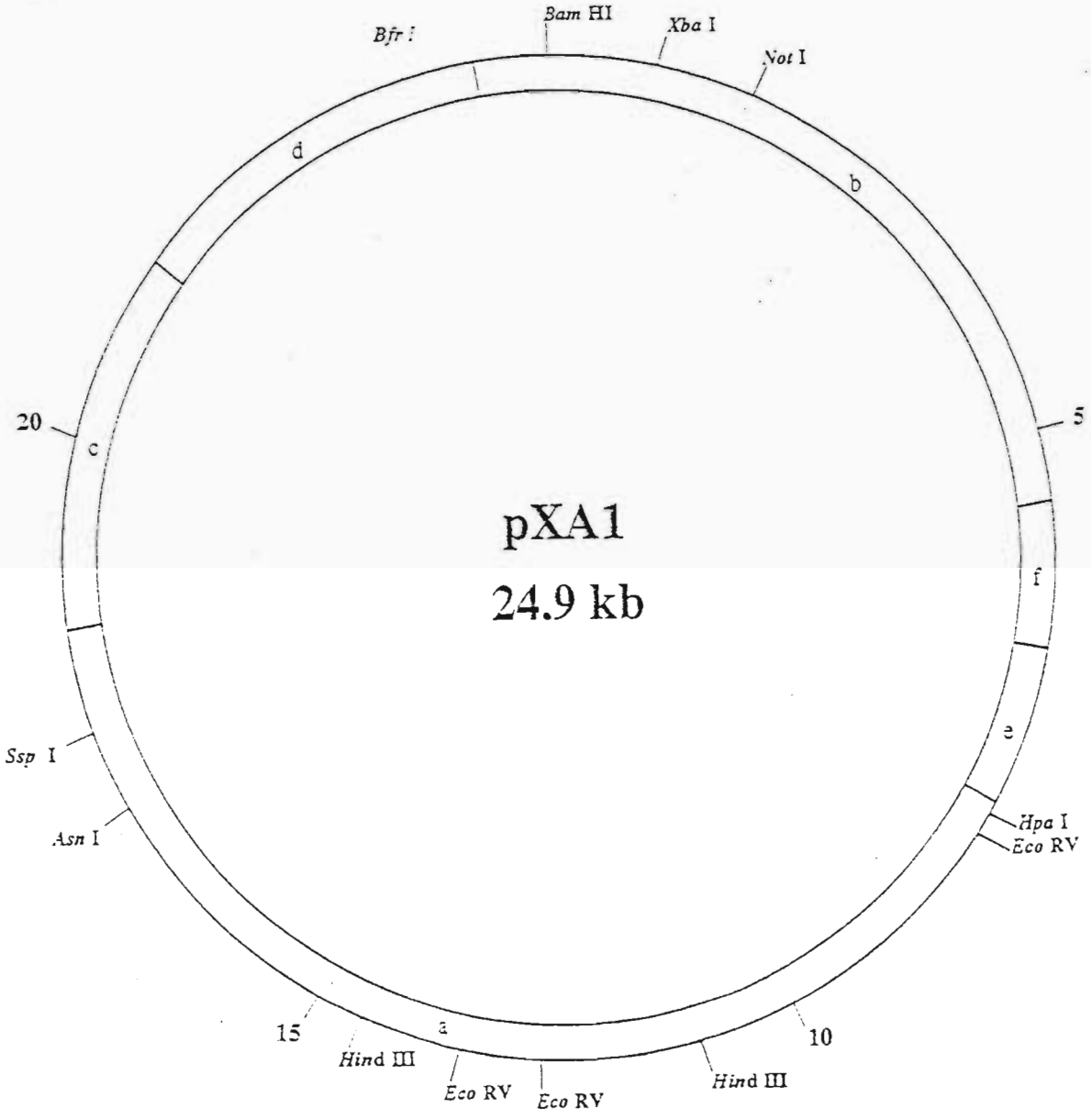

FIG. 2.10. Physical map of restriction endonuclease cleavage sites on pXA1. The *Bam*HI site was arbitrarily chosen as position 0. The inner ring represents the six *Eco*RI restriction fragments of pXA1. The positions of *Eco*RI fragments e and f are interchangeable.

2.3.5 Molecular Cloning of pXA1

The pXA1 restriction fragments cloned into *E. coli* are listed in Table 2.4. Five of the fragments are *Eco*RI restriction fragments. Even after repeated attempts, EIb, the 6.4 kb *Eco*RI restriction fragment, could not be cloned. This fragment was cleaved with *Bam*HI to form two fragments, BEIa (5.6 kb) and BEIb (800 bp). BEIb was then cloned into *E. coli* but BEIa could still not be cloned. Hence, almost 20 kb of the 25 kb plasmid was successfully cloned.

TABLE 2.4. pXA1 fragments cloned in *E. coli*

Fragment	Size	Recombinant plasmid designation
EIa	10.4 kb	pTX1
EIc	2.8 kb	pTX3
EId	2.6 kb	pTX4
EIe	1.5 kb	pTX5
EIf	1.2 kb	pTX6
BEIb	0.8 kb	pTX7

After large scale *Eco*RI cleavage of pXA1, electrophoresis of products on a low melting temperature gel (Fig. 2.11) and isolation of the fragments using the Geneclean kit, the individual fragments were analyzed on an agarose gel (Fig. 2.12) and found to be of a sufficiently high concentration.

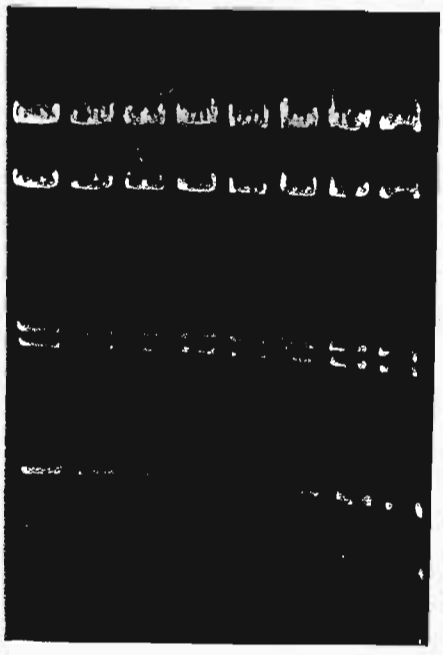

FIG. 2.11. Large scale cleavage of pXA1 with *Eco*RI and electrophoresis in a 1% low melting temperature agarose gel in 1 × TAE buffer.

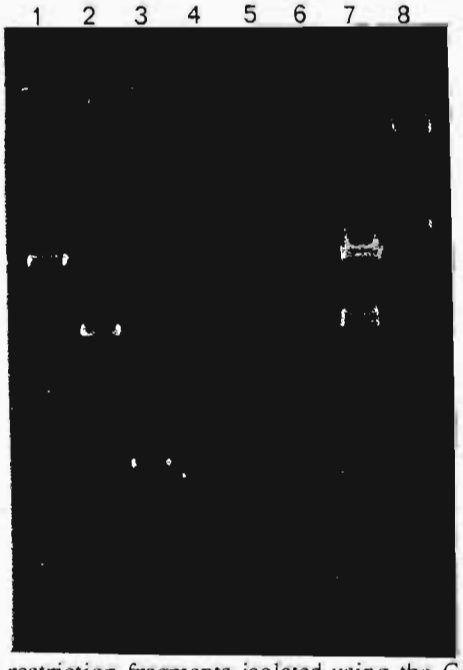

FIG. 2.12. *Eco*RI restriction fragments isolated using the Geneclean kit. Lane 1: EIa; lane 2: EIb; lane 3: EIc; lane 4: EId; lane 5: EIe; lane 6: EIf; lane 7: *Eco*RI digested pXA1; and lane 8: uncleaved pXA1.

Cloning of EIc, EId, EIe, EIf and BEIb proved to be simple due to their small size. However, cloning of EIa was difficult and was only accomplished after dephosphorylation of the cloning vector. As expected, dephosphorylation of pTZ19R significantly reduced the background of blue colonies observed on X-gal plates.

Further modifications of the plasmid isolation procedure of Birnboim and Doly (1979), used to rapidly screen white colonies for the presence of pXA1 inserts, yielded DNA that was of a sufficiently high purity to allow digestion by restriction endonucleases. Restriction of recombinant plasmids carrying pXA1 DNA fragments is shown in Fig. 2.13

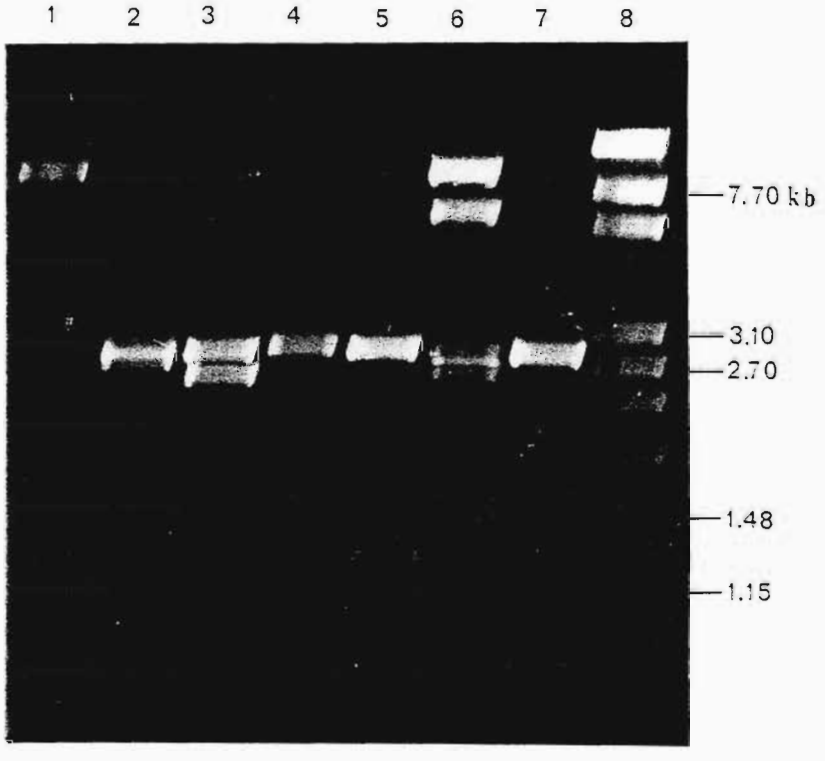

FIG. 2.13. Restriction of recombinant plasmids isolated from *E. coli*, showing the presence of pXA1 fragments. Lane 1: pTX1; lane 2: pTX3; lane 3: pTX4; lane 4: pTX5; lane 5: pTX6; lane 6: *Eco*RI digested pXA1; lane 7: *Eco*RI-linearised pTZ19R; and lane 8: DNA molecular weight marker IV.

2.3.6 Assaying for Expression of Cloned DNA

None of the assays used provided evidence for the expression of pXA1 fragments in *E. coli*.

2.3.6.1 SDS-polyacrylamide gel electrophoresis

The banding pattern of polypeptide components of the cellular proteins from the six *E. coli* cell types carrying pXA1 fragments were identical to the pattern observed for *E. coli* cells carrying the cloning vector only (Fig. 2.14). Due to the high copy number of pTZ19R, it was expected that expression of pXA1 fragments would result in a high concentration of the foreign protein which would have been detected on SDS-polyacylamide gels.

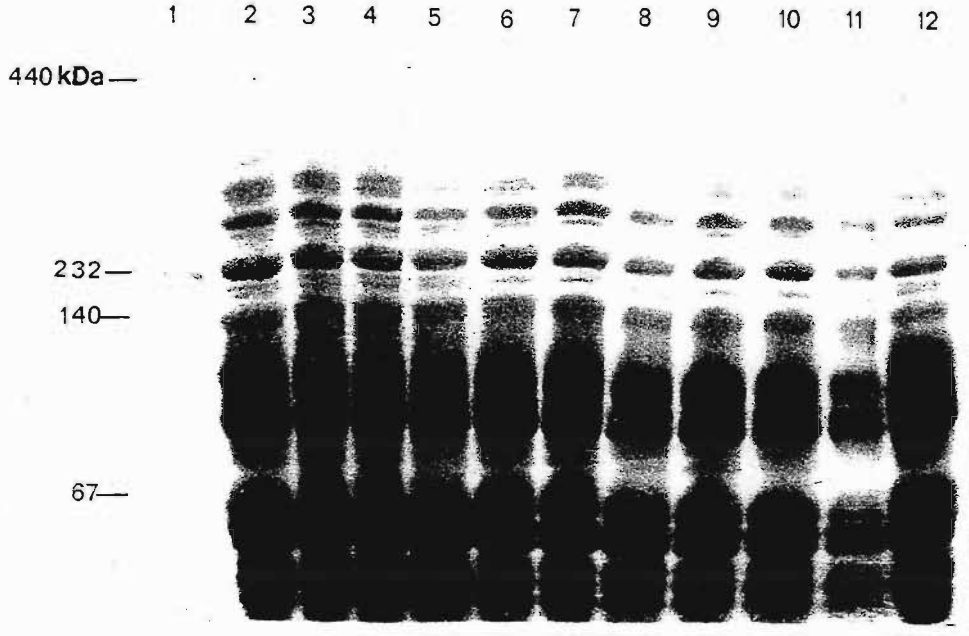

FIG. 2.14. Polyacrylamide gel of proteins isolated from *E. coli* cells carrying recombinant plasmids containing pXA1 fragments. Lane 1: High molecular weight protein marker; lane 2: pTX1; lane 3: pTX7; lanes 4 and 5: pTX3; lanes 6 and 7: pTX4; lanes 8 and 9: pTX5; lanes 10 and 11: pTX6; and lane 12: pTZ19R.

2.3.6.2 Albicidin production and resistance

All six recombinant *E. coli* cell types neither coded for albicidin production nor for resistance to albicidin (Fig. 2.15). The lack of albicidin production was observed as a failure to inhibit the growth of *E. coli* HB101. When testing for resistance to albicidin, the recombinant plasmids could not protect their *E. coli* hosts from the effects of albicidin produced by XA 86-1.

2.3.6.3 Biochemical assays

The sensitivity and resistance of the recombinant *E. coli* cells to the various antibiotics were identical to that of *E. coli* pTZ19R, implying that the pXA1 fragments did not code for resistance or sensitivity to these antibiotics. The only difference noticed was that the second control, i. e., *E. coli* DH5αF$'$ was sensitive to ampicillin whereas *E. coli* pTZ19R and the recombinant cells were not. This was due to the ampicillin resistance gene present on pTZ19R. The pXA1 fragments did not code for a sucrase as the *E. coli* recombinant cells were unable to grow on the minimal medium containing sucrose as the sole source of carbon.

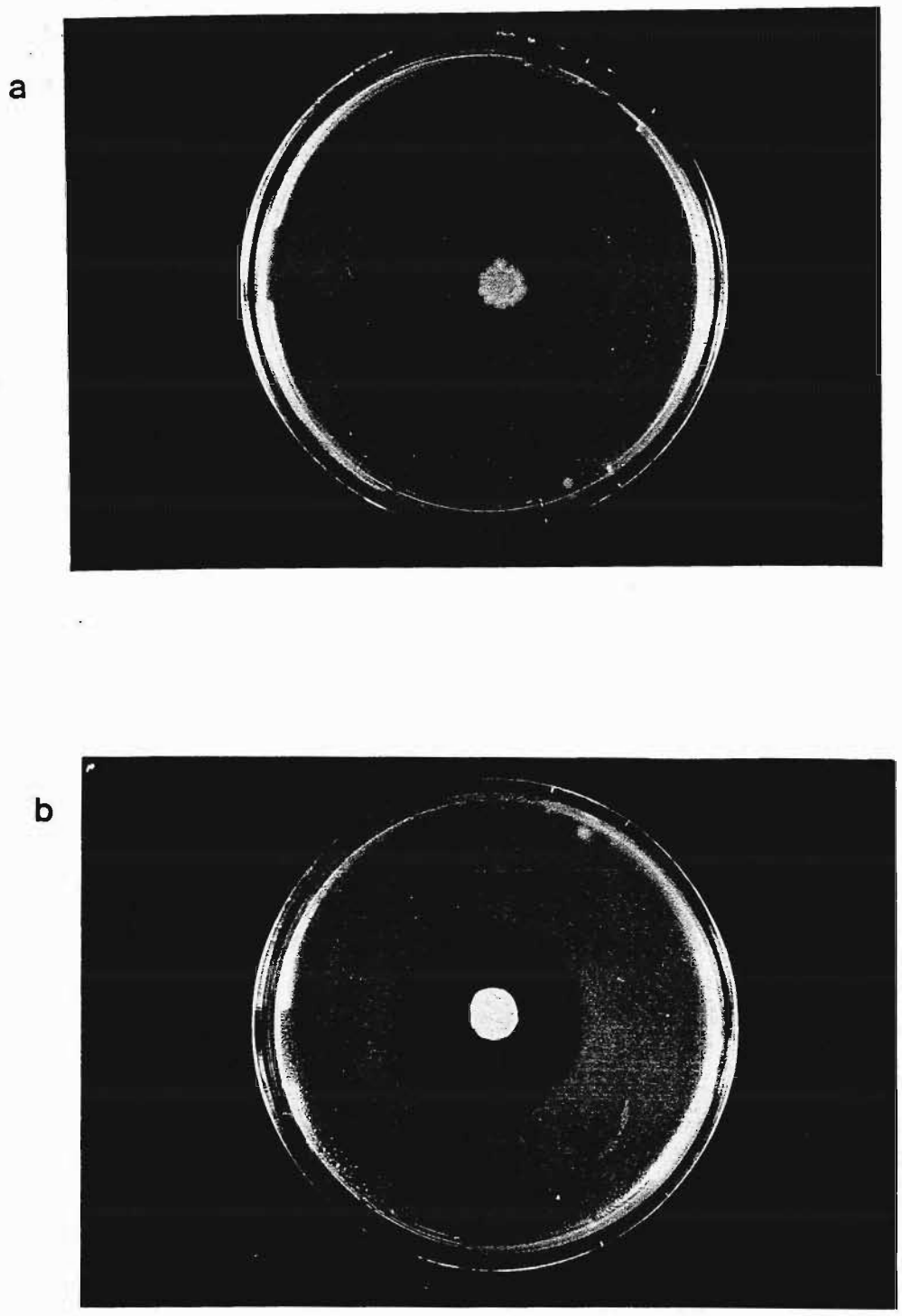

FIG. 2.15. Albicidin production assay (a) and resistance assay (b) using the *E. coli* clone carrying pTX1. The clone lacked the ability to either inhibit *E. coli* HB101 or code for resistance to albicidin produced by XA 86-1. All recombinant clones carrying pXA1 fragments were identical in this regard.

2.4 DISCUSSION

The presence of one or more plasmids in plant pathogenic bacteria appears to be the rule rather than the exception (Panapoulos and Peet, 1985). This statement is particularly true for the pathovars of *X. campestris*, among which plasmids are widely distributed (Lazo and Gabriel, 1987). Two previous reports by Birch and Patil (1987a) and Lazo and Gabriel (1987) demonstrated that plasmids were not present in Australian isolates and a Florida isolate of *X. albilineans*, respectively. All five isolates used in this study were previously suspected of harbouring plasmids (Permaul *et al.*, 1991; Singh and Pillay, 1990). This was based primarily on a DNA band that was common in all isolates. This study has shown that a plasmid is present only in the South African isolate, XA 86-1. The first evidence to suggest this, was the presence of multiple bands only for XA 86-1 after the plasmid isolation procedure. Secondly, the diffuse band present in the other four isolates did not co-migrate with the linear form of pXA1. The diffuse nature of these bands and the slight variations in their electrophoretic mobilities among the four isolates, suggest that these bands consist of sheared chromosomal DNA fragments. This was confirmed by restriction of the DNA with *Eco*RI. The DNA exhibited typical chromosomal DNA patterns when digested with an endonuclease. In contrast, the DNA from XA 86-1 showed typical plasmid DNA profiles. The presence of a diffuse chromosomal DNA band in plasmid preparations is not uncommon (Civerolo, 1985; Clark and Lawrence, 1986; Gonzalez *et al.*, 1984; Mazerai and Kerr, 1991; Mogen *et al.*, 1988; Morales and Sequeira, 1985). Chromosomal DNA contamination occurs due to breakage of bacterial chromosomes during plasmid DNA isolation. Larger chromosomal fragments are selectively removed during the procedure, but the smaller fragments mimic plasmid molecules.

Two of the three isomers of pXA1 were usually observed on agarose gels. The third isomer, OC DNA was present in low concentrations. The majority of the DNA isolated using any of the three isolation procedures described, consists of CCC DNA. This is consistent with most of the plasmids described in the literature. However, the banding pattern of the isomers of pXA1 differed from that observed for the majority of plasmids. CCC DNA molecules usually migrate the fastest, followed by linear DNA and then OC forms. The OC form of pXA1 migrated the slowest, but the order of the CCC and L forms were reversed. This pattern has also been reported for pCS1, a 48 kb plasmid found in *C. michiganense* subsp. *sepedonicum* (Clark and Lawrence, 1986; Mogen *et al.*, 1988).

The size of pXA1 was calculated to be 24.9 kb. This calculation involved addition of the sizes of restriction fragments produced by single and double digests of the plasmid. Small fragments (< 400 bp) would not have been detected on agarose gels, due to their small size and low staining-intensities. The accuracy of this technique could be compared with other techniques which measure the size of intact plasmid molecules, e.g., relative mobilities of CCC molecules and measurement of contour dimensions of plasmid molecules by electron microscopy (Clark and Lawrence, 1986). Analysis of the recognition sequences of the restriction endonucleases did not provide evidence about the GC content of pXA1, as the plasmid was cleaved by enzymes having both GC- and AT-rich recognition sequences. The construction of a restriction map for pXA1 facilitates further genetic manipulation of the plasmid, especially once genes are identified on the plasmid.

Approximately 80% of the plasmid was cloned into *E. coli*. Cloning of the four smaller *Eco*R1 fragments was relatively simple compared to the two larger fragments. Initial

difficulty in cloning these two fragments was due to their size. Recombinant plasmids greater than 10 kb are difficult to transform into *E. coli*. However, the concentrations of the reactants in the ligation reaction is of greater importance when large fragments are cloned. When the cloning vector and the insert DNA are of equal size, the possibility of forming recombinant molecules is greatest, as the compatible ends of the DNA have an equal chance of base-pairing. However, when larger inserts are cloned, the molar ratios of the reactants have to be adjusted in order to keep the concentrations of the ends of the DNA molecules equal. The molar ratios of EIa and EIb to the cloning vector varied between 1:1 and 2:1. Alkaline phosphate was used to dephosphorylate the cloning vector in order to prevent the ends of the cloning vector from ligating. This increased the amount of vector molecules that were available to base-pair with the inserts. Use of these two strategies to optimise the ligation reaction resulted in the successful cloning of EIa, the 10.4 kb *Eco*RI fragment of pXA1. However, EIb, the 6.4 kb *Eco*RI fragment could still not be cloned. Attempts were made to clone this fragment into *E. coli* DH5αF' cells that were resistant to albicidin. This possibility was explored as the size of the fragment could no longer explain the inability to clone the fragment, as the 10.4 kb fragment had already been cloned. To further emphasize this, EIb was digested by *Bam*HI into a 5.6 kb fragment, BEIa, and a 0.8 kb fragment, BEIb, and cloning of these fragments was attempted. The 0.8 kb fragment was easily cloned but the 5.6 kb fragment could still not be cloned. Cloning of BEIb was expected to be easier, not only because of its smaller size, but due to the fact that a directed cloning procedure was employed. Directed cloning, where the cloning vector and insert DNA are cleaved with different enzymes, usually increases the yield of recombinant molecules as the ends of the vector and insert are incapable of self-ligating. Cloning of EIb into albicidin-resistant *E. coli* cells was also unsuccessful. However, it has been proposed

by Birch *et al.* (1990), that resistance to albicidin in *E. coli* is membrane-dependant. This mechanism would therefore not protect *E. coli* cells against the toxin if it is being produced inside the cell. If EIb is suspected of coding for albicidin, it could be cloned into *Klebsiella oxytoca* in which albicidin resistance occurs by means of an albicidin-binding protein (Birch *et al.*, 1990).

Expression assays used in this study have failed to provide evidence about the role of pXA1 in XA 86-1. This could be due to the DNA fragments not being expressed in *E. coli*. Expression depends on host enzymes and also on the presence of a suitable promoter on the vector if the inserted genes lack their own promoters. However, a larger number of expression assays need to be performed, including screening for mRNA synthesis, before reaching this conclusion. Although plasmids are known to play a role in the ecological fitness of their hosts, a vast majority of the plasmids in plant pathogenic bacteria are still genetically cryptic (Coplin, 1989).

With interest in developing diagnostic methods to identify *X. albilineans*, it is possible that the use of plasmid profiles and plasmid DNA probes may be sufficient for the identification of XA 86-1. It is clear that XA 86-1 can be distinguished from the other four geographical isolates used in this study, on the basis of plasmid profiles. The specificity of a plasmid DNA probe will be discussed in Chapter 5. However, a larger number of geographical isolates and more South African isolates will have to be examined to determine the ubiquity of pXA1. In addition to aiding in their identification, these observations may have a taxonomic significance in differentiating these pathogens.

CHAPTER THREE

DNA FINGERPRINTING OF *X. albilineans* ISOLATES

3.1 INTRODUCTION

Several methods of DNA analysis have been used to compare and identify plant pathogenic bacteria. These include DNA-DNA hybridisation, RFLP analysis, hybridisation with specific DNA probes and plasmid profile analysis. Two recently discovered techniques, viz., genomic DNA fingerprinting using PFGE and random amplification of polymorphic DNA (RAPD), have been found to be useful in differentiating between very closely related bacteria. These two techniques were used to examine DNA relatedness among five isolates of *X. albilineans*, including the type strain and XA 86-1.

Genomic DNA fingerprinting with restriction endonucleases has traditionally been performed using conventional agarose gel electrophoresis and enzymes such as *Eco*RI. This results in several hundred to more than a 1 000 restriction fragments, which make resolution of individual fragments and their analysis difficult. The development of PFGE by Schwartz and Cantor (1984) and the discovery of rare-cutting restriction endonucleases led to less complex DNA profiles being generated, making this modified DNA fingerprinting an attractive method for comparing relatedness of bacterial isolates.

PFGE was developed to separate DNA fragments that were too large to be separated by

conventional electrophoresis. PFGE utilises more than one set of electrodes and each set of electrodes is positioned at different angles to the DNA samples in the agarose gels. During electrophoresis, each set of electrodes is alternatively switched on and off by a controller, hence the name "pulsed". The theory behind PFGE is that each time the direction of the electric field is changed, the DNA fragments must reorient and realign relative to the electric field. Because larger DNA fragments take longer to reorient than smaller ones, the larger the fragment, the longer it takes to migrate down the gel. Thus, DNA fragments are still separated according to size. The various types of PFGE include field inversion gel electrophoresis (FIGE), contour-clamped homogenous electric field electrophoresis (CHEF), rotating field electrophoresis (RGE), orthogonal field alternating field electrophoresis (OFAGE) and transverse alternating field electrophoresis (TAFE). TAFE was developed by Gardiner and Patterson (1988) and this system orientates the electric field transversely with respect to the agarose gel. Two pairs of electrodes are placed in front of and behind the vertical gel and when the electric field alternates between the electrodes, the DNA molecules zigzag down through the width of the agarose gel, not across the face of the gel as with the other pulsed field techniques. PFGE of *E. coli* DNA digested with the restriction enzymes *Not*I and *Sfi*I, which recognise rare 8 bp sequences, produces distinctive profiles consisting of less than 30 restriction fragments (Smith *et al.*, 1987). Since the recognition sequences of these two enzymes consist entirely of G and C nucleotides, they cut frequently in GC-rich genomes and are therefore not useful for DNA fingerprinting of most plant pathogenic bacteria (Grothues and Tümmler, 1987; McClelland *et al.*, 1987). Several enzymes that recognise 6 bp sequences were recently shown to cut infrequently in GC-rich genomes (Grothues and Tümmler, 1987; McClelland *et al.*, 1987).

The RAPD technique is a PCR-based assay that has recently been developed to detect polymorphisms in genomic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990). The technique relies on the presence of priming sites on the genome, close enough to permit PCR amplification using single primers of arbitrary nucleotide sequence. Hence, prior knowledge of the genome to be analysed is not required. RAPD analysis has been successfully used in examining DNA relatedness of species of animals, plants, fungi and bacteria (Welsh and McClelland, 1990; Williams *et al.*, 1990). Thus far, reports of its use to study genomic variation in plant pathogens are mainly concerned with fungal pathogens (Crowhurst *et al.*, 1991; Goodwin and Annis, 1991; Guthrie *et al.*, 1992; Leung *et al.*, 1992).

In this chapter, DNA fingerprinting of five *X. albilineans* isolates was attempted using TAFE and RAPD analysis in order to generate reproducible DNA fingerprints which may be useful in identification and differentiation of the isolates.

3.2 MATERIALS AND METHODS

3.2.1 **Preparation of DNA in Agarose Plugs**

SE : 75 mM NaCl

25 mM EDTA

pH 7.4

Lysis buffer : 0.5 mg/ml proteinase K

1% *N*-lauroylsarcosine

0.5 mM EDTA (pH 9.5)

TME : 10 mM Tris.HCl

10 mM $MgCl_2$

0.1 mM EDTA

pH 7.4

TE : 10 mM Tris.HCl

0.5 mM EDTA

pH 7.4

The five *X. albilineans* isolates (Table 2.1) were each grown to saturation in 10 ml Wilbrink's broth, at 28°C in an orbital shaker (2.2.1). Cells were pelleted at 10 000 × *g* for 10 min at 4°C, washed once in 10 ml SE buffer, and adjusted to an OD_{600} value of 2.5 in SE buffer. Equal volumes of the cell suspensions and molten (45°C) 2% low gelling temperature agarose (Seaplaque agarose, FMC Corporation) in TME buffer, were mixed together and dispensed into rectangular slots (4 × 3 × 12 mm). After placing the mixture at 4°C to solidify, agarose plugs were transferred to McCartney bottles. Bacteria embedded in the agarose were lysed and cellular proteins degraded by overnight incubation of the plugs at 56°C in 4 ml of lysis buffer. Plugs were subsequently washed with TE buffer and stored at 4°C in the same buffer. Unsheared total genomic DNA embedded in the plugs were analysed by electrophoresis in 0.7% agarose gels (2.2.2).

3.2.2 Cleavage of DNA in Agarose Plugs

Agarose plugs cut into half-blocks were equilibrated with the appropriate restriction buffers for 3 h on ice in Eppendorf tubes. DNA was cleaved with 20-25 U of *Dra*I, *Eco*RI, *Hin*dIII, *Not*I, *Pst*I, *Sfi*I, *Ssp*I and *Xba*I for 18-24 h at 37°C. In addition to the enzymes and restriction buffers, restriction reactions also contained 1.3 mM dithiothreitol and 0.13 mg/ml bovine serum albumin in a total volume of 155 μl. Reactions were stopped by placing the Eppendorf tubes on ice.

3.2.3 Transverse Alternating Field Electrophoresis

TAFE was performed using the Geneline II DNA Mapper (Beckman Instruments Inc.). Agarose blocks containing cleaved genomic DNA were transferred to the wells of 1% loe electroendosmosis agarose (LE agarose, Beckman Instruments Inc.) gels in 0.25 × TBE buffer (2.2.2), and sealed with 1% agarose. In addition, TAFE was also performed on CsCl-purified genomic DNA from the five *X. albilineans* isolates, cleaved with various restriction endonucleases (3.2.2). In these instances, wells were sealed with molten 2% agarose. Vertical agarose gels, measuring 15 × 15 × 0.75 cm, were placed in the electrophoresis tank containing 0.25 × TBE buffer pre-cooled to 15°C and electrophoresis was carried out at 15°C using various programmes in an attempt to optimise separation of DNA fragments. Programmes varied with regard to voltage, current strength, pulse times and total run times. After electrophoresis, gels were stained for 45 min in ethidium bromide (2 μg/ml) and destained in distilled water for 1-3 h. Gels were viewed on a UV

transilluminator and documented by photography as described previously.

3.2.4 Large Scale Preparation of Genomic DNA

Genomic DNA was prepared from the five *X. albilineans* isolates using a large scale procedure (Ausubel *et al.*, 1989). Bacteria were lysed using the detergent SDS and proteins were removed by digestion with the non-specific protease, proteinase K. Cell wall debris, polysaccharides and remaining proteins were removed by selective precipitation with hexadecyltrimethyl ammonium bromide (CTAB) and high molecular weight DNA was recovered by isopropanol precipitation. DNA was further purified on ethidium bromide-CsCl density gradients.

Each *X. albilineans* isolate was grown to saturation in 2 × 100 ml of Wilbrink's broth. Cultures were pelleted separately at 4 000 × *g* for 10 min at 4°C, resuspended gently in 9.5 ml of TE buffer and transferred to a 35 ml screw-capped polypropylene centrifuge tube. Cells were lysed and cellular proteins degraded by the addition of 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K and incubation for 1 h at 37°C. After the incubation period, 1.8 ml of 5 M NaCl was added and mixed thoroughly to adjust the salt concentration to >0.5 M. This prevented the precipitation of nucleic acids in the following step. 1.5 ml of CTAB/NaCl solution (10% CTAB; 0.7 M NaCl) was added, mixed thoroughly and incubated for 20 min at 55°C. Exopolysaccharides and residual proteins formed complexes with CTAB in this step. CTAB complexes were removed in a subsequent extraction with an equal volume of chloroform/isoamyl alcohol. This

extraction was carried out by repeatedly inverting the centrifuge tubes. Organic and aqueous phases were separated by centrifugation at 6 000 × *g* for 10 min at room temperature. The DNA-containing aqueous phase was transferred to fresh tubes using a wide-bored pipette to minimise DNA shearing and the DNA precipitated by the addition of 0.6 volumes of isopropanol. The stringy, white DNA precipitate was hooked onto the ends of heat-sealed pasteur pipettes and transferred to fresh centrifuge tubes containing 1 ml of 70% ethanol. After washing in ethanol, the DNA pellets were centrifuged at 10 000 × *g* for 5 min at 4°C. Supernatants were discarded and 3 ml of TE buffer was added to the pellets which were allowed to dissolve completely during overnight storage at 4°C.

DNA was further purified by ethidium bromide-CsCl density gradient ultracentrifugation. DNA solutions were added to 6.45 g of CsCl, partially dissolved in 3 ml of TE buffer. Solutions were mixed gently to dissolve the CsCl completely and 300 μl of a 10 mg/ml ethidium bromide solution was added. The mixtures were transferred to Quick-Seal centrifuge tubes which were filled with paraffin oil, balanced and sealed. After centrifugation at 150 000 × *g* (Ti75 rotor) for 24 h at 15°C, DNA bands were viewed with a UV lamp, and collected by puncturing the tubes with 15-G needles. Ethidium bromide was removed by repeated extractions with TE-saturated *N*-butanol. CsCl was removed by dialysis (overnight) against 2 l of TE buffer. The concentration and purity of the DNA was estimated by spectrophotometry and the DNA stored at -20°C.

3.2.5 Amplification of DNA Fragments using Arbitrary Primers

PCR amplification of DNA fragments was accomplished using 10-mer arbitrary primers listed in Table 3.1 (University of British Columbia RAPD Primer Synthesis Project).

TABLE 3.1. Sequences of 10-mer primers used in RAPD reactions

Primer	%G+C	Sequence (5′→ 3′)
UBC 208	80	ACGGCCGACC
UBC 228	80	GCTGGGCCGA
UBC 245	80	CGCGTGCCAG
UBC 251	70	CTTGACGGGG
UBC 262	80	CGCCCCCAGT
UBC 275	80	CCGGGCAAGC
UBC 283	80	CGGCCACCGT

CsCl-purified genomic DNA in TE buffer was precipitated with ethanol and resuspended in sterile deionised water, at a concentration of 5 ng/μl, to prevent possible inhibition of *Taq* DNA polymerase by EDTA present in the buffer. Amplification reactions were performed in 20 μl reaction volumes containing 1 × *Taq* incubation buffer (Boehringer Mannheim), 100 μM each of dATP, dCTP, dGTP and dTTP, 0.2 μM primer, 10 ng genomic DNA, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim). Before the addition of the polymerase, the mixture was heated at 94°C for 5 min and cooled

immediately on ice to denature genomic DNA. After addition of the enzyme, sterile mineral oil (50 μl) was overlaid onto the reaction mixture to prevent evaporation during PCR cycling. Amplification was performed in an Omnigene Thermal Cycler (Hybaid Ltd.). The programme comprised 30 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min, and extension of the primer at 72°C for 2 min. After the cycling steps were completed, the mixture was held at 72°C for 10 min to allow complete extension of amplified products. Amplified DNA fragments were analysed by electrophoresis in 2% agarose gels in 0.5 × TBE buffer. Sizes of the DNA fragments were calculated using the SW5000 Gel Documentation System (UVP Inc.).

3.3 RESULTS

3.3.1 Analysis of DNA in Agarose Plugs

Some of the genomic DNA migrated out of the plugs after agarose gel electrophoresis (Fig. 3.1). The majority of the DNA, however, remained in the plugs. This was most probably due to attachment of the native DNA molecules to the cell envelope and cell debris. Co-migration of the genomic DNA with the CCC form of pXA1 demonstrated the inability of 0.7% agarose gels to resolve large DNA molecules by conventional agarose gel electrophoresis. Two bands observed near the end of the gel probably consist of RNA molecules. These bands gradually disappeared upon storage of the plugs at 4°C. The amount of DNA present in the half-blocks was deemed sufficient for cleavage by restriction endonucleases.

FIG. 3.1 Agarose gel electrophoresis of unsheared genomic DNA from *X. albilineans* isolates, embedded in agarose plugs. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: pXA1, CCC and L forms.

3.3.2 TAFE Fingerprints

Various programmes were employed to optimise the run conditions necessary for the resolution of large DNA fragments by TAFE. However, none of these programmes resolved DNA fragments larger than 20 kb. Even a programme recommended by the manufacturer for separating phage λ concatemers ranging in size from 48.5-720 kb failed to resolve large DNA fragments (not shown) which were expected to fall within this range.

As expected, restriction of genomic DNA with enzymes such as *Eco*RI (not shown), *Hin*dIII

(Fig. 3.2) and *Pst*I (not shown) resulted in a streak consisting of a large number of restriction fragments. Similar streaks were observed after cleavage with the enzymes *Not*I and *Sfi*I (not shown) which recognise octanucleotide GC-rich target sequences. Despite the large restriction fragments not being resolved, slight differences were observed among the five isolates for DNA fragments less than 10 kb in size. Restriction of genomic DNA with *Hin*dIII and electrophoresis using the stated run conditions (Fig.3.2) resulted in unresolved high molecular weight DNA fragments migrating together with fragments in the 20-25 kb range. Apart from a high-intensity 3.8 kb fragment in lane 5 and a distinct triplet of bands in the 3.0-3.4 kb range in lane 4, the profiles were not useful in differentiating among the five *X. albilineans* isolates. The high-intensity band of XA 86-1 corresponds to the 3.8 kb

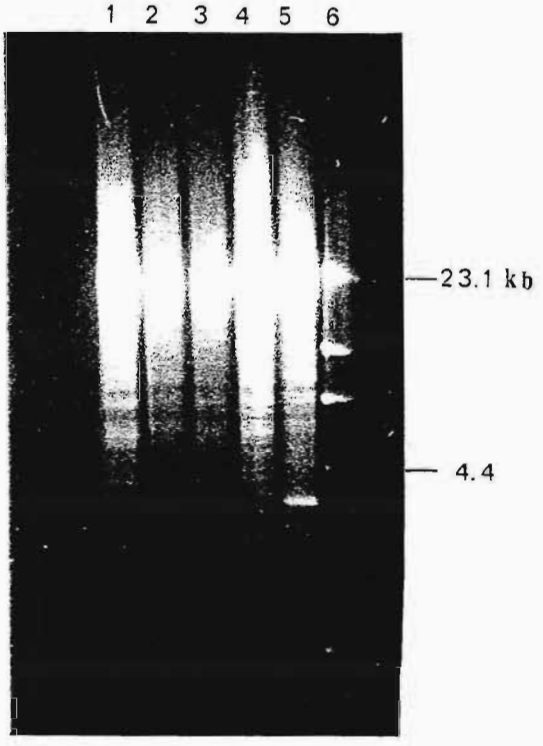

FIG. 3.2. TAFE profiles of CsCl-purified genomic DNA from *X. albilineans* isolates, cleaved with *Hin*dIII. The TAFE programme comprised three 6 h stages, with constant voltage of 100 V for the first stage and 200 V for the following two stages, and pulse times of 30, 60 and 90 s, respectively. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker II.

*Hin*dIII fragment of pXA1. Electrophoresis of genomic DNA cleaved with the rare-cutter *Dra*I (Fig. 3.3) resulted in fewer fragments under 10 kb in size. The majority of the DNA, consisting of large DNA restriction fragments, migrated as a single diffuse band. Differences were evident between PDDCC 196 and XA 86-1 (lanes 4 and 5). XA 86-1 possessed two high intensity bands, approximately 9.0 and 2.3 kb in size and polymorphisms between the two isolates were observed in the 3.7-4.4 kb region. The 9 kb fragment corresponded to the smaller *Dra*I restriction fragment of pXA1.

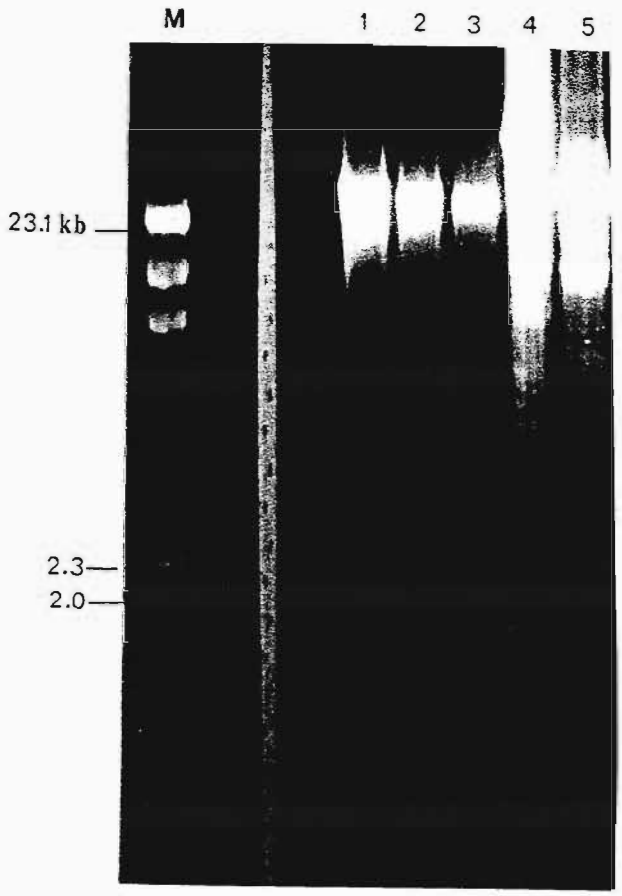

FIG. 3.3. TAFE profiles of CsCl-purified genomic DNA from *X. albilineans* isolates, cleaved with *Dra*I. The TAFE programme comprised three 6 h stages at a constant voltage of 100 V, with pulse times of 2, 3, and 4 min, respectively. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane M: DNA molecular weight marker II.

A programme used for electrophoresis of *Ssp*I-cleaved genomic DNA (Fig. 3.4) resulted in discrete fragments in the 1.5-5.5 kb size range. The absence of a "streak" in this region indicates that *Ssp*I did not cleave the genomic DNA frequently. However, the large restriction fragments were not resolved. Slight differences among the isolates were observed (Fig. 3.4).

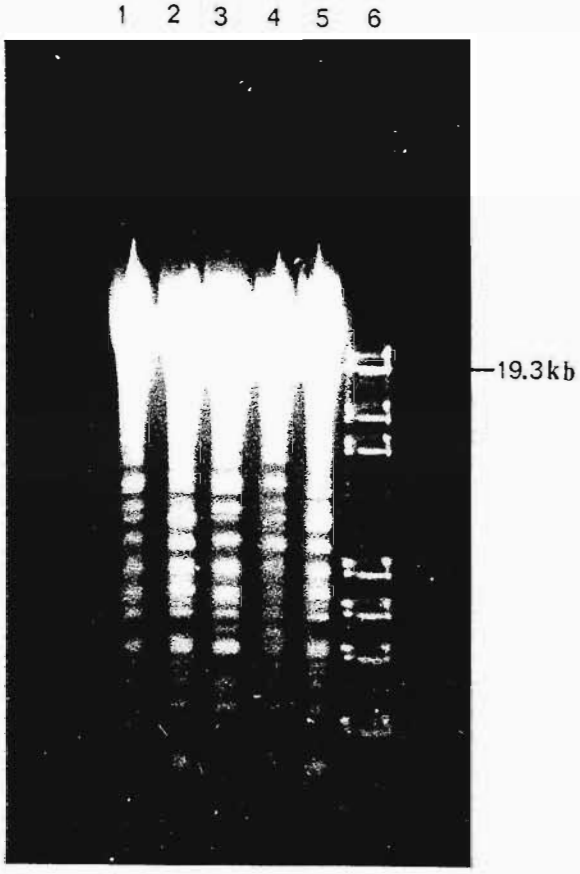

FIG. 3.4. TAFE profiles of *Ssp*I-cleaved genomic DNA, embedded in agarose plugs, from *X. albilineans* isolates. The TAFE programme comprised two 4 h stages and two 5 h stages at a constant voltage of 100, 150, 200, and 250 V, respectively and pulse times of 15 s, 30 s, 2 min, and 3 min, respectively. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker IV.

The same programme produced identical patterns for all five isolates after cleavage with *Xba*I (Fig. 3.5). The smear of unresolved fragments was higher up on the gel compared to those obtained for *Ssp*I cleavage. This meant that *Xba*I restriction resulted in comparatively larger restriction fragments. Consequently, even fewer fragments under 20 kb in size were observed.

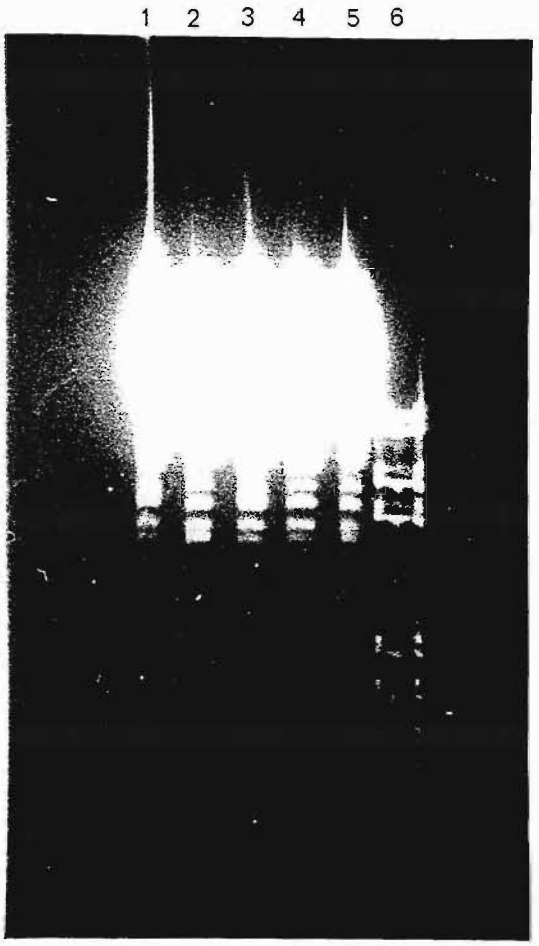

FIG. 3.5. TAFE profiles of *Xba*I-cleaved genomic DNA, embedded in agarose plugs, from *X. albilineans* isolates. The TAFE programme comprised two 4 h stages and two 5 h stages at a constant voltage of 100, 150, 200, and 250 V, respectively and pulse times of 15 s, 30 s, 2 min, and 3 min, respectively. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker IV.

3.3.3 RAPD Analysis

Sufficient DNA polymorphisms were observed in the RAPD profiles of the *X. albilineans* isolates to allow differentiation of the isolates. The two Mauritian isolates had similar profiles for most of the primers tested. The Australian isolate, type strain and South African isolate could be differentiated from each other and the two Mauritian isolates in all of their amplified DNA profiles. UBC primers 208, 228 and 251 led to amplification of reliable DNA markers in only two, one and four isolates, respectively (not shown). Amplification using primer 208 resulted in DNA markers only for isolates 2901 and LS155. Nevertheless, DNA polymorphisms were evident for these two isolates with this primer. A DNA fingerprint of PDDCC 196 only, consisting of three high-intensity bands, was observed after amplification with primer 228. Primer 251 resulted in amplification of high-intensity bands in all isolates except LS155. The DNA profiles of 2901 and XA 86-1 were similar, but DNA polymorphisms were evident between 2905 and PDDCC 196.

Four of the seven primers tested, viz., UBC 245, UBC 262, UBC 275 and UBC 283 resulted in reproducible amplification of DNA markers in all five isolates. RAPD profiles generated with primer 245 (Fig. 3.6) was useful for differentiating LS155 from the other isolates. The profiles of the two Mauritian isolates were identical (lanes 1 and 2). LS155 possessed four unique bands measuring 2.21 kb, 1.42 kb, 0.83 kb and 0.48 kb. This isolate also lacked a 1.5 kb fragment and a 0.94 kb fragment which were present in the other isolates. PDDCC 196 possessed three unique bands, 3.19 kb, 2.08 kb and 1.44 kb in size, but lacked a 1.03 kb fragment present in the profiles of the other isolates. The profile of XA 86-1 was identical to that of the Mauritian isolates with the exception of an

additional 1.39 kb fragment. Two bands, 1.1 kb and 0.63 kb in size. were present in the profiles of all five isolates.

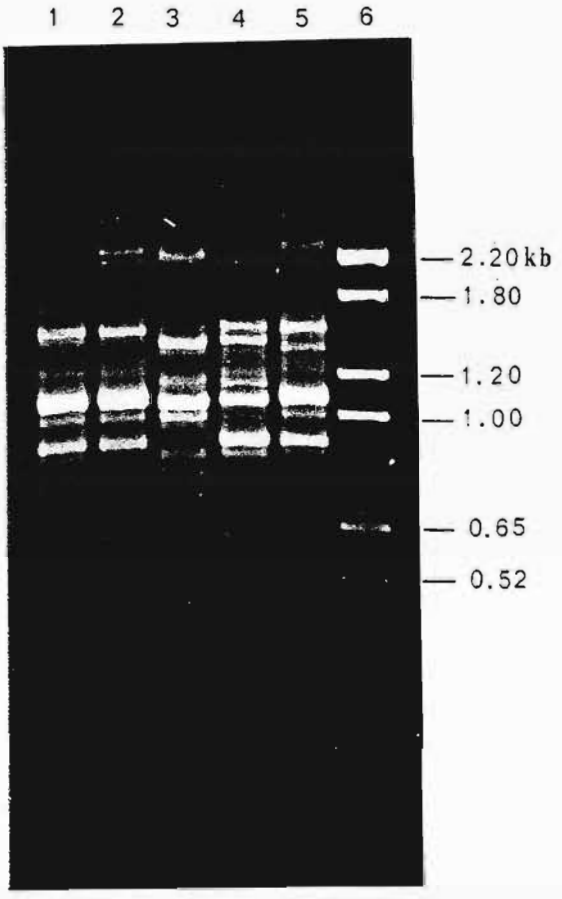

FIG. 3.6. RAPD profiles of *X. albilineans* isolates amplified with primer UBC 245. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker VI.

RAPD profiles generated with primer 275 (Fig. 3.7) was useful in differentiating between the two Mauritian isolates and also produced a distinctive profile for XA 86-1. The profiles of 2905 and LS155 were almost identical with 2905 having additional bands measuring 1.83 kb, 1.28 kb and 0.84 kb. 2901 could be differentiated from 2905 as it lacked the high-intensity bands measuring 1.6 kb and 1.2 kb which were major

amplification products of 2905. PDDCC 196 shared bands of 1.06 and 0.79 kb with 2901, 2905 and LS155 but had unique fragments of 0.56 kb and 0.43 kb. The profile of XA 86-1 consisted of only three bands measuring 2.37 kb, 1.13 kb and 0.53 kb which were unique to this isolate. The 1.13 kb fragment was the major product of the PCR amplification, followed by the 0.53 kb fragment.

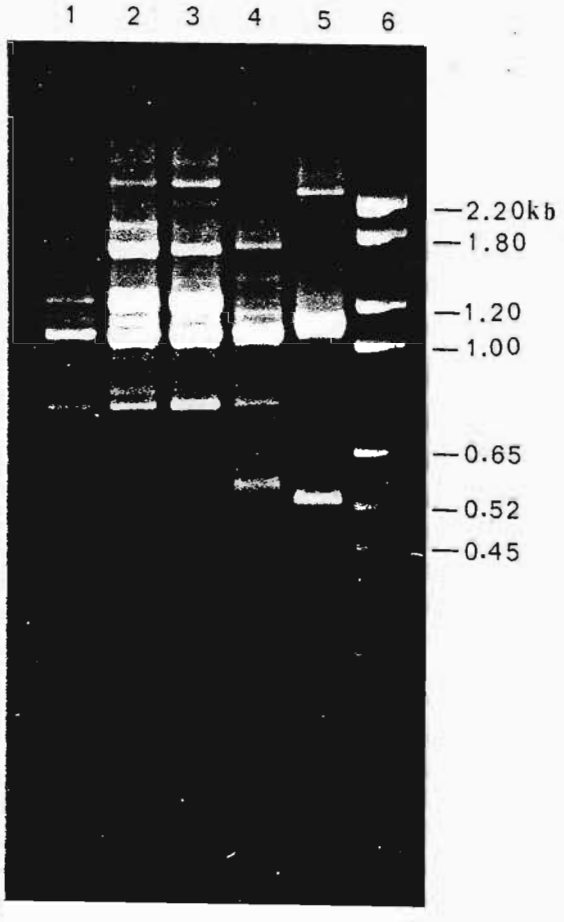

FIG. 3.7. RAPD profiles of *X. albilineans* isolates amplified with primer UBC 275. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker VI.

PCR amplification with primer 283 (Fig. 3.8) again produced identical profiles for 2901 and 2905 except for higher-intensity bands of 2.56 kb for 2905 and 0.92 kb for 2901. The

profiles of LS155, PDDCC 196 and XA 86-1 allowed differentiation of these isolates. LS155 possessed high-intensity fragments of 2.09 kb, 0.81 kb, 0.61 kb and 0.32 kb which were common to all isolates as well as a unique fragment of 0.28 kb. The major amplification product of XA 86-1, a 0.70 kb fragment, occurred only in this isolate. Similarly, a 1.02 kb fragment was unique to PDDCC 196.

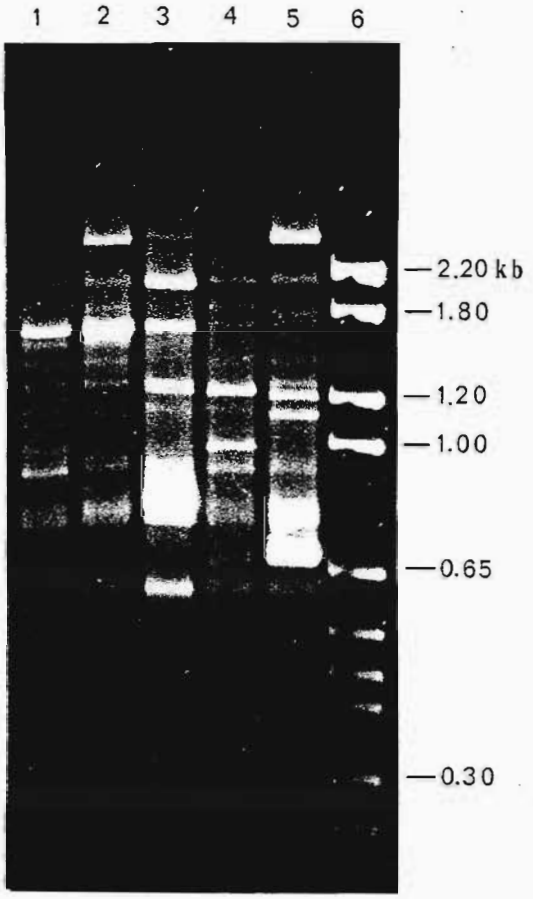

FIG. 3.8. RAPD profiles of *X. albilineans* isolates amplified with primer UBC 283. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker VI.

RAPD profiles generated with primer 262 (Fig. 3.9) differentiated the type strain, PDDCC 196 from the other isolates. This primer amplified a 0.78 kb fragment as the major

product in all the other isolates. Only low-intensity minor amplification products were observed for PDDCC 196.

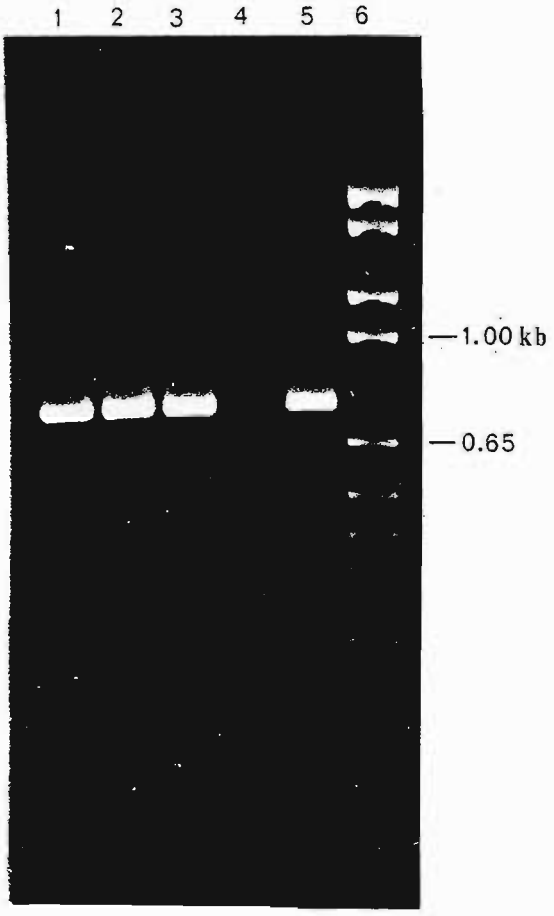

FIG. 3.9 RAPD profiles of *X. albilineans* isolates amplified with primer UBC 262. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; and lane 6: DNA molecular weight marker VI.

3.4 DISCUSSION

DNA fingerprints of the five *X. albilineans* isolates obtained by TAFE and RAPD analysis showed many similarities in terms of co-migrating DNA fragments. This was not surprising as the isolates belong to the same species and are therefore very closely related. However, it was possible to differentiate between isolates by comparison of their

respective DNA fingerprints. Compared to TAFE, RAPD fingerprints were more useful in this regard.

The separation of large restriction fragments by PFGE was not optimal. The most likely reason is that the programmes used were unable to resolve these fragments. Consequently, the fragments migrated together with ± 20 kb fragments to form a smear in this region of the gels. It is unlikely that the smear consists of uncleaved genomic DNA. Firstly, any inhibition of the restriction enzymes by agarose, present in the plugs, was tested by performing TAFE on cleaved CsCl-purified genomic DNA. Presence of the smear after restriction of the purified DNA indicated that it did not contain unrestricted DNA. Secondly, this smear was not observed after restriction with enzymes such as *Eco*RI, *Hin*dII and *Pst*I. These enzymes cleaved the DNA at such a high frequency that all the restriction fragments were under 20 kb in size. Therefore, presence of the DNA smear only when moderate or rare-cutting enzymes were used proved that it contained large restriction fragments rather than uncleaved genomic DNA. It was therefore possible to predict which enzymes would be useful for PFGE by screening for high molecular weight DNA fragments using conventional agarose gel electrophoresis. TAFE profiles obtained with the rare-cutters *Dra*I, *Ssp*I and *Xba*I showed very few fragments under 20 kb in size, with the majority of the DNA being of high molecular weight. Statistically, it was expected that *Dra*I and *Ssp*I, having recognition sequences TTTAAA and AATATT respectively, would cut the genome of *X. albilineans* (65 mol% GC) at any 60 kb. Grothues and Tümmler (1987) analysed *Dra*I and *Ssp*I restriction fragments of *P. aeruginosa* by FIGE and found that most fragments clustered in the 20-100 kb range. Fragments larger than 100 kb and smaller than 20 kb were obtained as pure species.

TAFE profiles of genomic DNA from *X. albilineans* also showed discrete fragments less than 20 kb in size. Furthermore, Cooksey and Graham (1989) found that *Xba*I produced restriction fragments of higher molecular weight than either *Dra*I or *Ssp*I in plant pathogenic pseudomonads and xanthomonads which was also evident in TAFE profiles of *X. albilineans*. These results suggest that restriction of *X. albilineans* genomic DNA with rare-cutting enzymes produced the expected number of fragments but that the large restriction fragments were not resolved by TAFE. The reason for these fragments not being resolved is most likely due to the sub-optimal run conditions for TAFE. The most important conditions that play a role in the separation of fragments include voltage applied, current strength, pulse times and run times. Generally, an increase in the pulse times allows separation of larger fragments. However, various programmes having pulse times ranging from 15 s to 4 min failed to resolve the large restriction fragments, including a programme designed to separate phage λ concatemers which spans the size range in which the restriction fragments were expected. Unfortunately, run conditions used by other researchers in separating bacterial genomic DNA restriction fragments could not be applied to TAFE due to differences in the design of the various types of pulsed field electrophoresis systems.

Slight differences were evident among the *X. albilineans* isolates upon comparison of the DNA fragments under 20 kb in size. However, these fragments represent only a small fraction of the genome and may therefore prove to be misleading. Restriction fragments of the plasmid pXA1 in XA 86-1 also contributed to some of the differences observed. Enzymes such as *Ssp*I and *Xba*I produced many co-migrating DNA fragments in all five isolates. Since these isolates are closely related, it is assumed that these fragments are

identical. It is possible that analysis of the DNA profiles of other xanthomonads might result in the identification of DNA fragments conserved only in *X. albilineans*. These conserved fragments could have potential as DNA probes for the specific detection of *X. albilineans* irrespective of geographic location.

Optimisation of the run conditions for TAFE that allows separation of all restriction fragments has many possible applications that can aid in the molecular characterisation of *X. albilineans* isolates. The size of the genome in *X. albilineans* can be accurately determined as direct visualisation of DNA restriction fragments by TAFE analysis should provide more reliable values than determinations from renaturation rates of DNA or velocity sedimentation combined with electron microscope examination (Grothues and Tümmler, 1987). Also, a long-range restriction map of the genome of *X. albilineans* can be generated using techniques similar to those used to construct a restriction map of pXA1. This physical map can ultimately be converted to a genetic map of the genome by determining the positions of genes of interest on the large restriction fragments by Southern blot analysis using cloned genes. In addition, preparative TAFE will allow the construction of sub-genomic DNA libraries from a single large restriction fragment. Finally, the genetic relatedness of *X. albilineans* isolates from different regions of the world could be assessed by comparison of their genomic restriction profiles. Identification of geographical isolates by their characteristic profiles would be useful in epidemiological studies and in tracing sources of infections.

RAPD fingerprints were found to be extremely useful in differentiating between the five *X. albilineans* isolates. Fingerprints unique to each isolate were reproducibly obtained and

these fingerprints can now serve as a means of identification of each isolate. In addition to identification of the isolates, analysis of RAPD markers were also useful in examining relatedness.

Analysis of RAPD fingerprints showed that the two Mauritian isolates were the most closely related of the five isolates. They possessed identical RAPD profiles for most of the primers tested. Although they could be differentiated using primers 251 and 275, they still shared many common bands with these two primers. The only primer that produced a unique profile for one of the two isolates was primer 208. The other three isolates possessed unique profiles for nearly all of the seven primers used. Of these isolates, LS155 appeared the most closely related to the two Mauritian isolates. The type strain, PDDCC 196, was the most distantly related isolate. It was the only isolate that did not possess a 0.78 kb RAPD marker after amplification with primer 262. It was surprising that the type strain did not possess this marker as it was the only major amplification product of the other four isolates, indicating a possible *X. albilineans*-specific marker. In addition, PDDCC 196 was the only isolate that possessed RAPD markers after amplification with primer 228 and it also possessed unique profiles for all other primers with the exception of 208 for which no DNA fragments were amplified. These profiles suggest that the type strain would form a separate group if this type of analysis was extended to constitute a representative sample of *X. albilineans* from around the world. Similarly, the profiles observed for XA 86-1 may also lead to its assignment into a group separate from that of the other four isolates.

The accuracy of RAPD markers in predicting genetic relationships has been demonstrated

previously (Bostock *et al.*, 1993; Fegan *et al.*, 1993). The results of RAPD analysis in this study supports those of Pillay *et al.* (1993) involving analysis of LPS from the same five isolates of *X. albilineans*. They reported three distinct patterns after electrophoresis of the LPS, with isolates 2901, 2905 and LS155 having similar profiles that were different to those of PDDCC 196 and XA 86-1. Immunoblotting also showed the same results and further demonstrated that although the IgG raised against the other isolates cross-reacted with the LPS of one another, this was not observed for the type strain. Although Seetal (1989) clustered 2901, LS155 and XA 86-1 into a single group and 2905 into a separate group, using data from agglutination reactions, all three studies suggested that PDDCC 196 belonged to a different sub-group in *X. albilineans*. These results raise the question as to whether the continued designation of PDDCC 196 as the type strain of *X. albilineans* is warranted, especially since NCPPB 2247 is the phenotypic centrotype of *X. albilineans* (Van den Mooter and Swings, 1990).

RAPD analysis has the advantage of being the least labour-intensive of the methods used to characterise and determine relationships between organisms. The technique requires only small amounts of DNA and is relatively easy to standardise between laboratories. It requires little knowledge of the molecular biology of the species being examined and no sequence information is necessary. Unlike PCR typing which is performed with pairs of primers from a known sequence, it is not confined to changes occurring in only a small part of the genome. Sources of DNA polymorphisms observed in RAPD profiles include deletions of a priming site and insertions that either increase the size of the amplification product or make the priming sites too distant to support amplification. Not all amplification products are the result of perfect pairing between the primer and the DNA

template. Williams *et al.* (1990) stated that the number of DNA fragments amplified from bacterial genomes could only be explained on the basis of mismatch between the primer and the DNA template. Low-intensity markers observed in the RAPD profiles of *X. albilineans* isolates are therefore the result of such mismatches. It was observed that these low-intensity fragments were not reproducibly amplified whereas the high-intensity markers were. It is therefore important that only RAPD markers that are reproducibly amplified are used to type organisms. It is possible to reduce the number of mismatches by increasing the annealing temperature in the PCR reactions. This increase in stringency would lead to elimination of the low-intensity bands and could result in simpler and more reproducible profiles. Although RAPD analysis has been used primarily to detect polymorphisms among the *X. albilineans* isolates, RAPD markers common to all isolates could also be used to develop *X. albilineans*-specific DNA probes. Analysis of the profiles produced for the five isolates suggest that RAPD analysis may provide an adequate means for assessing the genetic relatedness of *X. albilineans* isolates in a study extended to include a representative sample of isolates from all sugarcane growing regions.

CHAPTER FOUR

CONSTRUCTION OF A GENOMIC LIBRARY FOR XA 86-1

4.1 INTRODUCTION

A genomic library of *X. albilineans* refers to a collection of *E. coli* cells, each carrying a DNA fragment from *X. albilineans*. Each DNA fragment is ligated to a vector molecule and the fragments collectively represent the entire genome of *X. albilineans*. Construction of such a library simplifies the isolation of a DNA sequence of interest. *E. coli* colonies carrying a particular sequence can be identified by complementation, hybridisation with a DNA probe or by growth on a selective medium.

The most important factor in the generation of a useful recombinant DNA library, is the creation of a huge population of clones to ensure that the library contains at least one copy of every sequence of interest. Also, cloned DNA fragments should randomly represent the sequences present in the genome. The size of a library of random genomic DNA fragments which ensures representation of a particular sequence, is dictated by the size of the cloned fragments and the size of the genome. The probability of a sequence of interest being represented in a library can be estimated statistically based on Poisson distribution (Clarke and Carbon, 1976). The number of independent clones, N, needed to isolate a sequence with probability P is given by :

$$N = \ln(1-P)/\ln[1-(I/G)]$$

where I is the average size of the cloned fragments and G is the size of the genome, in base pairs.

Random genomic DNA fragments of a particular size range are usually produced by partial digestion with the restriction enzymes *Sau*3A and *Mbo*I which recognise a 4 bp sequence (GATC). These enzymes produce the most random collection of fragments as they cleave DNA frequently (every 256 nucleotides). Plasmid, bacteriophage and cosmid vectors can be used to construct a library. Compared to plasmid vectors, bacteriophage and cosmid vectors can accommodate larger inserts. Bacteriophage vectors can accommodate 10-20 kb inserts and cosmids can accommodate 20-40 kb inserts. The inserted DNA is introduced into *E. coli* cells by transduction. However, plasmids carrying inserts >10 kb are inefficiently transformed into *E. coli* cells. Thus, genomic libraries constructed using plasmid vectors comprise a larger number of clones.

The construction of genomic libraries for plant pathogenic bacteria has proved useful for the identification of genes required for pathogenicity, the hypersensitive response (*hrp* genes) and avirulence (*avr* genes). The standard technique used to identify such genes involves transposon mutagenesis to produce non-pathogenic mutants and screening of the library for recombinants able to restore pathogenicity to the mutants. Complementation of mutants has resulted in the identification of pathogenicity genes in *X. campestris* pv. *vesicatoria* (Seal *et al.*, 1990) and *X. campestris* pv. *glycines* (Hwang *et al.*, 1992), *hrp* genes in *Erwinia amylovora* (Bauer and Beer, 1991) and *P. syringae* pv. *syringae* (Huang *et al.*, 1988), and avirulence genes in *P. solanacearum* (Ma *et al.*, 1988) and *P. syringae* pv. *tabaci* (Salch and Shaw, 1988). Besides complementation, libraries can also be

screened using DNA probes. Hopkins *et al.* (1992) demonstrated the presence of a family of avirulence genes in *X. campestris* pv. *oryzae* by screening a genomic library with the avirulence gene *avrBs3*, from *X. campestris* pv. *vesicatoria*. They concluded that two genes present in *X. campestris* pv. *vesicatoria*, *avrXa7* and *avrXa10*, are members of an avirulence gene family in xanthomonads. Similarly, Todd *et al.* (1990) showed that *X. campestris* pv. *oryzae* had genes isofunctional with pathogenicity genes from *X. campestris* pv. *campestris*. The easiest method for screening of libraries is by positive selection on agar plates. Examples of phenotypes identified by this method include protease expression in *X. campestris* pv. *campestris* (Tang *et al.*, 1987) and sucrose activity in the ethanologenic gram-negative bacterium *Zymomonas mobilis* (Gunasekaran *et al.*, 1990).

The aim of the work presented in this chapter was to construct a genomic library for XA 86-1 in order to provide a ready source of cloned *X. albilineans* genes. This library would, in the future, be screened for specific genes of interest in *X. albilineans*. The plamid vector pEcoR252 was chosen for construction of the library since the vector contains in addition to an ampicillin-resistance gene, a gene coding for the restriction endonuclease *Eco*RI. Insertion of DNA fragments into the *Bgl*II site of the plasmid results in inactivation of the *Eco*RI gene. Hence, only *E. coli* cells harbouring recombinant plasmids are able to grow on ampicillin-containing media. *Sau*3A-generated genomic DNA fragments in the 3-5 kb range were used to construct the library.

4.2 MATERIALS AND METHODS

4.2.1 Large Scale Preparation of Genomic DNA

Genomic DNA from XA 86-1 was prepared on a large scale and purified by ethidium bromide-CsCl density gradient ultracentrifugation as described in section 3.2.3.

4.2.2 Partial Digestion of Genomic DNA

Complete digestion of genomic DNA with the 4 bp cutter *Sau*3A results in DNA fragments of less than 1 kb. In order to obtain DNA fragments in the 3 to 5 kb region, partial digestion was therefore necessary. Partial digestion may be achieved by either varying the enzyme concentration or the time of incubation. The former procedure was employed to establish conditions for partial digestion of genomic DNA.

A reaction mixture containing 30 μg genomic DNA and restriction enzyme buffer was prepared to a final volume of 150 μl. 30 μl of the mixture was dispensed into an Eppendorf tube (tube 1), 15 μl dispensed into tubes numbered 2-8 and the remainder dispensed into tube number 9 which served as a negative control. All tubes were chilled on ice during the subsequent serial dilution. 4 U *Sau*3A were added to tube 1 and mixed to give a final concentration of 0.67 U/μg DNA. 15 μl of this mixture was added to tube 2 to give a final concentration of 0.33 U/μg DNA. The two-fold serial dilution was continued through to tube 8. Tubes 1-8 were placed in a water bath set at 37°C for 1 h.

Reactions were stopped by inactivation of the enzyme at 70°C for 15 min. Each sample was analysed by electrophoresis in a 0.7% agarose gel and stained in ethidium bromide. Electrophoresis was carried out slowly (1.25 V/cm) until the bromophenol blue dye had just migrated out of the gel. DNA Molecular Weight Marker IV (Boehringer Mannheim) was used to estimate DNA size. The amount of *Sau*3A needed to produce maximum intensity of fluorescence in the 3-5 kb region of the gel was established. Intensity of fluorescence is related to the *total* number of DNA molecules. In order to obtain the maximum number of *different* DNA molecules in this size range, half the amount of *Sau*3A that produced maximum amount of fluorescence (Maniatis *et al.*, 1982), was used in subsequent large scale restriction digestion reactions.

After establishing the amount of enzyme required for production of the maximum number of molecules in the 3-5 kb size range, 100 μg of genomic DNA was partially digested with the appropriate number of units of *Sau*3A for 1 h at 37°C, and the enzyme inactivated by heating at 70°C for 15 min.

4.2.3 Isolation of Genomic DNA Fragments

Partially digested genomic DNA was electrophoresed in a 1% low gelling temperature agarose gel at 1.25 V/cm until the bromophenol blue dye had just migrated out of the gel. After staining in ethidium bromide, gel slices containing DNA fragments in the 3-5 kb region were excised and transferred to pre-weighed Eppendorf tubes. Genomic DNA fragments were isolated using the Geneclean kit, according to the protocol described in

section 2.2.6.1. An aliquot of DNA solution was separated by electrophoresis in an agarose gel in order to estimate concentration of the DNA. The remainder was stored at -20°C.

4.2.4 Linearisation and Dephosphorylation of the Cloning Vector

The plasmid cloning vector, pEcoR252, was linearised with *Bgl*II, which cleaves the plasmid in the middle of the gene coding for the endonuclease *Eco*RI. 1.5 μg of CsCl-purified pEcoR252 was cleaved with 5 U of *Bgl*II in a reaction volume of 15 μl, for 1 h at 37°C. 1 μl of the reaction mixture was analysed by agarose gel electrophoresis to verify that complete cleavage had occurred. After linearisation, the enzyme was inactivated and removed by phenol/chloroform extraction. In order to prevent self-ligation in the ligation reaction, the vector was dephosphorylated as described in section 2.2.6.2 and resuspended in TE buffer. 2 μl was analysed by agarose gel electrophoresis to estimate the concentration of the dephosphorylated vector and the DNA stored at -20°C.

4.2.5 Ligation of Genomic Fragments to the Cloning Vector

Compatible ends of equimolar (22.5 fM) amounts of *Bgl*II-linearised pEcoR252 and *Sau*3A-digested genomic DNA fragments were ligated using T4 DNA ligase. The ligation reaction mixture comprised the following:

2 μl pEcoR252 (50 ng)

3 μl genomic DNA fragments (75 ng)

3 μl H_2O

1 μl ligation buffer (10 X)

1 μl T4 DNA ligase (1 U)

Prior to addition of ligation buffer and DNA ligase, DNA molecules were heated at 45°C for 5 min and immediately chilled on ice to melt any cohesive termini that may have reannealed. The reaction mixture was incubated overnight at 25°C and the reaction terminated by the addition of 0.5 μl of 0.5 M EDTA (pH 8) and the mixture stored at 4°C.

4.2.6 Transformation

Competent *E. coli* DH5αF´ cells were prepared using a $CaCl_2$ procedure as described in section 2.2.6.3. Cells were thawed slowly on ice, the ligation reaction mixture diluted with an equal volume of TE buffer and 1 μl aliquots of the dilution added to 100 μl aliquots of competent cells. Recombinant plasmids in the ligation mixture were transformed into *E. coli* cells according to the procedure described in section 2.2.6.5. Colonies containing recombinant plasmids were selected on YT agar plates supplemented with ampicillin. 250 μl of the cell suspensions were spread on each petri-dish. Plates were incubated overnight at 37°C.

4.2.7 Storage of the Genomic Library

E. coli colonies carrying *X. albilineans* genomic DNA fragments were enumerated using a colony counter. Cells were washed off the plates with YT broth containing ampicillin. Glycerol was added to the cell suspension to a final concentration of 15%, the suspension mixed thoroughly, and stored in 0.5 ml aliquots at -70°C. Each tube theoretically represented the genomic library of XA 86-1, cloned in *E. coli.*

4.2.8 Plasmid Isolation

In order to confirm that *X. albilineans* genomic fragments were cloned in *E. coli*, plasmid DNA was isolated from 8 randomly selected clones. The modified alkaline lysis procedure of Birnboim and Doly (1979) described in section 2.2.6.6, was used to isolate recombinant plasmids. Plasmid DNA was analysed by electrophoresis of intact and *Bgl*II-restricted molecules in 0.7% agarose gels.

4.2.9 Screening of the Genomic Library

The genomic library was screened for cells carrying *X. albilineans* DNA fragments that coded for sucrose utilisation. Frozen *E. coli* cells were thawed, diluted and plated onto M9 minimal medium containing 0.2% sucrose and 50 μg/ml ampicillin. Plates were examined for colonies growing on sucrose after an overnight incubation at 37°C. To

verify their viabibity, a YT agar plate supplemented with ampicillin was also inoculated with the cells.

4.3 RESULTS

4.3.1 Genomic DNA Isolation

Genomic DNA from XA 86-1 appeared as a single band after ethidium bromide-CsCl density gradient ultracentrifugation. The concentration of the isolated DNA was estimated to be 0.355 μg/μl. The low OD_{260}/OD_{280} ratio of 1.51 indicated that the DNA preparation contained protein contaminants.

4.3.2 Partial Digestion of Genomic DNA

The amount of *Sau*3A needed to produce the maximum number of DNA fragments in the 3-5 kb region was obtained by cleaving 30 μg of genomic DNA with varying amounts of *Sau*3A and analysis of restriction products by agarose gel electrophoresis (Fig. 4.1). Maximum fluorescence in the 3-5 kb region occurred in lane 7. This corresponded to an enzyme concentration of 0.01 U/μg DNA. Therefore, the enzyme concentration used in large scale cleavage of genomic DNA (Fig. 4.2) was half of this value, i.e., 0.005 U/μg DNA.

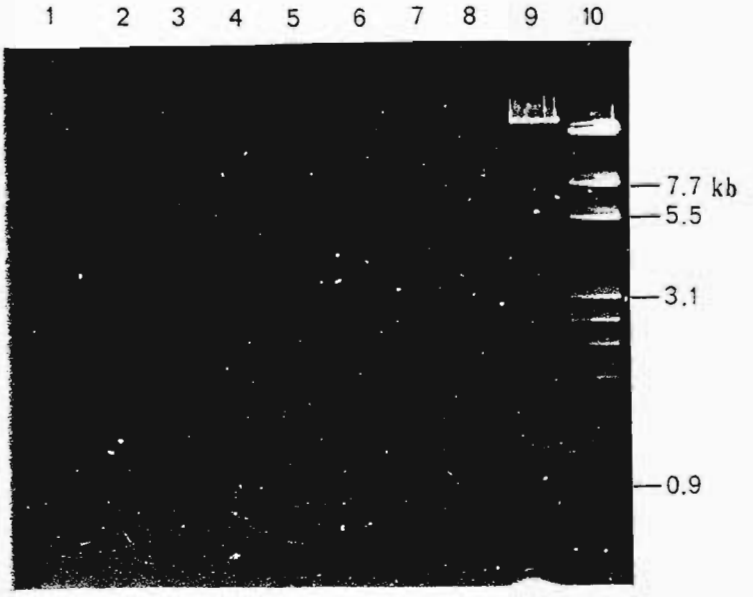

FIG. 4.1. Partial digestion of genomic DNA from XA 86-1 with varying amounts of *Sau*3A. Lanes 1-8: twofold serial dilutions of *Sau*3A ranging from 0.67 U/μg DNA to 0.005 U/μg DNA; lane 9: uncleaved genomic DNA; and lane 10: DNA molecular weight marker IV.

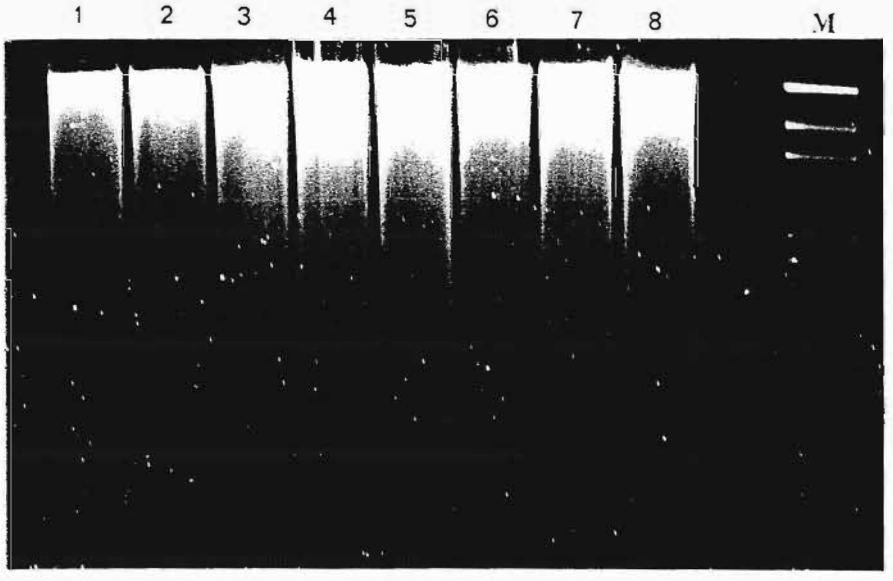

FIG. 4.2. Preparative agarose gel electrophoresis of products of large scale partial restriction of genomic DNA from XA 86-1, with *Sau*3A. Lanes 1-8: XA 86-1 DNA cleaved with *Sau*3A to produce fragments in the 3-5 kb range; and lane M: DNA molecular weight marker IV.

4.3.3 Cloning of Genomic DNA Fragments

The concentration of the dephosphorylated cloning vector and the 3-5 kb genomic DNA fragments was estimated to be approximately 25 ng/μl (Fig. 4.3). After ligation of the reactant molecules and transformation of recombinant plasmids, a total of 3 571 ampicillin-resistant *E. coli* colonies were obtained. Assuming that the size of the genome in *X. albilineans* is approximately the same as *P. fluorescens*, i.e,; 7.4 x 10^6 bp (Bak *et al.*, 1970), this therefore represents an 85% probability that a desired DNA sequence had been cloned.

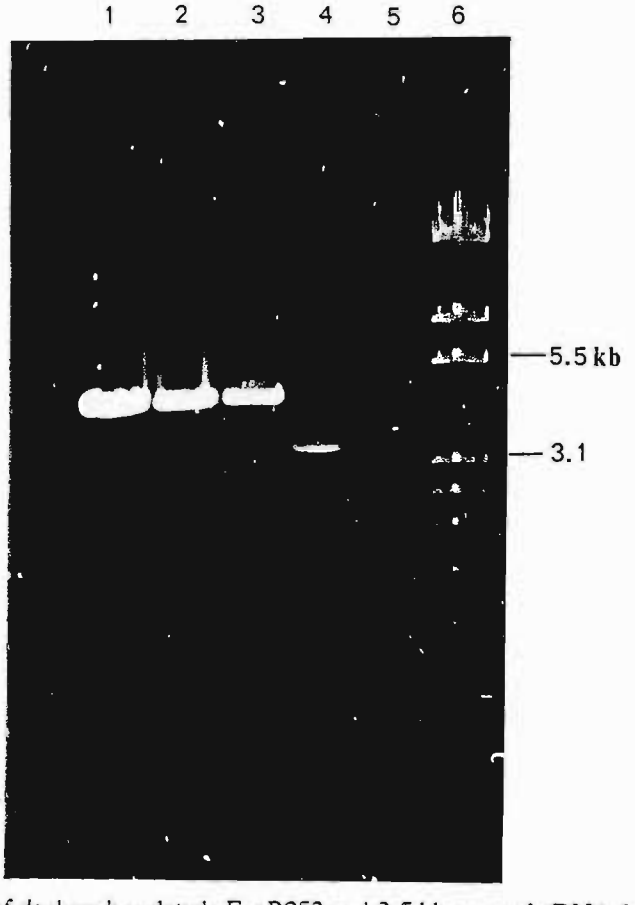

FIG. 4.3. Isolation of dephosphorylated pEcoR252 and 3-5 kb genomic DNA fragments and visual estimation of their concentrations. Lanes 1-3: 500 ng, 250 ng and 125 ng *Eco*R1-linearised pBR322; lane 4: dephosphorylated pEcoR252; lane 5: 3-5 kb genomic DNA fragments and lane 6: DNA molecular weight marker IV.

Cloning of genomic DNA fragments was verified by plasmid isolation from random clones and restriction endonuclease digestion of isolated plasmids. Plasmids isolated from all eight *E. coli* colonies migrated slower than the plasmid vector (Fig. 4.4). The larger size of these plasmids indicated that the vector was ligated to *X. albilineans* genomic DNA fragments. The two bands observed for the recombinant plasmids correspond to CCC DNA (lower band) and OC DNA (upper band). The fastest-migrating band in lane 9 consists of CCC pEcoR252. The other low-intensity bands corresponded to the linear form, open circular form and concatamers of the plasmid. *Bgl*II restriction of the recombinant plasmids resulted in cleavage of five of the eight plasmids (Fig. 4.5).

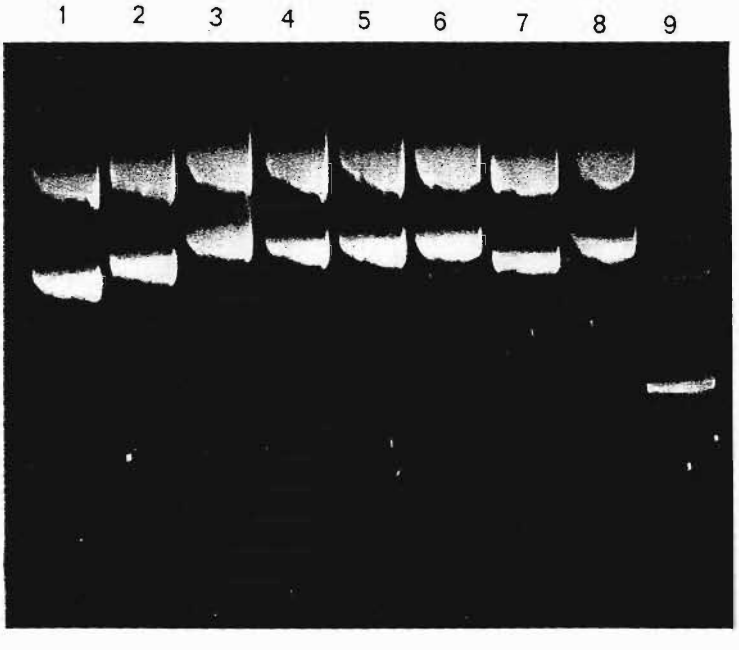

FIG. 4.4. Recombinant plasmids isolated from eight *E. coli* clones from the genomic library of XA 86-1. Lanes 1-8: plasmids isolated from the eight clones and lane 9: uncleaved pEcoR252.

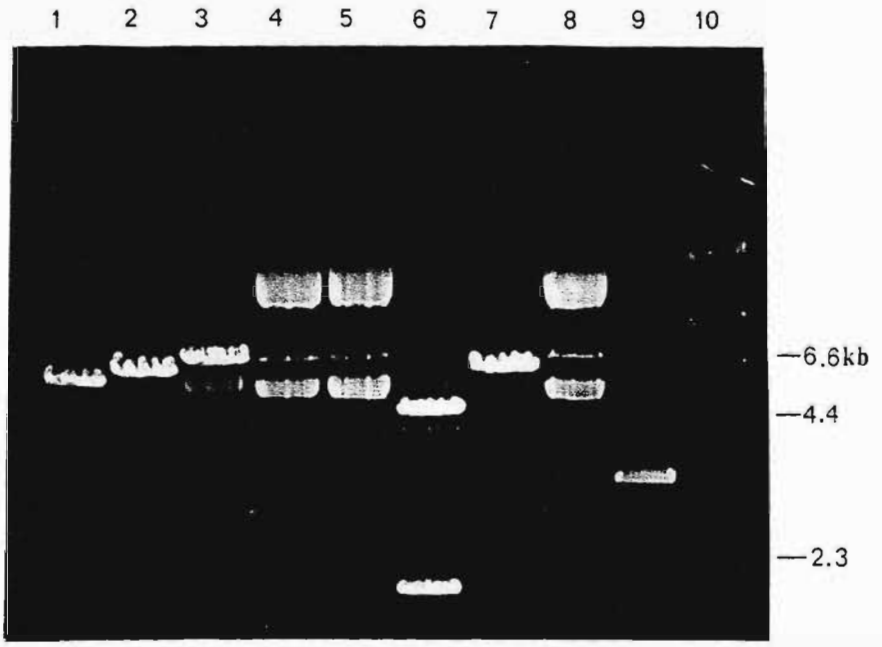

FIG. 4.5. *Bgl*II restriction of recombinant plasmids isolated from *E. coli* clones from the genomic library of XA 86-1. Restriction profiles verify that the plasmids consist of XA 86-1 genomic fragments ligated to pEcoR252. Lanes 1-8: plasmid isolated from eight *E. coli* clones; lane 9: *Bgl*II-restricted pEcoR252 and lane 10: DNA molecular weight marker II.

Although *Bgl*II and *Sau*3A produce protruding termini that can be ligated together, the *Bgl*II restriction site is conserved in only one of four possible hybrid molecules. In lanes 1, 2, 3 and 7 a high intensity band was observed which migrated slower than pEcoR252. Presence of this band indicated that these recombinant plasmids were cleaved at only one site by *Bgl*II. This suggests that either only one of the two *Bgl*II restriction sites was conserved after ligation of the *Bgl*II termini of the vector with the *Sau*3A termini of the genomic fragments or that the inserted DNA possessed a single *Bgl*II restriction site. The sizes of these linear molecules corresponded to the expected sizes of the recombinant molecules. The recombinant plasmids in lanes 4, 5 and 8 were not cleaved by *Bgl*II,

implying that both *Bgl*II sites were not conserved. The plasmid in lane 6 was cleaved at two sites by *Bgl*II, producing a 4.7 kb fragment and a 2.1 kb fragment. Since a band corresponding to the vector only was not evident, one or both of the sites are present in the genomic DNA insert. Hence, this plasmid carries a 3.5 kb insert, 1.4 kb of which remains attached to the cloning vector after *Bgl*II restriction.

4.3.4 Screening of the Genomic Library

None of the *E. coli* cells plated onto M9 medium were capable of growth on sucrose as a sole carbon source, indicating that they had not acquired a sucrase gene from XA 86-1.

4.4 DISCUSSION

One of the most important strategies in characterising genomes of bacteria is the construction of a genomic library. This initial step has been accomplished for XA 86-1. It is hoped that construction of the library will lead to the identification of specific genes of interest in *X. albilineans*. Of particular importance are genes involved in the production of albicidin and genes coding for resistance to albicidin.

The probability of isolating a desired DNA sequence from the library has been calculated to be 85%. This analysis assumes that the DNA was cleaved completely randomly prior to ligation to the vector. Such random cleavage is only possible by mechanical shearing

of DNA (Ausubel *et al.*, 1989). However, cloning of sheared DNA is not very efficient. Sufficiently random DNA fragments are, therefore, usually obtained by partial digestion with restriction enzymes that cleave DNA frequently. The second reason for using *Sau*3A was that it produced DNA fragments that were complementary to the ends of the cloning vector. DNA fragments in the 3-5 kb range were chosen as fragments in this range are efficiently cloned into the 3.3 kb vector. Although the cloning of larger fragments would have been desirable, this would have led to fewer transformants being produced.

A complete genomic library depends not only on the number of transformants obtained but also on the percentage of transformants harbouring recombinant plasmids. Use of a vector such as pEcoR252 and dephosphorylation of the vector should theoretically select for recombinant clones only. This was observed when eight random clones were tested for genomic inserts. The importance of the DNA concentrations in the ligation reaction is illustrated by the reports of Seal *et al.* (1990) and Todd *et al.* (1990) who obtained 97% and 70% recombinant clones, respectively, using similar cosmid vectors which do not prevent "empty" clones from growth.

Screening of the library for sucrase production in *E. coli* clones was unsuccessful. This may have been due to the desired DNA sequence not being represented in the library. Alternatively, the gene may be represented in the library but cannot be expressed in *E. coli*, due to the lack of a suitable promoter. Also, the size of the inserts may have resulted in *X. albilineans* operons and individual genes being cleaved and therefore inactivated. However, DNA libraries constructed with bacteriophage and cosmid vectors can accommodate larger inserts which reduces disruption of genes. There is also a greater

possibility of entire operons being cloned, making expression of foreign genes easier.

Future screening of the genomic library can lead to the identification of pathogenicity genes as well as genes that can serve as genetic markers on the genome of XA 86-1. Screening of the library by hybridisation with specific DNA probes can identify genes that code for non-selectable traits. Of special interest are genes coding for pathogenicity, the hypersensitive response and avirulence. It is possible that *X. albilineans* may possess *hrp* genes as Akhtar *et al.* (1988) observed a hypersensitive reaction after inoculation into tobacco plants. Strategies used to identify *hrp* genes in other plant pathogenic bacteria (Bauer and Beer, 1991; Seal *et al.*, 1990;) could be applied to identify such genes in *X. albilineans*. The first step would involve transposon mutagenesis to create mutants that are unable to induce the hypersensitive response. Screening of the library for clones that restore this phenotype in the mutant by conjugation would then lead to the isolation of *hrp* genes. Other clones such as those coding for albicidin resistance and ß-glucosidase, invertase and gelatinase enzymes can be isolated by screening for the relevant phenotypes, provided that the genes are expressed in *E. coli*. Construction of the genomic library therefore allows screening of any desired gene from *X. albilineans* in a host that is genetically well-characterised.

CHAPTER FIVE

DETECTION OF THE SOUTH AFRICAN ISOLATE OF *X. albilineans* WITH A PLASMID DNA PROBE

5.1 INTRODUCTION

Recent advances in molecular biology and biotechnology are being applied to the development of rapid, specific, and sensitive tools for the detection of plant pathogens. As a result of the specificity of nucleic acid probes, they have been applied for diagnostic purposes, including the detection of plant pathogens (Miller and Martin, 1988). Although, at present, the most commonly used labels are radioactive, the development of nonradioactive probes has greatly enhanced the applicability of nucleic acid probes as diagnostic tools.

The use of plasmid DNA probes for the detection of bacteria is advantageous since plasmid DNA sequences are usually present at a higher copy number than chromosomal DNA sequences. It is also easier to develop specific probes with plasmid DNA, simply because they have fewer restriction fragments. A plasmid DNA probe developed by Gilbertson *et al.* (1989) was sensitive enough to detect bacteria causing common bacterial blight of bean in bean leaves and debris. Therefore, the use of DNA probes in the identification of plant pathogenic bacteria does not require time-consuming isolation and characterisation steps using biochemical, physiological and pathogenicity tests. Also,

detection of plant pathogenic bacteria in host plants is more rapid using DNA probes compared to currently used techniques, most of which rely on the development of visual disease symptoms.

In this chapter, a DNA probe derived from pXA1 was used in the detection of *X. albilineans* to determine the specificity and sensitivity of the probe, using a nonradioactive technique.

5.2 MATERIALS AND METHODS

5.2.1 Minipreparation of Bacterial Genomic DNA from *Xanthomonas* spp.

Genomic DNA from *X. axonopodis* (*Xax*), *X. campestris* pv. *malvacearum* (*Xcm*), *X. campestris* pv. *pruni* (*Xcp*), *X. fragariae* (*Xf*) and *X. maltophilia* (*Xm*) was isolated using the CTAB-method (Ausubel *et al.*, 1989). The miniprep protocol was essentially a scaled-down version of the large scale procedure described in section 3.2.4, except that the DNA was not purified further in ethidium bromide-CsCl density gradients. Instead, DNA isolated from 1.5 ml of the bacterial cultures was spooled, washed in 70% ethanol, dissolved in 100 μl of TE buffer and stored at -20°C.

5.2.2 Preparation of Genomic DNA from *X. albilineans* Isolates for Hybridisation

Genomic DNA from the five *X. albilineans* isolates, embedded in agarose plugs, was prepared as described in section 3.2.1. For hybridisation studies, DNA was first restricted with *Eco*RI as described in section 3.2.2 and the fragments separated by agarose gel electrophoresis in 1% agarose gels. DNA fragments were then transferred to nylon membranes (Hybond-N, Amersham) using a vacuum blotting unit (Omeg Scientific).

5.2.3 Southern Blotting

Depurination solution	:	0.25 M HCl
Transfer solution	:	0.4 M NaOH
		0.6 M NaCl

Southern transfer of genomic DNA restriction fragments was performed by vacuum blotting using an alkaline transfer solution. The gel stack consisted of two pieces of Whatman 3MM paper, a piece of nylon membrane and the agarose gel. The blotting paper (same size as the gel) and the nylon membrane (3 mm larger than the gel on all sides) were pre-wetted in deionised water for 20 min. The pieces of blotting paper were placed in the middle of the porous support screen of the vacuum blotting apparatus and the nylon membrane was centred on them. The agarose gel was placed onto the membrane and any air bubbles trapped under the gel were removed. A rubber mask was

positioned around the gel, ensuring that a proper vacuum seal was obtained. The vacuum pump was subsequently adjusted to 15 cm of Hg and left on throughout the transfer process. Pre-treatment of the gel involved covering the surface of the gel with depurination solution for 10 min. This treatment resulted in breakage of large DNA fragments which made subsequent transfer to the membrane easier. After depurination of the DNA, the surface of the gel was continually covered with transfer solution for 90 min, ensuring that the gel did not dry out. After transfer, the gel and the nylon membrane were removed and the positions of the wells marked on the corresponding positions on the membrane which was then air-dried. The DNA was crosslinked to the membrane by exposure to UV light for 3 min, the membrane sealed in a plastic bag and stored at 4°C.

5.2.4 **Slot Blotting**

20 × SSC	:	3 M NaCl
		0.3 M Na_3-citrate
		pH 7.0
Transfer solution	:	0.125 × SSC

Slot blots were performed using the PR 600 Slotblot (Hoefer Scientific Instruments). A piece of nylon membrane was soaked in deionised water and placed in the Slotblot. Genomic DNA was diluted to the required concentrations, in a total volume of 50 μl, in transfer solution and denatured by boiling for 10 min and cooling immediately on ice. The freshly denatured DNA was loaded into the wells of the Slotblot apparatus which

consisted of slots measuring 6 mm × 0.8 mm. DNA samples were blotted onto the nylon membrane by applying a vacuum of 25 cm of Hg and the wells were washed with 3 × 1 ml aliquots of transfer solution. After the washing steps, the nylon membrane was removed and air-dried. DNA was crosslinked to the membrane by exposure to UV light for 3 min and used immediately for hybridisation to the plasmid DNA probe.

5.2.5 Lysis of *X. albilineans* Cells on Nylon Membranes

Denaturing solution	:	0.5 M NaOH
		1.5 M NaCl
Neutralising solution	:	0.5 M Tris.HCl, pH 8
		1.5 M NaCl
PBS	:	7 mM Na_2HPO_4
		3 mM $NaH_2PO_4.H_2O$
		145 mM NaCl
		pH 7.4

In order to test the sensitivity of the plasmid DNA probe, it was hybridised to DNA from a ten-fold serial dilution of cells, starting from 10^7 cells and going down to 10 cells. The cell concentration of a culture of XA 86-1 was determined using a bacterial counting chamber (Thoma, Weber Scientific International Ltd.), diluted to a concentration of 10^9 cells/ml with Wilbrink's broth and the OD_{600} value of the diluted cell suspension was measured. During subsequent experiments, a culture of XA 86-1 was merely diluted to

reach this value in order to obtain a cell concentration of 10^9 cells/ml. Ten-fold serial dilutions of this culture were carried out in PBS, until a concentration of 10 cells/ml was reached. One ml of the cell suspensions ranging from 10^7 cells/ml to 10 cells/ml were loaded into the wells of the Slotblot apparatus and filtered onto a nylon membrane. The wells of the Slotblot apparatus were washed with 1 ml of PBS and the nylon membrane was removed and air-dried. The cells on the membrane were lysed and the liberated DNA was bound to the membrane using a protocol adapted from Maniatis *et al.* (1982). Three pieces of Whatman 3MM paper were cut to fit neatly into the bottom of glass petri dishes (140 mm). The first piece was saturated with 10% SDS and the excess solution poured off. The nylon membrane was placed, right side up, on the SDS-impregnated 3MM paper for 3 min to lyse the cells, transferred to the second sheet of 3MM paper which had been saturated with denaturing solution and the DNA denatured for 3 min before the membrane was transferred to the third sheet of 3MM paper which had been saturated with neutralising solution. After 5 min, the membrane was removed and allowed to dry on a sheet of 3MM paper for 1 h. DNA was crosslinked to the membrane, as described previously, which was wrapped loosely in aluminium foil and stored under a vacuum at room temperature if it was not used immediately for hybridisation.

5.2.6 Cloning of the Plasmid Probe

The plasmid probe used for the detection of *X. albilineans* was the 800 bp *Bam*HI/*Eco*RI fragment of pXA1. Cloning of this fragment into the vector pTZ19R was described in section 2.2.6. The recombinant plasmid harbouring the 800 bp fragment was designated

pTX7. In order to confirm that pTX7 harboured the pXA1 fragment, the cloned fragment was hybridised to DNA fragments from digests of pXA1 with *Eco*RI and *Bam*HI/*Eco*RI.

5.2.7 DNA Labelling, Hybridisation and Detection of Hybrid DNA

DNA labelling and detection of hybrid DNA was carried out using the nonradioactive DIG DNA Labelling and Detection Kit (Boehringer Mannheim). The plasmid probe was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate (Dig-dUTP) which consists of dUTP linked to the steroid hapten digoxigenin via a spacer arm. After hybridisation to the target DNA, hybrids were detected by an enzyme-linked immunoassay using an antibody conjugate (anti-digoxigenin-alkaline phosphatase conjugate) and subsequent enzyme-catalysed colour reactions with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitro blue tetrazolium salt (NBT) as well as chemiluminescent reactions with Lumigen PPD.

5.2.7.1 **Labelling of the Plasmid DNA Probe**

dNTP Labelling Mixture (10×) : 1 mM each of dATP, dCTP and dGTP
0.65 mM dTTP
0.35 mM Dig-dUTP

The 800 bp *Bam*HI/*Eco*RI fragment from pTX7 was isolated from low melting point

agarose gels and purified using the Geneclean kit (section 2.2.6.1). DNA was denatured by boiling for 10 min and cooling immediately on ice. The labelling reaction comprised of approximately 100 ng of freshly denatured DNA, 2 μl of random hexanucleotide primers, 2 μl of dNTP labelling mixture and 1 μl of Klenow enzyme (2 U) in a total volume of 20 μl. The reaction mixture was incubated at 37°C for 20 h and stopped by the addition of 2 μl of 0.2 M EDTA (pH 8). Labelled DNA was precipitated with 2.5 μl of 4 M LiCl and 75 μl of cold 100% ethanol at -70°C for 1 h. DNA was pelleted by centrifugation at 15 800 × *g* for 15 min at 4°C, washed with 70% ethanol and dried under a vacuum. The dried pellet was dissolved in 50 μl of TE buffer and stored at -20°C.

5.2.7.2 **Hybridisation**

Hybridisation solution : 5 × SSC
1% blocking reagent
0.1% *N*-lauroylsarcosine, Na salt
0.02% SDS

The volumes of solutions used for hybridisation and detection reactions were calculated for a 100 cm^2 nylon membrane. Membranes containing bound DNA were pre-hybridised with 20 ml of hybridisation solution, in plastic bags, at 68°C for 3 h on a slowly rotating platform. The solution was replaced with 2.5 ml of hybridisation solution containing approximately 75 ng of freshly denatured labelled probe. Membranes were subsequently incubated overnight at 68°C. After hybridisation, membranes were washed 2 × 5 min

with 50 ml of 2 × SSC/0.1% SDS at room temperature and 2 × 15 min with 50 ml of 0.1 × SSC/0.1% SDS at 68°C and used immediately for detection of hybridised DNA.

5.2.7.3 Immunological Detection

Buffer 1	:	0.1 M maleic acid
		0.15 M NaCl
		pH 7.5
Buffer 2	:	1% blocking reagent in buffer 1
Buffer 3	:	0.1 M Tris.HCl
		0.05 M $MgCl_2$
		pH 9.5
Colour solution	:	45 μl NBT solution
		35 μl X-phosphate solution
		10 ml buffer 3
Lumigen solution	:	0.1 mg/ml Lumigen PPD in buffer 3

All incubations were performed at room temperature and except for the colour and Lumigen reactions, with gentle shaking. For colour detection, nylon membranes were washed briefly in buffer 1 and incubated for 30 min with 100 ml of buffer 2. Membranes were then incubated for 30 min with 20 ml of buffer 2 containing 150 mU/ml of the antibody conjugate, followed by 2 × 15 min washes with 100 ml of buffer 1. Prior to detection of hybrid DNA with 10 ml of colour solution, membranes were equilibrated with

20 ml of buffer 3. Colour reactions were performed in the dark and were terminated by washing the membranes in TE buffer after the desired bands were detected. Membranes were subsequently stored in TE buffer until they were photographed.

Chemiluminescent detection was accomplished by incubating the membranes for 5 min in 10 ml of Lumigen solution. Excess liquid was removed by blotting the membranes on a dry sheet of Whatman 3MM paper for a few seconds and the damp membranes were sealed in plastic bags and incubated at 37°C for 15 min to activate the alkaline phosphatase enzyme conjugated to the anti-digoxigenin antibody. Hybrid bands were detected by exposing the membranes to X-ray film for 15 min at room temperature.

5.3 RESULTS

5.3.1 Cloning of the Plasmid Probe

The 800 bp fragment cloned in pTX7 was confirmed as the 800 bp *Bam*HI/*Eco*RI fragment of pXA1 (Fig. 5.1). It hybridised to both forms of pXA1 (lane 2), the 6.4 kb *Eco*RI fragment (lane 3) which it forms a part of (Fig. 2.10) and the 800 bp *Bam*HI/*Eco*RI fragment of pXA1 (lane 4). The probe did not hybridise to any of the other five *Eco*RI fragments or to the 5.6 kb *Bam*HI/*Eco*RI fragment. A low-intensity signal, not corresponding to any of the *Eco*RI restriction fragments was observed in lane 3. The position of this band corresponds to a 3.4 kb partial digestion product consisting of the 2.6 kb *Eco*RI fragment joined to the plasmid probe (Fig. 2.10). This band was not observed when the probe was hybridised to fragments from the *Bam*HI/*Eco*RI digest of pXA1 (lane 4).

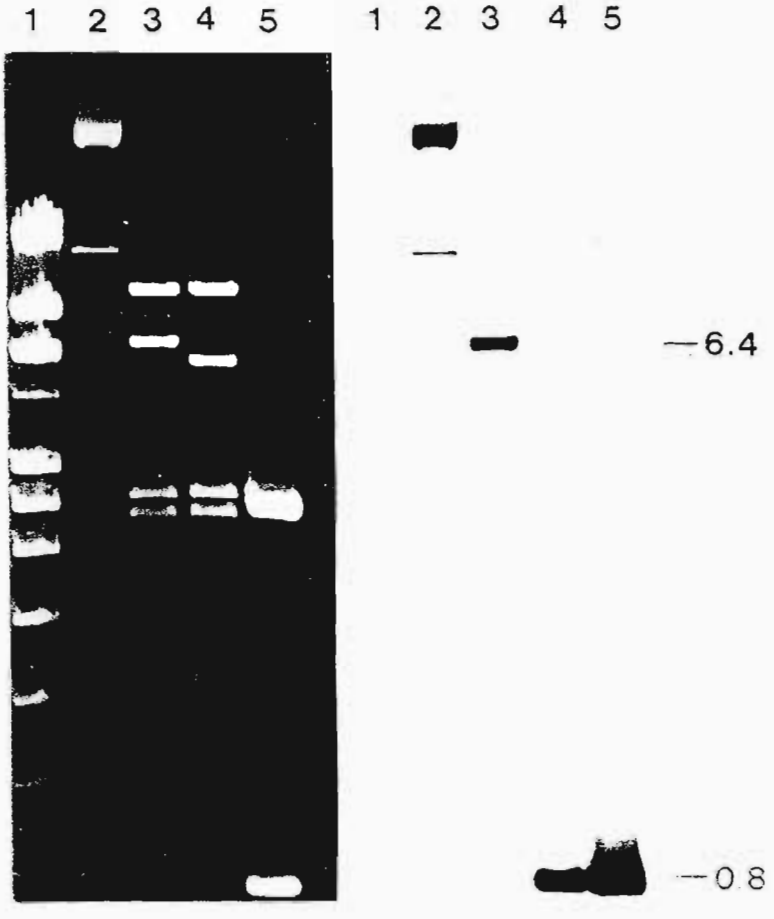

FIG. 5.1. Hybridisation of the plasmid probe to *Eco*RI and *Bam*HI/*Eco*RI digests of pXA1. Lane 1: DNA molecular weight marker IV; lane 2: uncleaved pXA1; lane 3: *Eco*RI-cleaved pXA1; lane 4: *Bam*HI/*Eco*RI-cleaved pXA1; and lane 5: *Bam*HI/*Eco*RI-cleaved pTX7.

5.3.2 Specificity of the Probe

The plasmid probe hybridised to total genomic DNA from XA 86-1 only and not to DNA from the other four isolates of *X. albilineans* tested (Fig. 5.2). The probe also hybridised to the 6.4 kb *Eco*RI fragment of pXA1 (lane 6). The presence of hybridisation bands in identical positions in lanes 5 and 6 indicated that the probe had hybridised to plasmid

DNA that was present in the preparation of total genomic DNA from XA 86-1 cells, and not to chromosomal DNA.

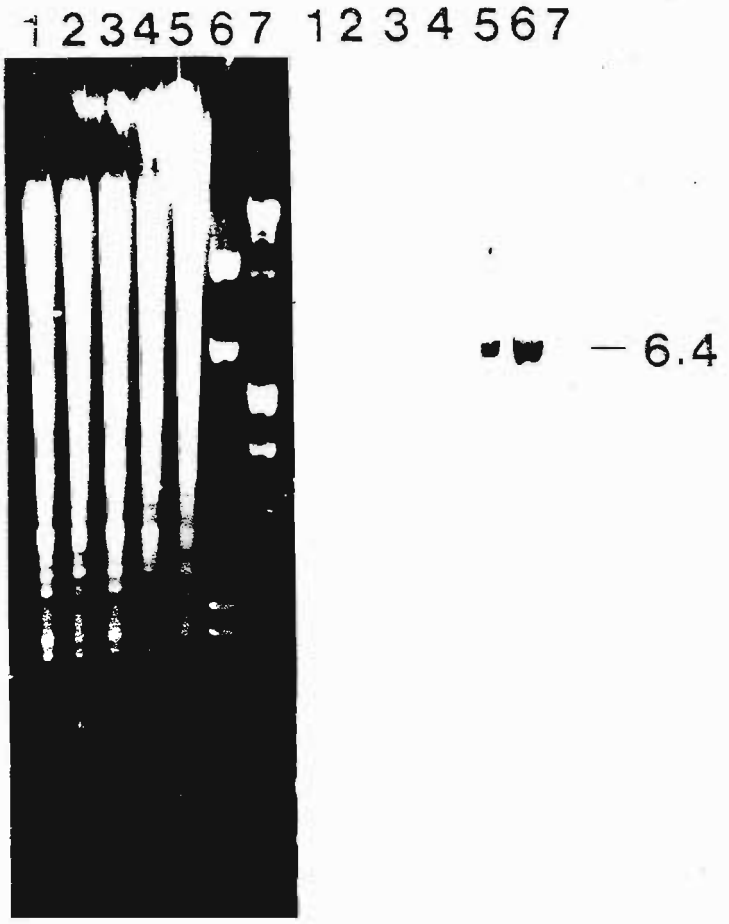

FIG. 5.2. Specificity of the plasmid DNA probe for XA 86-1 when hybridised to *Eco*RI-cleaved total genomic DNA, in agarose plugs, from five *X. albilineans* isolates. Lane 1: 2901; lane 2: 2905; lane 3: LS155; lane 4: PDDCC 196; lane 5: XA 86-1; lane 6: *Eco*RI-cleaved pXA1; and lane 7: phage λ DNA cleaved with *Eco*RI and *Hin*dIII.

In addition to being specific for XA 86-1, the plasmid probe also did not hybridise to DNA from any of the five *Xanthomonas* isolates tested (Fig. 5.3). Hybridisation of the

probe to genomic DNA, ranging from 1 μg to 10 ng, from XA 86-1 produced bright signals indicating the presence of plasmid DNA. The probe also hybridised to pXA1 DNA ranging from 100 ng to 1 ng. Faint signals obtained for DNA from the five *Xanthomonas* isolates were regarded as "background" signals.

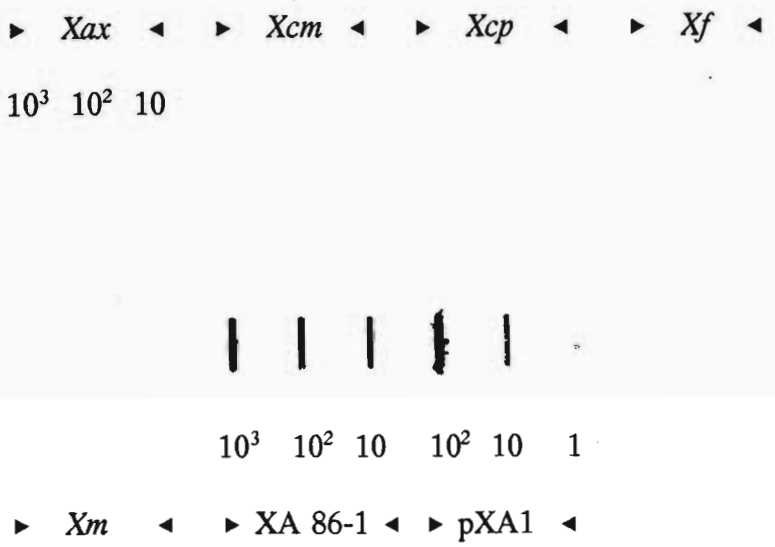

FIG. 5.3. Specificity of the plasmid probe for XA 86-1 when hybridised to genomic DNA from five *Xanthomonas* isolates, representing four *Xanthomonas* species. All slots were loaded with 1 000, 100 and 10 ng DNA except those containing pXA1 which were loaded with 100, 10 and 1 ng DNA.

5.3.3 Sensitivity of the Probe

The detection limit of the probe was found to be 10^5 cells using both colour and chemiluminescent detection of hybridised DNA. Colour detection (Fig. 5.4) yielded sharp, distinct hybridisation signals. Chemiluminescent detection (Fig. 5.5) which was very rapid, yielded more intense signals than colour detection for the equivalent number

of cells. Detection using both types of assays resulted in high background signals for slots that contained fewer than 10^5 cells. Apart from background signals, hybridisation of the plasmid probe to DNA from PDDCC 196 cells did not occur (panel B, Fig. 5.5).

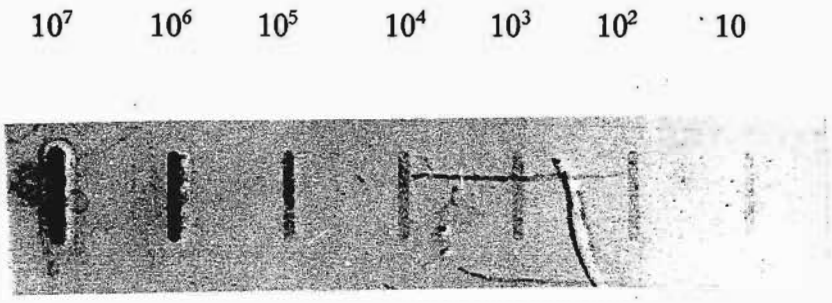

FIG. 5.4. Sensitivity of the plasmid probe for the detection of XA 86-1 cells using colour detection. Bacterial cells ranging from 10^7 to 10 cells/slot were lysed on the nylon membranes.

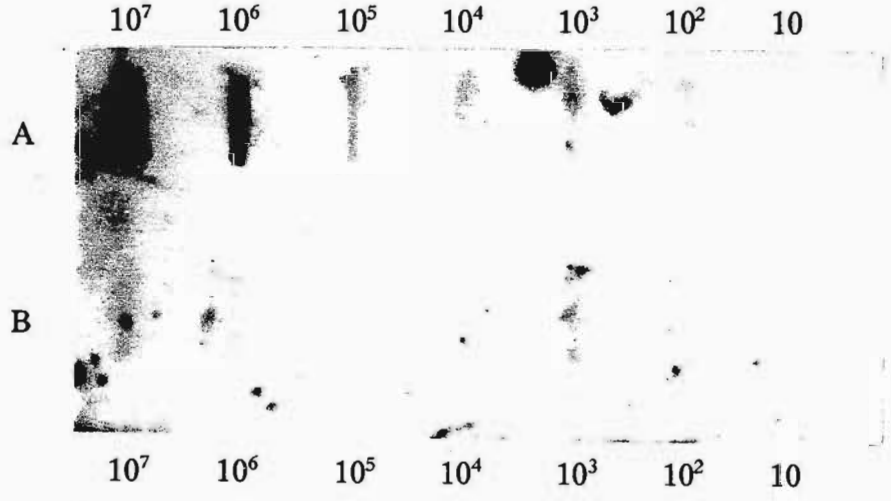

FIG. 5.5. Chemiluminescent detection of XA 86-1 cells (panel A) ranging from 10^7 to 10 cells/slot. Hybridisation of the plasmid probe to PDDCC 196 cells did not occur (panel B).

5.4 DISCUSSION

Specific detection of XA 86-1 was achieved using an 800 bp plasmid DNA probe derived from pXA1. Since plasmid isolation procedures have shown that pXA1 was present only in the South African isolate of *X. albilineans*, it was not surprising that the probe was specific for this isolate. Ideally, a DNA probe useful for the detection of *X. albilineans* should hybridise to DNA from all isolates of the pathogen. However, this probe is nevertheless useful as a strain-specific detection tool.

Hybridisation of the probe to pXA1 restriction fragments revealed that the DNA sequence of the probe was not repeated on the plasmid. Hybridisation of the probe to an approximately 3.4 kb partial restriction product of *Eco*RI-digested pXA1 was surprising since this product was expected only in *Bam*HI/*Eco*RI digests of pXA1. However, this band was not present in other blots of *Eco*RI-digested pXA1 or in blots of cleaved genomic DNA and therefore could only be due to a partial digestion product that contains probe sequences.

In addition to occurring at one site only on the plasmid, probing of total genomic DNA has shown that the probe sequence is not repeated on the chromosome of XA 86-1. A signal corresponding to a 6.4 kb fragment in the blot of XA 86-1 total genomic DNA was due to the 6.4 kb *Eco*RI fragment of pXA1. The intensity of the signal obtained suggested that the plasmid was present in XA 86-1 at a moderate copy number. Absence of signals from the other four *X. albilineans* isolates provided conclusive evidence that pXA1 was not present in an integrated form on the bacterial chromosomes of these isolates.

pXA1 was also not detected in five other *Xanthomonas* isolates, representing four *Xanthomonas* species, including *X. axonopodis* which is the *Xanthomonas* species that is most closely related to *X. albilineans* (Van den Mooter and Swings, 1990). This suggests that pXA1 is probably unique to XA 86-1 and that a DNA probe derived from this plasmid would be highly specific for this isolate, even when tested against other xanthomonads.

Unlike the plasmid probe of Gilbertson *et al.* (1989) that coded for repetitive sequences in plasmid and chromosomal DNA and could detect 10^3 cells, the 800 bp probe hybridised to only a single site which was on pXA1. The detection limit was found to be 10^5 cells. However, the true end-point of the sensitivity assays was masked by high background signals in slots containing lower numbers of bacteria. It was subsequently discovered that these background signals were most probably due to nonspecific binding of the probe or the antibody conjugate to cellular proteins present on the nylon membranes after lysis of the cells. These high background signals can therefore be reduced or eliminated by including a second pre-hybridisation step to degrade proteins with proteinase K. Another strategy that could be used to improve the detection limit would be to use a larger plasmid probe, e.g., the 4.2 kb *Eco*RV fragment. This would result in the incorporation of a larger number of digoxigenin molecules into the probe and thus lead to a more sensitive detection system. The sensitivity of the assay can also be improved by using a technique that would concentrate the bacteria in a smaller area on the nylon membrane. The resultant signals would be more intense and would therefore permit detection of even fewer bacteria. These modifications to the technique could result in a detection system that would be more sensitive than the ELISA technique developed by Pillay *et al.* (1992) which detected *X. albilineans* cells at a concentration of 10^4 cells/ml.

The sensitivity of the probe is sufficient for the detection of XA 86-1 in diseased plants since *X. albilineans* can reach cell densities of up to 2×10^7 cells/cm^2 of diseased leaves (Birch and Patil, 1987a). Detection of XA 86-1 in sugarcane plants using the plasmid DNA probe would not require isolation of the organism or an enrichment process, but would involve direct detection using a technique such as the squash blot where leaf disks are simply squashed onto nylon membranes to force out the bacteria. Detection using this technique would be rapid and results would be available within 24-30 h. This technique would, however, only be useful in early detection of the pathogen if bacterial populations within the leaf are high enough to permit detection during the early stages of the disease. It is unlikely that detection limit of 10^5 cells is sufficient for the detection of latent infections.

The plasmid probe developed in this study is useful in that it is strain-specific and can be used for the detection of XA 86-1 and also for identification of this organism. However, a DNA probe useful for the early detection of leaf scald disease should be able to detect all isolates of the pathogen. DNA sequences common to all isolates, identified using DNA fingerprinting techniques, and preferably repetitive sequences, would make useful probes. Nonradioactive detection using DNA probes has potential for use in the early detection of *X. albilineans* since it is a rapid, specific and sensitive technique.

CHAPTER SIX

GENERAL DISCUSSION

Molecular characterisation of *X. albilineans* isolates has shown variation among isolates from different geographical regions. The presence of a plasmid unique to the South African isolate differentiated it from the four others used in this study. DNA fingerprinting, used to differentiate the five *X. albilineans* isolates, provided evidence for the possible existence of sub-groups of the pathogen. A detection assay developed using a plasmid DNA probe can be used for the specific detection of the cultured South African isolate. The findings of this study may explain why outbreaks of leaf scald disease have occurred in sugarcane varieties that were previously resistant to the disease.

It has been previously reported that plasmid isolation procedures showed that only the South African isolate harboured a plasmid of approximately 25 kb (Pillay *et al.*, 1993). However, the possibility still exists that the other four isolates possess the plasmid in an integrated form on the bacterial chromosome, a situation reported for isolates of *C. michiganense* subsp. *sepedonicum* which lacked an autonomous form of the plasmid pCS1 (Mogen *et al.*, 1988). This study has confirmed that XA 86-1 is the only isolate that harbours pXA1 in an autonomous form. Furthermore, hybridisation studies with a pXA1-derived DNA probe has shown that the other *X. albilineans* isolates do not contain the plasmid in an integrated form. Birch and Patil (1987a) and Lazo and Gabriel (1987) have reported that plasmids were absent in *X. albilineans* isolates, but these studies described results of plasmid isolation procedures from only two isolates and one isolate, respectively.

Therefore, final conclusions regarding the ubiquity of pXA1 can only be drawn after examination of a larger number of *X. albilineans* isolates, including several South African isolates.

Like most plasmids present in plant pathogenic bacteria, pXA1 is a genetically cryptic plasmid as it has not yet been associated with any known function(s). The existence of these cryptic plasmids raises the question as to whether plasmids always need to confer a selective advantage in order to be maintained in a host. Coplin (1989) stated that the conclusion that carriage of plasmids imposes an unnecessary metabolic burden on bacteria was drawn from early plasmid studies with resistance plasmids introduced into laboratory strains and not indigenous plasmids from natural populations. Reports of plasmids from plant pathogenic bacteria support the newer concept that indigenous plasmids are stable in their natural hosts. With pXA1, this certainly seems to be the case as no apparent selective pressure was needed for stable maintenance of the plasmid by XA 86-1. It is tempting to speculate on the role of pXA1 in pathogenicity and, in particular, the production of albicidin since a 6.4 kb fragment of the plasmid could not be cloned in *E. coli*, indicating that it could have coded for a lethal product, e. g., albicidin. However, coding of this fragment for other products, e.g., restriction endonucleases, would have produced identical results. Also, it has now been confirmed that genes coding for production of albicidin in at least four of the isolates used in this study are located on their respective chromosomes as they do not possess extrachromosomal DNA.

The existence of cryptic plasmids in plant pathogenic bacteria may reflect that the assays used by plant pathologists in screening for functions encoded by these plasmids are biased

towards certain easily identifiable traits only. Coplin (1989) argued that the "secret" life of bacterial pathogens as saprophytes, when not present in host plants, has been ignored and that these so-called cryptic plasmids may code for biochemical pathways that allow survival of the pathogens in less favourable environments. Although this could be true for XA 86-1 since it possesses a plasmid, other *X. albilineans* isolates have been shown to survive for only a few hours in the soil (Rott *et al.*, 1986). There is a possibility that pXA1 may be indirectly involved in pathogenicity by coding for genes involved in host range and race/cultivar specificity as six *avr* genes have been reported to be clustered on a 90 kb plasmid found in *X. campestris* pv. *malvacearum* (De Feyter and Gabriel, 1991). It is likely that *X. albilineans* does contain *avr* genes since Hopkins *et al.* (1992) concluded that two genes, *avrXa7* and *avrXa10*, are members of a family of *avr* genes in xanthomonads. *Hrp* genes must also be present in *X. albilineans* since Akhtar *et al.* (1988) reported a hypersensitive response in tobacco plants inoculated with the pathogen. The occurrence of pXA1 in XA 86-1 raises the intriguing possibility that genes encoding race/cultivar specificity can be transferred between isolates of *X. albilineans* in a manner similar to that observed for antibiotic-resistance plasmids in bacteria pathogenic to humans. It has previously been reported that the resistance of a sugarcane cultivar to leaf scald disease varies in different countries (Egan, 1970; Koike, 1992; Ricaud and Ryan, 1989). Since gene-for-gene interactions between pathogen and host plant determines resistance or susceptibility, resistance of a particular cultivar to leaf scald disease in a certain country may be due to isolates of *X. albilineans* in that country not possessing the host-range genes necessary for pathogenicity. Plasmid-mediated transfer of genes involved in race/cultivar specificity may therefore explain recent outbreaks of leaf scald disease in sugarcane varieties that were previously resistant to the disease.

DNA fingerprinting using RAPD analysis provided evidence for the existence of sub-groups of *X. albilineans*. Grouping of the five *X. albilineans* isolates showed correlation with previous studies using serological tests (Seetal, 1989) and LPS profiles (Pillay *et al.*, 1993). Recently, two reports also described sub-grouping of *X. albilineans* isolates using DNA fingerprinting (Davis *et al.*, 1993; Schenk *et al.*, 1993). However, a comprehensive study involving a larger number of isolates needs to be performed to establish distinct sub-groups in *X. albilineans*. In the same study, a DNA fingerprinting technique should be standardised for *X. albilineans* to allow typing of isolates not included in this study. Rott *et al.* (1986) divided 28 *X. albilineans* isolates into 3 serovars and 6 lysovars, but their technique cannot be used by other researchers as it was designed to merely compare isolates and not type them. DNA fingerprinting of *X. albilineans* isolates used in this study showed that the type strain, the South African isolate and the 2 Mauritian isolates may belong to separate sub-groups. The Australian isolate was grouped together with the 2 Mauritian isolates by Pillay *et al.* (1993) but DNA fingerprinting showed that sufficient polymorphisms existed among these 3 isolates to warrant possible exclusion of the Australian isolate from this group. It was also found that the type strain, PDDCC 196, was not representative of the species and it is proposed that its designation as the type strain be withdrawn. Designation of NCPPB 2247 as the phenotypic centrotype of *X. albilineans* (Van den Mooter and Swings, 1990) suggests that this strain is more representative of the species.

A reliable system to detect the presence of *X. albilineans* in sugarcane plants is necessary to help prevent further outbreaks of the disease in sugarcane-growing regions. The currently used method relies on the development of visual symptoms of the disease. This

method is usually effective in detecting the presence of the causal organism, except when the disease is latent. Since most countries cultivate sugarcane varieties that have high levels of resistance to leaf scald disease, latency in commercial crops is usually not serious. However, latent infections can allow the passage of *X. albilineans* isolates through quarantine barriers between countries and thus destroy the varietal resistance developed there. Techniques such as the immunofluorescence assay, ELISA and the use of DNA probes can be effectively used for the early detection of the disease. The plasmid DNA probe developed for the detection of XA 86-1 demonstrated that this method is specific enough to allow efficient detection of the causal organism. Field trials will have to be conducted to determine how early in the infection cycle the probe is able to detect XA 86-1. However, serological and DNA probe-based methods are not reliable for the detection of latent infections due to the low numbers of bacteria present in such situations. The advent of PCR has made detection of less than 10^2 cells possible and therefore may be the only reliable assay that will detect latent infections. A pre-requisite for such a detection system is the identification of a DNA sequence that is highly specific for the pathogen, as the technique has to be performed under stringent conditions to prevent amplification of plant DNA. The obvious choice of such unique sequences in *X. albilineans* are genes coding for albicidin production, which are not found in any other organism. Such a detection system could be used to prevent the spread of *X. albilineans* isolates between countries by effectively intercepting any isolates that would normally escape detection due to latent infections.

The existence of sub-groups in *X. albilineans* lends support to the hypothesis of Egan (1969; 1970) that strains of the pathogen exist. Further studies need to be performed to

correlate the presence of sub-groups with the different types of pathogenicity or avirulence genes that the isolates possess. This would lead to the identification of pathotypes of *X. albilineans* that are pathogenic on certain varieties only. Recent outbreaks of leaf scald disease (Chen *et al.*, 1993; Davis *et al.*, 1993; Koike, 1992) were probably due to introduction of new strains of the pathogen into an area where the disease was controlled by the cultivation of resistant varieties. In order to prevent further outbreaks, a sensitive detection system has to be developed to reliably detect latent infections. This would protect resistant varieties from new strains of the pathogen. The existence of different strains of *X. albilineans* has important implications for the sugarcane industry as varietal resistance is the only effective means of controlling leaf scald disease.

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