STUDIES ON THE CAUSAL AGENT OF LEAF SCALD DISEASE IN SUGARCANE

by

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Chapter 1. INTRODUCTION AND LITERATURE SURVEY

1.1 INTRODUCTION

Leaf scald disease, described as "the fastest spreading major disease of sugarcane in recent times" (Egan, 1969a) and "one of the most destructive" (Dowson, 1957), remains as elusive and difficult to detect under both natural and experimental situations today as it has been since it was first recognized in the 1920's. The validity of these statements at the present time becomes obvious when the recorded distribution of the disease (Abbott, 1953; Babu, 1979; Edgerton, 1955; Martin & Robinson, 1961; Ricaud *et al.*, 1989) and the severity of outbreaks in various countries over the previous two to three decades are examined. These include outbreaks in the territories of Australia (Fox, 1961; Hughes, 1969,1972; Persley, 1973a,1975), Barbados (Walker, 1968), Brazil (Masuda & Tokeshi, 1978), British Guiana (Hutchinson & Robertson, 1953), Guyana (Bisessar, 1970; Khan, 1971), Hawaii (Anon., 1953a), India (Egan, 1979; Satyanarayana & Rao, 1980; Waraitch, 1979; Waraitch & Singh, 1978), Jamaica (Shaw, 1981; Thompson, 1982a), Kenya (Sheffield, 1969), Malaysia (Pan, 1984), Mozambique (Pinto, 1972; Ricaud, 1969b), Nigeria (Persley, 1976; Zummo, 1976), South Africa (Thomson, 1969a,b), Swaziland (Thomson, 1969a), Trinidad (Ogier & Goberdhan, 1970), United States of America (Coleman, 1968; Hughes, 1978) and Uruguay (Koike, 1982).

The prolific nature of the disease in sugarcane growing countries world-wide is, therefore, evident and is further substantiated by the fact that in 1950 leaf scald had been recorded in nine countries, by 1974 in twenty-eight (Hughes, 1978) and in 1989 forty-four countries (Ricaud & Ryan, 1989).

Within the last decade the need to define the principles underlying the elusive and destructive nature of the disease has stimulated a resurgent interest in the causal agent, *Xanthomonas albilineans*, and its interaction with the host plant, *Saccharum officinarum*. However, until the mechanisms of the infection process are better understood and means for its control are found, leaf scald disease will remain a problem to the sugarcane-producing countries world-wide.

1.2 HISTORY, DISTRIBUTION AND ECONOMIC IMPORTANCE

The two workers independently accredited with the first descriptions of leaf scald disease are Wilbrink and North, respectively, documenting the disease in Java in 1920, and Australia in 1926 (Edgerton, 1955; Martin & Robinson, 1961). Although the origins of the disease remain obscure, early investigators in the Eastern Hemisphere countries of the Indian and mid-Pacific Ocean (including Australia, Fiji, Java, Mauritius and the Philippines) had described symptoms of leaf scald but attributed these to other known diseases

or to weaknesses in sugarcane varieties (Abbott, 1953; Edgerton, 1955; Egan, 1970). Leaf scald has subsequently spread to the Western Hemisphere and now occurs in every major sugar-cane growing region in the world (Hughes, 1978), as shown in Table 1 (Ricaud *et al.*, 1989). This wide distribution is partly the result of exchange programmes between countries, using susceptible or tolerant sugarcane varieties harbouring the pathogen, at a time when quarantine measures were not strictly enforced (Ricaud & Ryan, 1989).

Table 1. A list of sugarcane producing countries in which leaf scald disease occurs (Ricaud *et al.*, 1989)

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

The impact of leaf scald disease on the sugar economy is best described as a three-pronged one. Firstly, should a crop largely comprise varieties of sugarcane susceptible to leaf scald, a heavy infection has the potential to destroy the crop completely - a situation which existed in Australia when the disease was first recorded (Edgerton, 1955). Secondly, should the infection not be so severe as to cause

crop failure, there is a reduction in cane and sugar yields as a result of slower stalk growth (Hutchinson & Robertson, 1953), stalk death and poor juice quality (Madan *et al.*, 1982; Singh *et al.*, 1981) with up to a 40% reduction in sucrose content (Thompson, 1982a). Costs are also incurred in replanting clear spaces created by the death of stalks within a field (Hughes, 1961; Martin & Robinson, 1961). Finally, indirect financial losses also occur when promising, but highly susceptible, varieties are discarded during seedcane selection trials (Ricaud & Ryan, 1989), or are substituted with less productive resistant ones. Often the varieties of sugarcane, resistant to leaf scald, have smaller stalks and provide lower yields of sugar (Abbott, 1953; Edgerton, 1955). However, with improvements in sugarcane selection and breeding programmes the quality of resistant sugarcane varieties has greatly improved (Hughes, 1961; Nuss, 1976; Waraitch, 1979).

1.3 PATHOLOGY

There is firm agreement among all authors on the development of characteristic symptoms during the course of leaf scald disease. In this regard several well documented reviews have appeared through the years (Abbott, 1953; Dowson, 1957; Edgerton, 1955; Hughes, 1961,1978; Martin & Robinson, 1961; Ricaud & Ryan, 1989).

Two distinct symptomatic forms of leaf scald are evident, a chronic form and an acute form. At one stage, prior to the etiology of the disease having been established, these forms were often regarded as separate diseases (Martin & Robinson, 1961). A third form of the disease, often undescribed but equally characteristic, is the occurrence of a symptomless latent infection (Ricaud & Ryan, 1989). Although each form of the disease can occur independently, various combinations of the forms are usually more prevalent (Hughes, 1961).

1.3.1) Chronic form

The most typical external symptom of the chronic form, and of the disease itself, is the presence of narrow (from <1 mm to 3 mm wide), white or creamish "pencil-line" streaks (Edgerton, 1955; Ricaud & Ryan, 1989) on the leaf blades and sheaths (Hughes, 1978) located over and around the fibrovascular bundles (Edgerton, 1955). These follow the direction of the main veins, arise at an angle to the midrib and usually end as a purple tinge in the leaf sheath (Hughes, 1961; Martin & Robinson, 1961). The specific name of the pathogen, *X. albilineans* (Latin: *albus*=white; *lineans*=striping), is derived from the appearance of this streak (Bradbury, 1984; Ricaud & Ryan, 1989). Sometimes, instead of definite streaks, the entire leaf blade or shoot may become chlorotic (Abbott, 1953; Birch & Patil, 1983; Edgerton, 1955; Ricaud & Ryan, 1989). Chlorosis represents a more serious infection and usually results in stalk

stunting, occasionally followed by death of the plant. This is a situation typical of young shoots arising from diseased stalks (Hughes, 1961).

As the leaves age, the streaks become broader and more diffuse although the fine pencil-line is still evident in the centre of the streak. These streaks can be confused with those due to other diseases, e.g., gummosis and chlorotic streak. However, the distinguishing feature remains the central white pencil-line which may be reddened in areas but still is characteristic of leaf scald (Ricaud & Ryan, 1989; Martin & Robinson, 1961). At this stage, necrosis becomes evident - the margins of leaf tips begin withering and this progresses along the length of the streak giving a scalded appearance, whence the name of the disease (Martin & Robinson, 1961). The withering process is usually accelerated under conditions of water stress, ultimately leading to stalk death (Edgerton, 1955). Another prominent external feature of the chronic phase is the development of the lateral buds into side shoots. This usually starts at the base of the stalk with shoots often showing well developed pencil-lines and/or chlorosis (Abbott, 1953; Hughes, 1961; Martin & Robinson, 1961; Ricaud & Ryan, 1989).

The mechanism of induction of chlorosis in leaves has been described. Birch & Patil (1983), in an ultrastructural study of the disease, observed the lack of differentiation

of chloroplasts in leaf tissue adjacent to infected xylem vessels - resulting in the pencil-line striping. In a series of experiments (Birch & Patil, 1985, 1987a,b), the factor responsible for inhibiting chloroplast development was isolated and identified as "a family of antimicrobial compounds, including albicidin" which was produced by *X. albilineans* in infected tissue. Inhibition of plastid differentiation was manifested at the DNA replication level, followed by a partial inhibition of protein synthesis (Birch & Patil, 1987b). Spontaneous loss of the ability to produce the inhibitor was found to occur at high rates (Birch & Patil, 1987a) and these non-inhibitor producing bacteria could be isolated from sugarcane showing no symptoms of leaf scald disease.

1.3.2) Acute form

This form of the disease is characterised by the sudden wilting and death of a single stalk or an entire plant. Plants thus affected are usually mature and do not show any other symptoms of the disease (Abbott, 1953; Hughes, 1961; Martin & Robinson, 1961; Ricaud & Ryan, 1989). Even isolation of the pathogen from stalks, afflicted with this form of the disease, is difficult and is only possible either when the acute form is diagnosed in its early stages (Hughes, 1961), or from shoots which may develop at the base of the affected stalk (Roth & Thomson, 1970). Dry weather, as in the case of the chronic form of the disease, appears

to favour the expression of acute stage symptoms (Dowson, 1957; Persley, 1973a,b).

1.3.3) Latent infections

The mechanism of the latent infection is not well understood (Ricaud & Ryan, 1989). Although stress imposed under conditions of drought are reported to favour the expression of chronic and acute phases of the disease (Babu, 1979; Bisessar, 1970; Persley, 1973a,b, 1975; Zummo, 1976), Ricaud & Ryan (1989) describe unsuccessful attempts by Ryan & Birch (unpublished) to induce symptoms in artificially infected plants by temperature and moisture stress. The unpredictable nature of this phase is aptly summed up in the statement by Sheffield (1969) who reported these results following the quarantine holding of two varieties of sugarcane: "For years we have never found symptoms on these ratoons and I wondered if we were only wasting effort and greenhouse space by keeping them. However, the practice has been thoroughly vindicated. Leaf scald has been found on two occasions on canes imported from Barbados." Symptoms appeared after four months on the one occasion and after nine on the second.

1.3.4) Internal symptoms

These have been best described when stalks were split longitudinally (Abbott, 1953; Edgerton,1955; Hughes, 1961; Martin & Robinson, 1961). A characteristic red

discolouration of vascular bundles occurred in leaves and stalks (more pronounced at the nodes) showing symptoms of the chronic form only. No internal symptoms were evident in plants affected by the acute phase except at points where side shoots developed (Hughes, 1961; Martin & Robinson, 1961). Birch & Patil (1983), in an ultrastructural study, reported an absence of differentiated chloroplasts in chlorotic tissue although protoplastids and etioplasts were present. Tightly packed bacterial cells were localized in xylem vessels, pit cavities and the spaces between xylem vessel and bundle sheath cell walls.

1.4 TRANSMISSION, DETECTION AND CONTROL

One of the areas of paramount interest to sugarcane pathologists the world over is to identify all possible means of transmission of leaf scald disease and to constantly devise and re-evaluate means for its detection and control. Thus the transmission, detection and control factors are inextricably linked whenever methods for keeping the disease in check are considered.

Transmission must be considered at two levels: the local and global perspectives. Globally, at one stage, dissemination of leaf scald occurred primarily as a result of the exchange of diseased material between countries. However, with more stringent quarantine controls having been imposed (Edgerton,

1955), sugarcane varieties with the potential to carry the disease are closely screened and rated (on the basis of susceptibility or resistance) before release for commercial cultivation. The problem experienced at this level is the development and use of sufficiently sensitive techniques for detecting the disease, especially when it occurs as a symptomless latent infection during screening trials. Methods for detection in use at present still rely almost entirely on the appearance of symptoms during the chronic phase, expression of the acute phase of the disease (Egan, 1969b; Ricaud, 1969a, 1971; Thompson, 1982b; Wismer, 1969), and isolation of the pathogen (Masuda & Tokeshi, 1978; Shaw, 1981; Waraitch & Singh, 1978). Serological means of detection, by immunofluorescence (Leoville & Coleno, 1976) and micro-agglutination (Ricaud *et al.*, 1978), although sensitive, have proven uneconomical and sometimes inconsistent, with successful diagnoses ranging between 66% and 93% of known infected material.

At the local level, leaf scald is transmitted by infected setts and contaminated harvesting implements (Hughes, 1961; 1978; Martin & Robinson, 1961; Persley, 1973a; Ricaud & Ryan, 1989; Roth & Thomson, 1970; Ryan, 1976; Thomson, 1969b). Diseased setts are obtained from plants which are already infected with the chronic or acute phases, or from "healthy" plants harbouring a latent infection. Transmission by this means is usually over long distances

(Ricaud & Ryan, 1989), e.g., between farms within a locality. Within a crop, leaf scald can be rapidly spread using contaminated harvesting implements; one contaminated harvesting knife would be capable of infecting several plants (Hughes, 1961; Ricaud & Ryan, 1989; Roth & Thomson, 1970). Transmission by other means has also been reported, although none have been substantiated. These include transmission by flood water and strong winds (Persley, 1973a,b, 1975), soil leachate (Hughes, 1968) and rats and insects (Egan, 1970).

Control of local outbreaks and transmission of the disease has been effected by restricting the transportation of diseased material from place to place (Babu, 1979), encouraging better farming practices and higher standards of supervision (Ogier & Goberdhan, 1970), the use of resistant sugarcane varieties (Ryan *et al.*, 1980; Thomson, 1969b), sterilization of harvesting implements (Roth & Thomson, 1970) and the rogueing of diseased plants within a field where these comprise 5% or less of the total crop (Thomson, 1969a). The latter has limited value because of the occurrence of symptomless infected plants (Hughes, 1961, 1978; Thomson, 1969b). The use of heat for treating setts prior to planting is unreliable. Bailey (1976), in an experiment evaluating three methods, found that hot air treatment for 8 h at 54 C was unable to eliminate leaf scald; hot water treatment for 2 h at 50 C exerted some

control over the disease; and hot water treatment for 1 h at 52 C, repeated after 24 h, impaired sett germination.However, small quantities of planting material could be successfully treated by soaking in cold water for 24 to 48 h followed by hot water at 50 C for 3 h before planting (Persley, 1973a,b).

1.5 ALTERNATIVE HOSTS

Edgerton (1955) and Martin & Robinson (1961) reported that leaf scald did not occur naturally in any other plant besides sugarcane. There are, however, three reports to the contrary. Persley (1975) and Ryan (1976) recorded the occurrence of leaf scald in blady grass (*Imperata cylindrica* var. *major*) in Australia from which *X. albilineans* was isolated, but whether sugarcane could be infected with these isolates was not determined. The third report of leaf scald on *I. cylindrica* showed that the isolate could not infect sugarcane (Dowson, 1957). Artificial inoculations of other grasses have resulted in varying degrees of symptom expression (Edgerton, 1955; Martin & Robinson, 1961; Ricaud & Ryan, 1989; Ryan *et al.*, 1981). All symptoms were typical of those normally found on sugarcane.

Chapter 2. PATHOGENICITY OF STRAIN XA 86-1 AND CHARACTERIZATION OF ALL STRAINS

2.1 INTRODUCTION AND OBJECTIVES OF STUDY

The most important problems with leaf scald disease relate to difficulties in detecting the disease, frequently as a result of the total absence of typical diagnostic symptoms (Ricaud *et al.*, 1978) and symptom variability (Martin & Robinson, 1961), leading to the confusion of symptoms with those of other diseases (Edgerton, 1955), e.g., chlorotic streak and ratoon stunting disease (Masuda & Tokeshi, 1978). These problems are directly the result of the ability of the causal agent to occur as a symptomless latent infection in susceptible varieties of sugarcane. The states of latency and symptom expression are known to be regulated by environmental and climatic conditions (Martin & Robinson, 1961) although the exact conditions and their role remain undefined (Hughes, 1978; Rott & Chagvardieff, 1984) and have been the subject of speculation (Hughes, 1961; Persley, 1973a,b, 1975; Zummo, 1976).

Egan (1969a, 1970) hypothesized that several strains of *X. albilineans* exist. This hypothesis was based on the susceptibility of specific varieties of sugarcane to the disease in certain countries and their resistance in other countries. Varietal responses to the disease have been

extensively studied (Khan, 1971; Ogier & Goberdhan, 1970; Thompson, 1982a,b; Walker, 1968) with no clearly evident conclusions concerning either pathogenicity or varietal specificity (Persley, 1973b). Isolates of *X. albilineans* have also been compared using various experimental criteria, e.g., cultural and biochemical characteristics and bacteriophage typing (Ricaud & Ryan, 1989), DNA base composition (De Vos & De Ley, 1983), polyacrylamide gel electrophoresis of soluble proteins (El-Sharkawy & Huisingh, 1971), pectolytic activity (Dye, 1960), the presence of specific enzymes (Hayward, 1977) or enzyme systems (Whitaker *et al.*, 1981) and toxin production (Birch & Patil, 1983, 1985, 1987a,b).

The objectives of this study were two-fold: In addition to examining the pathogenicity of the South African strain of *X. albilineans*, this study, using the type species and four geographical isolates of the bacterium, also tests Egan's (1969a, 1970) hypothesis that several strains of *X. albilineans* exist. The criteria used in this section of the evaluation include cell morphology, the cultural, physiological, biochemical and growth characteristics and the polypeptide composition of the outer envelopes. Characteristics of the outer envelope of this bacterium have not been previously described.

2.2 MATERIALS AND METHODS

2.2.1) Bacterial strains

The four geographical isolates of *Xanthomonas albilineans* studied were from:

(i) Australia, strain LS155 from Nambour, supplied by the Bureau of Sugar Experiment Stations, Queensland;

(ii) Mauritius, strains 2901 and 2905, respectively from Belle Rive (a superhumid zone) and Medine (a subhumid zone) supplied by the Mauritius Sugar Industry Research Institute;

(iii) South Africa, strain XA 86-1 from Pongola, isolated from material provided by the South African Sugar Association Experiment Station, Mount Edgecombe.

The type strain, PDDCC 196, was obtained from the Plant Diseases Division Culture Collection, Department of Scientific and Industrial Research, New Zealand.

Other bacteria used for comparative purposes in cell membrane characterization and serological studies included *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Xanthomonas campestris* pathovar *zeae* (obtained from the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg).

(a) Isolation and purification of XA 86-1

Several methods have been used for the isolation and subsequent purification of *X. albilineans* from infected tissue. Broadly, these methods may be grouped as follows:

(i) Homogenisation or maceration of tissue together with sterile distilled water (Baudin & Chatenet, 1981; Bertoni & Mills, 1987; Daniels *et al.*, 1984; Khan, 1971; Maas et al., 1985; Persley, 1972; Schaad, 1980; Timmer *et al.*, 1987);

(ii) Expression of sap by "crushing" cut portions of infected areas showing symptoms of the disease (Dye, 1980; Khan, 1971; Persley, 1972);

(iii) Diffusion of sap from the tissue into sterile distilled water with intermittent agitation (Mulrean & Schroth, 1981; Timmer *et al.*, 1987), or without agitation (Masuda & Tokeshi, 1978; Persley, 1972; Schaad & Forster, 1985; Thompson, 1982a).

The liquid obtained from each of these extraction procedures was streaked onto dried agar plates. For *X. albilineans* the two recommended media used were Wilbrink's agar (Dowson, 1957; Martin & Robinson, 1961) and Yeast Extract Sucrose Peptone (YSP) agar (Dye, 1980).

On the basis of these methods, three techniques were developed for the isolation of strain XA 86-1 from diseased material. These techniques took into account the manner of disinfection, the part of the plant sampled and the

treatment of tissue in the process of extracting the pathogen.

For the first technique, infected sugarcane stalks and leaves were washed in tap water and disinfected using, successively, 10% (aq.) solutions of Germotol and sodium hypochlorite. Under sterile conditions, cross-sectional cuts were made across infected stems and leaves. Freshly exposed cut surfaces were then adpressed onto dried plates of Wilbrink's agar (sucrose 10,0 $g.l^{-1}$; peptone 5,0 $g.l^{-1}$; K_2HPO_4 0,5 $g.l^{-1}$; $MgSO_4.7H_2O$ 0,25 $g.l^{-1}$; Na_2SO_3 0,05 $g.l^{-1}$; agar 15,0 $g.l^{-1}$) and YSP agar (yeast extract 5,0 $g.l^{-1}$; sucrose 20,0 $g.l^{-1}$; peptone 10,0 $g.l^{-1}$; agar 15,0 $g.l^{-1}$). Disinfected material in sterile distilled water was also homogenised (Baudin & Chatenet, 1981; Khan, 1971) in a Waring Blender for 10 x 2 min and the resulting liquid streaked on agar plates.

The second technique involved disinfecting the leaves of diseased plants with a 10% (aq.) solution of Germotol. These were aseptically cut into 10 mm^2 pieces and sequentially passed through a series of 5 beakers: a 10% (aq.) Germotol solution, then 95% alcohol for 1 min followed by three washes in separate beakers containing sterile distilled water. Cross-sectional cuts were made across these pieces and freshly exposed tissue was adpressed onto the surfaces of dried agar plates. In addition, 1 to 2 mm^2

sections were plated with the cut surfaces in contact with agar medium. Finally, segments of leaf tissue were squashed and the expressed sap was streaked on agar (Khan, 1971).

For the third technique the sampling points included leaves with pencil-line symptoms, and complete etiolation, in plants showing symptoms of the disease (Masuda & Tokeshi, 1978; Thompson, 1982a), or new shoots and leaves of asymptomatic plants previously infected and suspected to be carriers of the disease. These were disinfected in a series of 10%, 5% and 1% aqueous sodium hypochlorite solutions (5 min each) and washed in 3 changes of sterile distilled water (10 min for the first two and 15 min for the third). Individual leaf segments were aseptically cut into 1 mm^2 pieces and left in sterile petri plates containing 2 ml sterile distilled water at room temperature for 3 h (Ricaud *et al.*, 1978). A loopful of "diffusate" from each petri plate was then streaked on Wilbrink's and YSP agar plates (Persley, 1972; Thompson, 1982a).

Each technique was employed for isolations in the initial stages of the experiments detailed below, until the most successful technique was identified and used for the remainder of the experiments. All inoculated plates were incubated at 26 C and examined daily over a 7 d period.

(b) Maintenance of stock cultures

The preservation of *X. albilineans* as short-term (or working) and medium- to long-term stock cultures was necessary because of the frequent need for cultures at specific growth phases. These cultures were also required to be genetically similar to the original isolates. Several methods were evaluated for their efficiency and ease of operation in fulfilling these needs. Short-term storage methods included regular subculturing (Gherna, 1981), agar slant cultures (Heckly, 1978) and storage in sterile distilled water (Perez, 1970; Tsiantos, 1986). The medium- to long-term storage methods were storage under mineral oil (Heckly, 1978; Perez, 1970), freeze-drying (Heckly, 1978), rapid- or snap-freezing (Moore & Carlson, 1975) and ultra-freezing (Gibson & Khoury, 1986).

Cultures streaked on petri plates and agar slants were stored at room temperature, and at 4 C, after incubation and growth at 26 C. Suspensions of 7 and 14 d cultures (exponential and stationary phase cells, respectively) were prepared in sterile, double-distilled water dispensed as 1 ml aliquots into cryotubes (supplied by Weil Organisation) and also stored at room temperature and 4 C.

For the medium- to long-term storage methods, where cells were subjected to sub-zero temperatures, cryoprotective agents were incorporated with the suspending media. An

exception was the storage of slant cultures overlayed with sterile mineral oil at 4 C. The cryoprotective agents used were sucrose, glycerol, skim milk and sodium glutamate. For freeze-drying, snap-freezing and ultra-freezing, the following suspending media were used:

- YSP medium supplemented with 5% glycerol; and
- a mixture of 10% skim milk and 1% sodium glutamate.

One ml aliquots of the media were dispensed into cryotubes for snap- and ultra-freezing, and into freeze-drying ampoules all of which were autoclaved at 121 C for 15 min.

In the media described above, cell suspensions of approximately 10^{12} to 10^{13} cells.ml^{-1} were prepared from 14 d cultures grown as confluent layers on agar plates. Samples for ultra-freeze were initially kept in ice before freezing and storage at -70 C. Those for snap-freeze were immersed into liquid nitrogen at -196 C until frozen, and also stored at -70 C in a biofreezer (Forma Scientific, supplied by Labotec).

All preserved samples were examined at 1-2 month intervals to monitor viability of the cultures.

2.2.2) Pathogenicity trials

Dye (1980) noted that the final identification of phytopathogenic *Xanthomonas* species could only be

accomplished by means of pathogenicity tests on host plants. This is essentially a fulfilment of Koch's Postulates for a pathogen known to be associated with a particular disease. In this experiment, the pathogenicity of only the South African isolate (XA 86-1) was tested.

(a) Experimental plants and conditions

Six varieties of healthy sugarcane stalks were provided by the South African Sugar Association Experiment Station, Mount Edgecombe. These varied in their responses to leaf scald disease: varieties L 76 and Co 1001 were most susceptible; N 6 and CB 36/14 were of intermediate resistance; and NCo 310 and NCo 376 were resistant to the disease.

All stalks were cleaned by removing any attached leaves and disinfecting in separate aqueous solutions of 1% Germotol and 5% sodium hypochlorite. One-bud setts were prepared using cutting tools disinfected with 95% ethanol. Ten setts from each variety, with the best developed buds and from different positions on a stalk (Thompson, 1982a; Walker, 1968), were selected for potting. Setts were either immersed in a 1% Aretan solution (aq.)(control and post-planting inoculation setts treated to inhibit fungal growth), or inoculated prior to planting.

The potting medium comprised equal parts of loam, river sand and vermiculite placed into plastic potting bags. Germination of the setts occurred within 11 d and the plants were maintained under greenhouse conditions with natural lighting, temperatures between 22 C to 27 C and a relative humidity ranging from 45% to 60%.

(b) Inocula

X. albilineans XA 86-1 was grown on Wilbrink's agar at 26 C for 7 d. Following purity checks cells were harvested, washed and resuspended in sterile distilled water to give a concentration of approximately 1 x 10^{11} cells.ml^{-1} (Baudin & Chatenet, 1981). Inoculum cultures with ages ranging from 48 h (Satyanarayana & Rao, 1980), 4-5 d (Waraitch & Singh, 1978), 5-7 d (Khan, 1971) to 14 d (Thompson, 1982b) have all proven successful in pathogenicity trials.

Diseased plant tissue used for the isolation of XA 86-1 was homogenised, as described for the first isolation technique (Section 2.2.1)(a)), and also employed as inoculum in these trials.

(c) Inoculation techniques

The success rate of any one inoculation technique for showing good leaf scald disease symptoms varies depending upon prevailing conditions in the environment (Bisessar, 1970; Martin & Robinson, 1961; Thompson, 1982a; Walker,

1968), the cane variety being tested (Wismer, 1969), the particular inoculation method employed (Thompson, 1982b), methods of production and maintenance of the inoculum (Ricaud, 1969a, 1971; Thompson, 1982a,b; Waraitch & Singh, 1978) and the time of day for inoculation (Egan, 1969b; Satyanarayana & Rao, 1980). In this regard, Wismer (1969) recommended the use of at least two different methods of inoculation during pathogenicity trials.

Of the methods that have gained favour among the various groups of researchers, the method of choice was usually the one which resulted in the highest success rate for symptom expression. Pre-planting and post-planting inoculation techniques are available. The post-planting methods are:
(i) The Aluminium cap method, developed by Koike (1965), where stalks were decapitated above the growing point, inoculum applied and the cut stalk covered with an aluminium cap to maintain humidity. A modification to the method was the substitution of the aluminium cap with a plastic hood (Roth & Thomson, 1970);
(ii) Plants grown under laboratory conditions (callus regeneration) with the stem tips cut off and the severed ends soaked in a bacterial suspension prior to planting in test tubes (Rott & Chagvardieff, 1984);
(iii) The hypodermic syringe method, where stalks were inoculated by injecting a suspension of bacteria near the growing point (Martin & Robinson, 1961), at the base of the

stalk (Baudin & Chatenet, 1981), or into the leaf vein of a plant grown in culture (Rott & Chagvardieff, 1984);
(iv) Applying a suspension of bacteria onto the cut ends of setts prior to planting by:

- spraying (Thompson, 1982b; Timmer *et al.*, 1987);
- paint brush (Martin & Robinson, 1961; Ryan *et al.*, 1980); or,
- soaking (Rott & Chagvardieff, 1984).

The pre-planting method involves the immersion of setts in a suspension of either a pure culture of bacteria (Bisessar, 1970; Thompson, 1982a), or a suspension of homogenised diseased tissue (Walker, 1968; Wiehe, 1951).

The two inoculation techniques used in this study comprised a pre-planting and a post-planting method. Setts for pre-planting inoculation were prepared as described above (Section 2.2.2)(a)) without immersing in Aretan, soaked overnight in sap extracted from diseased tissue (Section 2.2.1)(a)) and planted in the greenhouse.

Healthy sugarcane plants at the 7-8 leaf stage served as post-planting inoculation experimental material. These were cleaned by removing any surrounding dead leaf material and leaves present below the growing point. Cleaned stalks were cut at the growing point using surface sterilized scissors and 1 ml of bacterial suspension, or sterile distilled water (control plant), pipetted onto the cut surface in two

applications of 0,5 ml each. The growing points were sealed, in plastic bags containing a drop of water to maintain humidity, for 24 h.

In summary therefore, ten setts were prepared for each of the six sugarcane varieties as follows:

- two setts per variety served as controls, one being left undisturbed while the other was cut at the growing point and sterile distilled water applied to the cut surface. Plants subjected to the latter treatment were then treated in a similar manner to the post-planting inoculated test plants.
- four setts per variety were used for the pre-planting inoculation experiments; two being inoculated using pure cultures and two using suspensions of homogenised diseased tissue.
- four setts per variety were used for the post-planting inoculation experiments; two being inoculated using pure cultures and two using suspensions of homogenised diseased tissue.

Experiments were monitored daily for the first two months and thereafter at fortnightly intervals.

2.2.3) Whole cell characteristics of all strains

(a) Colony form, cell morphology and staining reactions

An examination of the colony characteristics and cell morphology of cultures up to 14 d old was carried out after growth at 26 C on YSP and Wilbrink's agar.

(i) Colony descriptions

Colony descriptions were based on those of Smibert & Krieg (1981). Features such as colony size, form, elevation, margin, texture, colour and consistency were examined.

(ii) Cell morphology and staining

Cell dimensions were measured by light and electron microscopy. Bacteria were suspended in a 25% (w/v) solution of gelatin and sizes determined using a Wild Ocular Micrometer (Wild, Switzerland) mounted onto a Zeiss Standard KF2 Microscope (Zeiss, West Germany). Cell sizes and numbers and arrangement of flagella were established by electron microscopy as described by Schaad (1980). Suspensions of bacteria grown on agar slants and in petri plates were stained with 1% phosphotungstic acid (pH 6,8) for 30 s and examined on collodion coated copper grids (300 mesh) using a Philips EM 301 Transmission Electron Microscope at accelerating voltages between 60-80 kV.

Capsule formation was determined by the Duguid method (Doetsch, 1981). A loopful of culture was mixed with a loopful of nigrosin on a glass coverslip. This was pressed down firmly onto a microscope slide and viewed with the high-dry and oil-immersion lens systems. Clear areas around the refractile cells indicated the presence of capsules.

For the Gram stain, by the modified method of Schaad (1980), air-dried and fixed smears were stained for 1 min each in crystal violet (Hucker's) and Gram's iodine, destained for 30 s in 95% Ethanol and counterstained with Gram's safranin. Smears were washed in water between each staining step and blotted dry before viewing.

(b) Pigments

All strains of *X. albilineans* used in this study were pigmented. Shake cultures were prepared in YSP and Wilbrink's broth using a Braun Certomat H/HK incubation hood and Certomat U orbital shaker (120 rev.min^{-1}; 26 C; 24 to 72 h) . Cultures were checked for purity and harvested by centrifugation for 10 min at 10000 x *g* and 4 C. Using the methods of Starr (1981) and Starr & Stephens (1964) for the extraction of pigments, 50 g absolute methanol (reagent grade) was added per 1 g wet weight of cells. Containers were placed in a waterbath (approximately 90 C) and alcoholic cell suspensions allowed to boil for 20 min, or until the cells had lost their colour. This crude (total) pigment extract

was centrifuged at 10000 x *g* for 10 min to remove all cell debris. The methanolic extract was scanned, using 1 cm path-length quartz cuvettes in a Beckman DU-8 Spectrophotometer, equipped with a DU-8 Wavelength-Scan Compuset (Beckman Instruments Inc., USA) to determine the pigment absorption maxima. Pigment extractions using other organic solvents (e.g., acetone, benzene, diethyl ether and petroleum ether b.p. 40-60) were also attempted.

(c) Biochemistry and physiology

All of the biochemical and physiological tests described below were replicated seven times; while the anaerobic growth, sodium chloride and pH tolerance and optimum growth tests were replicated three times.

Inoculum was prepared by making a suspension of 5-7 d old cells (grown on agar), in sterile distilled water, to give a final concentration of approximately 10^9 cells.ml^{-1}. 0,1 ml of this suspension was used to inoculate each tube. Inoculum, similarly prepared, was used for all subsequent inoculations of broth media in test tubes, unless otherwise indicated.

(i) Media, substrates and reagents

- *Carbohydrate and Organic Acid Basal Medium* - modified from Dye's Medium C (Bradbury, 1984; Dye, 1962) and YS Broth Base (Dye, 1962; 1980):

$NH_4H_2PO_4$	0,5 $g.l^{-1}$
K_2HPO_4	0,5 $g.l^{-1}$
$MgSO_4.7H_2O$	0,2 $g.l^{-1}$
Yeast extract	1,0 $g.l^{-1}$
Bromocresol purple	0,7 ml of a 1,5% (w/v) alcohol solution
Bacto-agar	15,0 $g.l^{-1}$

The concentrations of carbohydrates and organic acids were 1% and 0,5% (w/v) respectively. The pH of the medium before sterilizing was adjusted to 6, the mid-range for bromocresol purple. Sugars, except for arabinose, cellobiose, fructose, galactose, glucose, lactose, melibiose, ribose, trehalose and xylose, which were all filter sterilized, were autoclaved at 121 C for 5 min. Organic acids were autoclaved at 121 C for 15 min. All sugars and organic acids were incorporated in the media prior to sterilization. Modifications to the media were as follows:

-the omission of 5 $g.l^{-1}$ NaCl from Dye's Medium C. Dye (1966) recommended this modification to allow vigorous growth in cultures of *X. albilineans*; and,

-the reduction of the quantity of yeast extract from 5 $g.l^{-1}$ to 1 $g.l^{-1}$ in YS Broth Base.

Slants were inoculated by stabbing the butt and streaking the slant surface (Azegami *et al.*, 1987; Schroth & Hildebrand,

1980). Tubes were incubated at 26 C and observed at 2, 4, 7, 14, 21 and 28 d.

Acid production was shown by a change in colour of the indicator to yellow. Alkaline substances, when produced, caused a colour change to purple. Gas production would be indicated by splitting, or cracking, of the agar.

- *Urease Production* - from a modified Urea Agar Base (Christensen, 1946; Cook, 1948; Jeffries, 1964; Skerman, 1967; Stuart *et al.*, 1945):

Peptone (from meat)	1,0	$g.l^{-1}$
Glucose	1,0	$g.l^{-1}$
KH_2PO_4	2,0	$g.l^{-1}$
Phenol red	0,012	$g.l^{-1}$
Bacto-agar	12,0	$g.l^{-1}$

The agar base was autoclaved at 121 C for 15 min. When the medium had cooled to 45-55 C, filter sterilized urea solution was added to a final concentration of 2% (w/v). The medium was dispensed aseptically in 10 ml aliquots into sterile Macartney bottles and slants prepared. Heavily inoculated and uninoculated tubes, prepared by inoculating with full loopfuls of cells lifted from colonies on plates, were incubated at 26 C for 28 d. Control tubes without urea were also inoculated and incubated with these tests.

Urea is hydrolysed to carbon dioxide and ammonia. The ammonia formed causes an increase in alkalinity, indicated

by a red colour in the medium, and is evidence of urease activity (Jeffries, 1964). This method is ideal for detecting weak urease producing microorganisms because the agar base is weakly buffered (Cook, 1948; Stuart *et al.*,1945).

- *Nitrate Reduction and Denitrification* – using Nitrate Agar (Anon., 1953b; Smibert & Krieg, 1981):

Bacto-beef extract	3,0 $g.l^{-1}$
Bacto-peptone	5,0 $g.l^{-1}$
Potassium nitrate	1,0 $g.l^{-1}$
Bacto-agar	12,0 $g.l^{-1}$

Tubes of sterile medium were slanted so that deep butts formed upon solidification of the medium. These were inoculated by streaking over the surface of the slant and stabbing into the butt. Cultures were incubated at 26 C (together with uninoculated controls) and examined after 2, 4, 7, 14, 21 and 28 d.

Gas production was indicated by splitting of the agar. The presence of nitrites was tested by adding 1 ml each of alpha-naphthylamine (0,6 g *N,N*-dimethyl-1-naphthylamine dissolved in 100 ml of a 5 N acetic acid solution by gentle heating in a fume hood) and sulphanilic acid (0,8 g sulphanilic acid dissolved in 100 ml of a 5 N acetic acid solution as described above) solutions to the tubes. Development of a pink or red colour was a positive

indication of nitrate conversion to nitrite. With no colour development, zinc powder was added to the tubes. Zinc chemically reduces the nitrate in the medium to nitrite and a red colour develops. If no colour was produced after the addition of zinc, then all the nitrite had been reduced to ammonia or gaseous nitrogen. Uninoculated control tubes were similarly examined.

- *Hydrogen Sulphide Production from Cysteine* (Dye, 1968; Schroth & Hildebrand, 1980; Smibert & Krieg, 1981):

$NH_4H_2PO_4$	0,5 $g.l^{-1}$
K_2HPO_4	0,5 $g.l^{-1}$
$MgSO_4.7H_2O$	0,2 $g.l^{-1}$
Yeast extract	5,0 $g.l^{-1}$
L-cysteine	0,5 $g.l^{-1}$

Five ml of medium was dispensed per test tube and autoclaved at 121 C for 15 min. Dried, sterile filter paper strips previously soaked in 10% (aq.) neutral lead acetate were included before incubating the inoculated media; these strips were suspended approximately 5-10 mm above the level of the liquid without making contact with the medium. All tubes, including uninoculated media containing suspended strips, were incubated at 26 C and observed at 2, 4, 7, 14, 21 and 28 d.

Blackening of the lower portion of the strips indicated the production of H_2S; uninoculated media would show no change to the lead acetate paper strips.

- *Indole Production* (Anon., 1953b; Blazevic & Ederer, 1975; Smibert & Krieg, 1981):

Bacto-tryptone	10,0 $g.l^{-1}$

Aliquots of 5 ml Bacto-tryptone solutions were dispensed into test tubes and autoclaved at 121 C for 15 min. Inoculated tubes were incubated at 26 C with shaking (120 $rev.min^{-1}$ in a Braun Certomat incubation hood and orbital shaker). This test was used to determine the ability of the microorganism to degrade the amino acid tryptophan resulting in the formation of the by-product indole.

Tubes were examined after 2, 4, 7, 14, 21 and 28 d for indole production as follows:
To each tube 0,5 ml Kovac's reagent (para-dimethylamino-benzaldehyde 3,0 g; amyl alcohol 75 ml; concentrated HCl 25 ml) was added and gently shaken. The development of a red colour in the reagent layer indicates indole production.

- *Methyl Red and Voges Proskauer Test* (Anon., 1965,1986; Blazevic & Ederer, 1975; Skerman, 1967; Smibert & Krieg, 1981):

Bacto-peptone	7,0 $g.l^{-1}$
Glucose	5,0 $g.l^{-1}$
K_2HPO_4	5,0 $g.l^{-1}$

Aliquots of 5 ml of medium were dispensed into test tubes and autoclaved at 121 C for 15 min. Tubes were inoculated and incubated at 26 C with shaking as above.

After 2, 4, 7, 14, 21 and 28 d tubes were analysed using the following tests:

Methyl red test - To each tube was added 0,25 ml methyl red solution (0,1 g methyl red was dissolved in 300 ml 95% ethanol and made up to 500 ml with distilled water). Development of a magenta red colour indicated a positive reaction, yellow being negative, while pink or pale red was indeterminate.

Voges Proskauer test - To 1 ml of culture was added 0,6 ml of a 5% (wt/vol) solution of alpha-naphthol dissolved in absolute ethanol. This was mixed thoroughly and followed by the addition of 0,2 ml 40% (aq.) KOH. Tubes were mixed once more and left in a slanted position to expose a large surface area to the atmosphere (reaction is oxygen dependent). Upon examination after 15 and 60 min a strong red colour at the surface of the medium indicated a positive reaction.

The methyl red test distinguishes between organisms (mixed acid fermenters) which oxidize glucose to produce and maintain a high acidity level, and those which produce a lower level of acidity in the medium (2,3 butanediol fermenters) which eventually reverts to neutrality owing to

production of the neutral product. Specifically, the Voges Proskauer test determines whether or not organisms which metabolize glucose to pyruvate and organic acids convert these to acetolactate and then to non-acidic or neutral end-products, especially acetylmethylcarbinol (acetoin) and 2,3 butanediol.

- *Oxidation-Fermentation Test* (Anon., 1986; Bruce *et al.*, 1983; Schaad, 1980; Smibert & Krieg, 1981):

-Modified Hugh and Leifson Basal Medium*

Bacto-peptone (pancreatic digest of casein)	2,0 $g.l^{-1}$
K_2HPO_4	0,3 $g.l^{-1}$
Bromothymol blue	0,08 $g.l^{-1}$
Bacto-agar	3,0 $g.l^{-1}$

*medium was modified by excluding 5,0 $g.l^{-1}$ NaCl

-Vaspar Preparation
A 1:4 (v/v) mixture of molten paraffin wax (Merck, melting point 46-48 C) and molten vaseline (Unilab, white). Autoclave mixture at 121 C for 15 min.

A total volume of 900 ml of basal medium was prepared, heated to boiling to dissolve the agar and the pH adjusted to 7,1. The preparation was divided into four aliquots of 225 ml each and autoclaved at 121 C for 15 min. When the medium had cooled to 45-50 C, 25 ml each of filter-sterilised 10% glucose, lactose and sucrose was added to three separate flasks, and 25 ml sterile distilled water added to the fourth. These were mixed thoroughly and 2 ml of each medium aseptically dispensed into separate sterile 15 x 150 mm test tubes. Tubes were cooled rapidly by

immersing into cold water and 1 ml of molten Vaspar preparation was layered onto the solidified agar. This was cooled again and 1,5 ml of the same medium layered onto the solid Vaspar.

Each organism was used to inoculate four tubes, three containing each of the different sugars (glucose, lactose and sucrose) and the fourth lacking carbohydrate. These were replicated seven times. Inoculation was effected using a straight wire, immersed in a suspension of cells in sterile distilled water, ensuring that both layers of the medium were penetrated. All tubes were incubated at 26 C and analysed at 2, 4, 7, 14, 21 and 28 d. In addition to the control lacking carbohydrate, a second control (uninoculated medium with carbohydrate) was also incubated.

Results were analysed as follows: fermentative metabolism would cause acidic conditions to develop below the Vaspar seal and in the aerobic layer above, both becoming yellow; oxidative metabolism, however, would result in acidic conditions only in the aerobic layer which would change to yellow. Should neither layer become acidified, then the organism would be unable to catabolize the carbohydrate. The indicator in both controls should remain unchanged.

- *Ornithine Decarboxylase and Arginine Dihydrolase Tests* (Anon., 1986; Blazevic & Ederer, 1975; Skerman, 1967; Thornley, 1960):

Peptone (from meat)	5,0	$g.l^{-1}$
Yeast extract	3,0	$g.l^{-1}$
Sucrose	1,0	$g.l^{-1}$
Bromocresol purple	0,016	$g.l^{-1}$

Add 5,0 $g.l^{-1}$ l-ornithine and l-arginine to separate flasks.
Adjust pH of the medium to 6,7.

Aliquots of 5 ml per test tube were dispensed and autoclaved at 121 C for 15 min. Tubes (including controls lacking ornithine and arginine) were inoculated, overlayed with sterile liquid paraffin and incubated at 26 C for 28 d. The medium should initially become yellow, because of acid production from glucose metabolism, then violet following degradation of ornithine or arginine. Control tubes would remain yellow: otherwise results would be invalid.

- *Lysine Decarboxylase Sulphydrase Test* (Edwards & Fife, 1961; Johnson *et al.*, 1966):

Peptone (from meat)	4,5	$g.l^{-1}$
Peptone (from soya)	2,0	$g.l^{-1}$
Yeast extract	3,0	$g.l^{-1}$
Sucrose	1,0	$g.l^{-1}$
L-lysine	10,0	$g.l^{-1}$
Sodium thiosulphate	0,2	$g.l^{-1}$
Ammonium iron(II) sulphate	0,2	$g.l^{-1}$
Bromocresol purple	0,032	$g.l^{-1}$
Bacto-agar	6,0	$g.l^{-1}$

The medium was heated to dissolve the agar, pH adjusted to 5,6 and dispensed 5 ml per test tube. Tubes were autoclaved

at 121 C for 15 min, immediately overlayed with sterile liquid paraffin and allowed to solidify in a vertical position. Inoculation was by a central stab through the liquid paraffin layer to the bottom of the tube. Incubation was at 26 C for 28 d.

Organisms with lysine decarboxylase activity neutralize the medium as a result of cadaverine production due to the decarboxylation of lysine. This changes the indicator colour to violet. Species which reduce thiosulphate cause an additional blackening of the violet medium as a result of iron sulphide precipitation. Lysine decarboxylase negative organisms do not increase the pH value of the medium and the indicator colour remains unchanged.

- *Asparagine as Sole Source of Carbon and Nitrogen* (Dye, 1962):

Four inorganic solutions were prepared, viz.,

1 =	K_2HPO_4	8,0	g
	KH_2PO_4	2,0	g
	Distilled water	100	ml
2 =	$MgSO_4.7H_2O$	2,0	g
	$FeSO_4$	0,5	g
	NaCl	1,0	g
	$MnSO_4$	0,02	g
	N_2-free H_2SO_4	1 drop	
	Distilled water	100	ml
3 =	Na_2MoO_4	0,02	g
	Distilled water	100	ml

4 = $CaSO_4$, saturated solution in distilled water.

Ten ml of each solution were mixed in the order 3, 4, 2 and 1, filtered and added to 960 ml distilled water in which was dissolved 2,0 g l-asparagine. This was dispensed as 5 ml quantities into test-tubes. All glassware used during preparation of the medium was acid soaked, rinsed thoroughly in several changes of distilled water, autoclaved and dried before use. Inoculated tubes were incubated at 26 C and examined for growth after 2, 4, 7, 14, 21 and 28 d. Tubes showing growth were streaked out onto YSP and Nutrient agar plates to verify purity of the cultures.

- *Aesculin Hydrolysis* (Dye, 1962,1980):

$NH_4H_2PO_4$	0,5 $g.l^{-1}$
K_2HPO_4	0,5 $g.l^{-1}$
$MgSO_4.7H_2O$	0,2 $g.l^{-1}$
Yeast extract	5,0 $g.l^{-1}$
Ferric ammonium citrate	0,5 $g.l^{-1}$
Aesculin	1,0 $g.l^{-1}$

The pH of the medium was adjusted to 6,8 and 5 ml amounts dispensed into test tubes before autoclaving at 121 C for 15 min. Tubes were inoculated and incubated at 26 C for 28 d with shaking.

Results were read as follows: aesculin utilization - development of a dark brown colour; complete hydrolysis of aesculin - no fluorescence under ultra-violet light in addition to the dark brown colour.

- *Starch Hydrolysis* (Dye, 1962,1980; Smibert & Krieg, 1981): The carbohydrate and organic acids basal medium (excluding bromocresol purple) was supplemented with 0,2% soluble starch and the medium sterilized at 115 C for 10 min. Dried plates were streaked once across the surface and incubated at 26 C for 28 d.

Analysis for starch hydrolysis (beta-amylase activity) was carried out at 2, 4, 7, 14, 21 and 28 d by flooding the plates with a solution of iodine. Unhydrolysed starch formed a blue colour with iodine, while complete hydrolysis resulted in a clear zone in the medium surrounding the colony. Reddish-brown zones around colonies denoted partial hydrolysis to dextrins (alpha-amylase activity).

- *Gelatin Hydrolysis* (Anon., 1953b; Blazevic & Ederer, 1975; Kelman & Dickey, 1980; Smibert & Krieg, 1981):
Method 1 - The carbohydrate and organic acids basal medium (excluding bromocresol purple) was supplemented with 0,4% gelatin and autoclaved at 121 C for 15 min. Dried plates were inoculated by streaking once across the surface and incubating at 26 C for 28 d. Plates were examined weekly for gelatin liquefaction, as a result of gelatinase activity, by flooding with a solution of mercuric chloride ($HgCl_2$ 15 g; conc. HCl 20 ml; distilled water 80 ml). Unhydrolysed gelatin formed a white opaque precipitate with

the reagent, while hydrolysed gelatin appeared as a clear zone around the streak.

Method 2 - Nutrient gelatin (Difco Laboratories, USA) was dissolved by heating and 8 ml amounts dispensed into test tubes and sterilized at 121 C for 15 min. Tubes were stab inoculated and incubated at 26 C for 6 weeks. These were examined weekly for gelatin liquefaction by refrigeration for 30 min. The medium became solid when liquefaction had not occurred.

- *Casein Hydrolysis* (Dye,1980; Smibert & Krieg, 1981):
Sterile double strength carbohydrate and organic acid basal medium (excluding bromocresol purple) was added to an equal volume of sterile 10% skim milk (autoclaved at 121 C for 5 min) at 50 C and mixed thoroughly. Dried plates were inoculated by streaking once across the agar surface and incubated at 26 C for 28 d. Clear zones surrounding the bacterial growth would indicate casein hydrolysis, verified by flooding the plates with a solution of mercuric chloride (see Gelatin Hydrolysis).

- *Catalase Test* (Blazevic & Ederer, 1975; Dye, 1962; Skerman, 1967; Smibert & Krieg, 1981):
One drop of 3% H_2O_2 was placed onto a clean microscope slide containing a suspension of bacteria from an agar plate. This suspension was prepared using a non-metallic

instrument, e.g., a wooden stick or glass rod. The suspension was examined immediately, and again after 5 min, for effervescence caused by the liberation of gaseous oxygen, indicating the presence of the enzyme catalase.

- *Oxidase Test* (Blazevic & Ederer, 1975; Moore et al., 1980; Skerman, 1967; Smibert & Krieg, 1981):
Test reagent - 0,1 g tetramethyl para-phenylenediamine hydrochloride was dissolved in 10 ml distilled water. The reagent was prepared fresh prior to use. A small area of filter paper was soaked with 2-3 drops of reagent onto which a test colony was smeared. Oxidase positive colonies turned dark purple within 5-10 seconds.

- *Litmus Milk Reactions* (Anon, 1965; Seeley & VanDemark, 1972; Smibert & Krieg, 1981)

Skim milk powder	100 $g.l^{-1}$
Litmus	0,75 $g.l^{-1}$

The medium was mixed to give a thin cream and 5 ml aliquots were distributed into test tubes. Tubes were sterilized by autoclaving at 121 C for 5 min.

Following inoculation as above and incubation at 26 C, tubes were examined at 2, 4, 7, 14, 21 and 28 d. Reactions occurring in litmus milk include:

(i) Acid production as a result of lactose fermentation to lactic acid causing the medium to become pink; increased acidity results in curdling of the casein (acid clot) and if gas is produced during this fermentation, bubbles and furrows form in the curd ("stormy clot").
(ii) The oxidation of lactose results in the formation of butyric acid, lactic acid, CO_2 and H_2 - the excess hydrogen reduces the litmus and the medium turns white (litmus reduction), usually in the bottom portion of the tube.
(iii) Alkaline reactions either do not change the colour of the medium, or change it to a deeper blue - this reaction is the result of the partial degradation of casein into shorter polypeptide chains and a simultaneous release of alkaline end-products.
(iv) Proteolysis occurs when casein is degraded to amino acids by organisms which cannot utilize lactose as an energy source - litmus changes to deep purple in the upper portion of the tube and the medium appears translucent, brown and wheylike.
(v) Renin production results in a soft semisolid clot insoluble in alkali. Unlike the acid clot, this clot flows when the tube is tilted.

- *Triple Sugar-Iron Agar* (Anon, 1986; Smibert & Krieg, 1981):

Peptone (from casein)	15,0 $g.l^{-1}$
Peptone (from meat)	5,0 $g.l^{-1}$
Meat extract	3,0 $g.l^{-1}$
Yeast extract	3,0 $g.l^{-1}$

NaCl	5,0 $g.l^{-1}$
Lactose	10,0 $g.l^{-1}$
Glucose	1,0 $g.l^{-1}$
Sucrose	10,0 $g.l^{-1}$
Ammonium iron (III) citrate	0,5 $g.l^{-1}$
Sodium thiosulphate	0,3 $g.l^{-1}$
Phenol red	0,024 $g.l^{-1}$
Agar	12,0 $g.l^{-1}$

The medium was dissolved and dispensed into test tubes so that upon slanting butts with a height of at least 3 cm were formed. Cultures were streaked onto the slant and the butts stab inoculated. Tubes were incubated at 26 C and examined at 2, 4, 7, 14, 21 and 28 d.

The reactions after incubation were interpreted as follows:

- Acid butt and alkaline slant (yellow butt, red slant) indicated that glucose was fermented but not sucrose or lactose;
- Acid butt and acid slant (yellow butt, yellow slant indicated the fermentation of lactose and/or sucrose;
- Alkaline butt and alkaline slant (red butt, red slant) indicated that none of the sugars were fermented;
- Gas production indicated by bubbles in the butt. Large amounts of gas would break the agar or push it upward;
- H_2S production indicated by blackening of the butt as a result of H_2S reacting with the ferrous ammonium sulphate to precipitated sulphide.

- *Simmon's Citrate Agar* (Anon, 1965,1986; Blazevic & Ederer, 1975; Smibert & Krieg, 1981):

$MgSO_4.7H_2O$	0,2	$g.l^{-1}$
$NH_4H_2PO_4$	1,0	$g.l^{-1}$
NaCl	5,0	$g.l^{-1}$
K_2HPO_4	1,0	$g.l^{-1}$
Sodium citrate	2,0	$g.l^{-1}$
Bromothymol blue	0,08	$g.l^{-1}$
Agar	15,0	$g.l^{-1}$

Dried plates of the medium were lightly inoculated and incubated at 26 C for 28 d.

Citrate utilization resulted in the medium becoming alkaline, causing a colour change from green to blue. Organisms unable to utilize citrate were either inhibited by the medium, or did not cause a colour change when growth occurred.

(ii) Anaerobic growth

Growth under anaerobic conditions was tested by incubating log-phase cultures on Wilbrink's and YSP agar plates in sealed Oxoid anaerobic jars (Oxoid Ltd., England). Each jar contained an Oxoid gas generating kit for anaerobic systems and an Oxoid anaerobic indicator for the incubation period of 14 d.

(iii)Sodium chloride and pH tolerance

Suspensions of log phase cultures, at concentrations of approximately 10^{12} cells.ml^{-1}, were prepared in sterile distilled water. Tubes containing 5 ml aliquots of Wilbrink's and YSP broth at varying pH's and sodium chloride concentrations were inoculated with 0,1 ml of these suspensions and incubated at 26 C for 48 h, with shaking as above. Control tubes of both media, lacking sodium chloride and at pH 7 (the mid-range for optimum growth of all isolates), were included in the ranges tested, namely:

- % sodium chloride: 0,01; 0,05; 0,1; 0,2; 0,3; 0,4; 0,5; 0,6; 0,8; 1,0; 1,5; 2,0; 3,0 and 4,0.
- pH: 5,4; 5,6; 5,8; 6,0; 6,2; 6,4; 6,6; 6,8; 7,0; 7,2; 7,4; 7,6; 7,8 and 8,0.

Following incubation, the turbidity of each culture was determined spectrophotometrically (A_{600}) using a Beckman DU-8 spectrophotometer. Sodium chloride tolerance was expressed as a percentage of the optical density (A_{600}) in the control tubes.

(iv) Optimum growth temperature

The optimum growth temperature for each strain was determined using a Model 675 Temperature Gradient Incubator (Scientific Industries, Inc.) equipped with L-shaped culture tubes. Growth in the range from 10 C to 45 C was examined. Tubes containing 15 ml of Wilbrink's broth were inoculated

with 0,1 ml of an exponential phase suspension of bacteria prepared in sterile distilled water at a concentration of approximately 1 x 10^9 cells.ml^{-1}. After incubation for 48-72 h with shaking (shaker setting of 3), the turbidity at each temperature was measured spectrophotometrically (A_{600} as above).

2.2.4) Cell membrane characterization

(a) Isolation and preparation

(i) Crude membranes

All five strains of *X. albilineans* were grown on Wilbrink's and YSP media incubated at 26 C. Log-phase bacterial suspensions, prepared in sterile distilled water, were seeded on Wilbrink's and YSP agar plates to form a lawn of bacteria after 3-5 d. Broth cultures (500 ml aliquots in 1 l Erlenmeyer flasks) were incubated with shaking at 120 rev.min^{-1} for 3-5 d.

Following purity checks using the Gram stain and streak plate methods, petri dish cultures were harvested by scraping off the lawn of bacteria using a sterile bent glass rod and suspending the cells in 10 mM Tris-HCl at pH 8. These were washed once by centrifuging at 10000 x *g* for 10 min at 4 C (Beckman J2-21 centrifuge using a JA-17 rotor). The washed cells were resuspended in Tris buffer to give an

absorbance of 10 at 600 nm (Beckman DU-8 Spectrophotometer). After purity checks, broth cultures were harvested by centrifuging at 10000 x *g* for 10 min at 4 C. Cells were resuspended in Tris buffer and adjusted to the same cell concentration as above.

Bacteria were lysed by two cycles through a French pressure cell (SLM-Aminco Instruments, Inc., USA) at 16000 psi. On average, the percentage lysis after the first and second cycles were 85,0% and 93,5%, respectively. This was determined spectrophotometrically by comparing the absorbances of lysed and unlysed suspensions at 600 nm.

French pressed suspensions were centrifuged at 10000 x *g* (10 min, 4 C) to remove any remaining intact bacteria. The supernatant was then centrifuged at 160000 x *g* (av.) for 60 min at 4 C (Beckman L5-50 Preparative Ultracentrifuge) to pellet the crude membrane preparation. The pellets were resuspended in Tris buffer and stored at -20 C.

(ii) Fractions

Prior to sucrose density gradient centrifugation, frozen crude membrane preparations were thawed and washed once in Tris buffer. Membrane band separation was optimized using the following stepped sucrose density gradient:

% sucrose (w/w)	volume (ml)
55	1,0
50	2,0

45	3,0
40	3,0
30	3,0

Each gradient was loaded with 1 ml of crude membranes in 25% sucrose in 10 mM Tris-HCl (pH 8) and centrifuged at 150000 x *g* (av.) for 16 h at 4 C (Beckman L5-50 Preparative Ultracentrifuge). After centrifugation, gradients were fractionated using an Isco Density Gradient Fractionator Model 640 and the absorbance profiles, at 280 nm, of each gradient obtained (Isco Model UA-5 Absorbance Monitor).

After refractometric density determinations of selected fractions at 20 C (Atago Refractometer), the fractions constituting the different membrane bands were pooled, washed, and pelleted by centrifugation at 160000 x *g* (av.) for 60 min at 4 C. All pellets were resuspended in Tris buffer and stored at -20 until further use.

(b) Morphology and composition

(i) Electron microscopy

Crude membranes were examined by electron microscopy to establish their morphological characteristics. Samples for transmission electrion microscopy were negatively stained with 1% phosphotungstic acid (pH 6,8) for 30 s, placed onto collodion coated grids and examined using a Philips EM 301 Transmission Electron Microscope.

Membrane preparations for scanning electron microscopy were placed onto Nucleopore polycarbonate filters (0,22 µm pore size) and excess fluid drained by weak vacuum suction. Filters containing the membranes were fixed in 3% glutaraldehyde in 0,05 M cacodylate buffer (pH 6,8) for 1 h, then washed twice in cacodylate buffer (10 min each wash). Dehydration was effected in a series of ethanol dilutions of 10%, 30%, 50%, 70%, 90% and twice in 100%, 10 min each. The material was critical point dried with CO_2 in a Hitachi HCP Critical Point Drier, gold-coated and viewed on a Jeol Scanning Electron Microscope.

(ii) SDS-polyacrylamide gel electrophoresis

The modified Laemmli discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system was used to determine the polypeptide composition of all membrane preparations (Laemmli, 1970; Laemmli & Favre, 1973). In order to establish molecular masses of individual polypeptides, and to effect comparisons between the membrane bands and crude membrane preparations, electrophoregrams were scanned on a Beckman DU-8 spectrophotometer, equipped with a slab-gel compuset accessory.

The following stock solutions were prepared for use in these experiments:

- Acrylamide/Bis
  acrylamide 30,00 g
  N,N'-methylene-bis-acrylamide 0,80 g
  The solutions were dissolved in distilled water and made up to 100 ml, filtered if turbid, and stored at 4 C in the dark.

- *NNN'N'*-tetramethylethylenediamine (TEMED)
  A 1:100 dilution was prepared in distilled water prior to use.

- Ammonium persulphate
  A 10% (w/v) aqueous solution was prepared prior to use.

- Sodium dodecyl sulphate*
  100 g SDS was dissolved in distilled water by gentle heating and mixing, and made up to 500 ml.

- Separation buffer, pH 8,8 (5x stock)
  1,875 M Tris-HCl 227,15 g
  0,5% SDS* 25,00 ml
  Dissolved in approximately 800 ml distilled water, pH adjusted to 8,8 with HCl (conc.) and made up to 1 l with distilled water.

- Stacking buffer, pH 6,8 (10x stock)
  1,25 M Tris-HCl 75,7 g
  1% SDS* 25,0 ml
  Dissolved in approximately 400 ml distilled water, pH adjusted to 6,8 with HCl (conc.) and made up to 500 ml with distilled water.

- Electrode buffer, pH 8,3 (10x stock)
  0,25 M Tris-HCl 60,60 g
  1,92 M glycine 288,28 g
  1% SDS* 100,00 ml
  Dissolved in approximately 1500 ml distilled water, pH adjusted to 8,3 and made up to 2 l with distilled water.

- Ethanol-water
  A 20% solution made up in distilled water.

- Sample disintegration buffer (2x)
  1 M Tris-HCl, pH 6,9 6,25 ml
  20% SDS* 10,00 ml
  mercaptoethanol 5,00 ml
  glycerol 10,00 ml
  distilled water 8,75 ml
  0,02% bromophenol blue
  (filtered) 10,00 ml
  Aliquots of 1 ml were dispensed and stored frozen.

- Staining and destaining solutions (volume in ml)

	1	2	3	4	5
isopropanol	625	250	-	-	-
glacial acetic acid	250	250	250	250	250
distilled water	1550	1975	2250	2250	2125
Coomassie brilliant blue R250 (1% solution)	75	25	7,5	-	-
glycerol	-	-	-	-	125

Gels were prepared as follows:

- Separation gel

5x separation buffer	5,00 ml
acrylamide/bis	10,40 ml
TEMED	0,70 ml
distilled water	8,75 ml

The solution was carefully mixed, degassed for 30 s and 0,14 ml of the ammonium persulphate solution added. The solution was mixed again and 20 ml poured per gel slab assembly. This was immediately, but carefully, overlaid with the ethanol-water solution, allowed to polymerize and stand overnight. Polymerization was carefully monitored to ensure that the process was completed within 10 to 16 min. Within this period, polymerization occurred uniformly throughout the gel. Precautions to be taken when pouring gels, included: adding the ammonium persulphate only when the gel was ready to be poured; solutions had to be mixed carefully to avoid frothing; to prevent mixing when overlaying with ethanol-water; and carefully monitoring the polymerization time by sucking up some gel solution into a Pasteur pipette.

- Stacking gel

The ethanol-water overlay was poured off, the separation gel rinsed with 1x stacking buffer and excess rinse buffer removed. The stacking gel solution comprised:

10x stacking buffer	1,00 ml
acrylamide/bis	1,30 ml
TEMED	0,50 ml
distilled water	7,10 ml

The solution was mixed, degassed for 30 s and 0,11 ml ammonium persulphate added to it. The solution was carefully mixed again, a small amount used to rinse the separation gel surface and the gel solution poured into the assembly. The well-forming comb was carefully inserted so as not to trap any air-bubbles. Polymerization was monitored as before, and the gel allowed to stand for 1 h.

Samples and protein standards were prepared by mixing equal volumes with the sample disintegration buffer. These were heated in a boiling water bath for between 3 to 5 min. Between 3 to 25 microlitres of sample was applied to each well, using a microsyringe, and electrophoresed at 75 V (15 - 20 mA) for 30 min and 125 V (approximately 30 mA) for the rest of the run. The runs were stopped when the tracking dye reached 1 cm from the bottom of the gel.

Gels were fixed and stained in the staining solutions listed above, successively for 4 h to overnight in solution 1, and 2 to 4 h in each of solutions 2, 3 and 4. Gels to be preserved were placed into solution 5 for 4 h before drying. All gels were scanned and photographed as described above.

2.3 RESULTS

2.3.1) Isolation and purification of XA 86-1

All three isolation techniques (section 2.2.1(a)) were used to take samples during the initial stages of this work. A combination of effective surface sterilization and the use of selected areas on diseased tissue were found to be the most effective means of isolating XA 86-1 in the shortest time possible (3 to 5 d), with little or no contamination. Of the methods developed, the third technique was the most successful and consistent in this regard. The best sampling

sites were the symptom-bearing parts of diseased material and newly emerged shoots on symptomless plants and diseased material. In addition, the rigorous disinfection routine proved to be invaluable in eliminating the faster growing contaminating bacteria and fungi which facilitated easier purification of XA 86-1. For these reasons, and for ease of operation and consistency in the results obtained, this technique was used solely in all subsequent routine isolations of XA 86-1.

The random sampling approach of the first technique was not feasible for the following reasons:

- it was labour-intensive since various areas of diseased plants were analysed, not all yielding isolates of the pathogen;
- the method of disinfection was inadequate because within 1 to 4 d the faster growing bacteria and fungi would dominate; after 7 d all plates were entirely overgrown. The slower-growing XA 86-1 colonies, usually punctiform after 3 d, could be isolated with some difficulty and at least three subcultures were required to purify isolates;
- extracting the pathogen by adpressing sections of freshly exposed tissue onto agar, and homogenisation proved to be cumbersome because of the large amounts of material usually sampled.

The second technique was an improvement on the first with a more effective disinfection procedure and reduced labour time as a result of restricting the sampling material only to the leaves of diseased plants. Fewer contaminating organisms tended to develop on the isolation plates and XA 86-1 was usually obtained in pure culture from 50% to 60% of the samples analysed. The leaf squash (Khan, 1971) and sap exudation (Masuda & Tokeshi, 1978) techniques yielded excellent results, the former when sufficient sap could be extracted from symptom-bearing leaves. A modification to the procedure of Khan (1971), a squash in one drop of sterile distilled water, created the problem of unnecessary contamination. Unless effectively disinfected, samples analysed by the sap exudation method also resulted in the contamination of agar plates.

2.3.2) Maintenance of stock cultures

Short-term working stocks maintained on agar slants and petri plates kept at room temperature required subculturing at 2- to 3-week intervals. Cultures kept for longer periods tended to grow more slowly when transferred to fresh media and colonies varied in appearance and consistency. Some colonies appeared smaller and more deeply pigmented. Plate and slant cultures kept at 4 C were transferred to fresh media every 6- to 8-weeks and the colonies that developed

appeared similar to those arising from cells freshly isolated from diseased material.

Conversely, cell suspensions in distilled water stored at 4 C remained viable for up to 6 months only, while the room temperature suspensions were viable for between 8 to 9 months. The survival of exponential phase (7 d) cultures was better than that of the stationary phase (14 d) cultures - shown by the larger numbers of colonies developing upon culturing on fresh media. Perez (1970) noted that bacteria stored in distilled water remained virulent even after 18 months storage, with no loss of pigments after plating. Although the effect of storage on virulence was not examined, the method of distilled water storage was adopted for all short-term working stocks.

The traditional methods of freeze-drying and storage under mineral oil produced inconsistent results. Cultures stored under mineral oil remained viable for 14 months, and resulted in low cell recovery rates. Similar results were obtained for cultures freeze-dried in YSP medium supplemented with 5% glycerol. More successful in terms of the period of viability and cell recovery rates were the snap-freeze and ultra-freeze techniques, using the suspending media described earlier (Section 2.2.1)(b)), and lyophilization in the skim milk/sodium glutamate mixture. A viability check at 30 months showed all cultures to be

viable, even with repeated freezing and thawing of the snap- and ultra-frozen material.

2.3.3) Pathogenicity trials

Growth of the setts, after 11 d, was noted as the emergence of the first leaves above soil level. All control plants germinated and none expressed any form of leaf scald disease throughout the experimental period.

The pre-planting inoculation technique, using sap extracted from diseased tissue, resulted in a variety of symptoms, not all of which were typical of leaf scald disease. These were produced on the 27% of setts which germinated. Setts which did not germinate were examined at two months following planting and were found to have decayed, probably as a result of fungal infections. Shoots of variety N 6, which gave rise to chlorotic leaves, died off soon after emergence of the first and second leaves; however, the pencil-line symptoms appearing on shoots of CB 36/14 persisted for 2 weeks following germination, before disappearing completely. Successful isolations of XA 86-1 were made from all of these specimens.

Post-inoculation setts generated a more consistent pattern of symptoms within the first two months after infection, followed by a disappearance of all symptoms thereafter

(Table 2). Symptoms ranged from the typical pencil-line streaks (Fig. 1a) and withered leaf-tips (Fig. 1b) of the chronic phase, to ultimate stalk death from the acute form of the disease. A fortuitous set of circumstances produced evidence of infection in the more resistant varieties at a later stage. On the basis of reports by Babu (1979), Bisessar (1970), Persley (1973a,b, 1975) and Zummo (1976) all plants were water stressed after two months to induce symptom expression, however, none were produced - which was in agreement with the unpublished report of Ryan & Birch (Ricaud & Ryan, 1989). Thereafter, a mechanical failure in the greenhouse temperature and humidity control systems,

Table 2. Symptom expression on post-planting inoculated setts maintained under greenhouse conditions

Variety Rating	Susceptible		Intermediate		Resistant	
Variety	Co 1001	L 76	N 6	CB 36/14	NCo 310	NCo 376
Time span	S Y M P T O M S					
2-4 weeks	pencil-lines + widespread chlorosis	p e n c i l - l i n e s				
5-6 weeks	wilted leaf-tips and necrosis	------pencil-lines becoming indistinct-------				
7-8 weeks	all leaves necrotic	-no pencil-lines on new and existing leaves-				
6 months	dead	dead	dead	p e n c i l - l i n e s		none

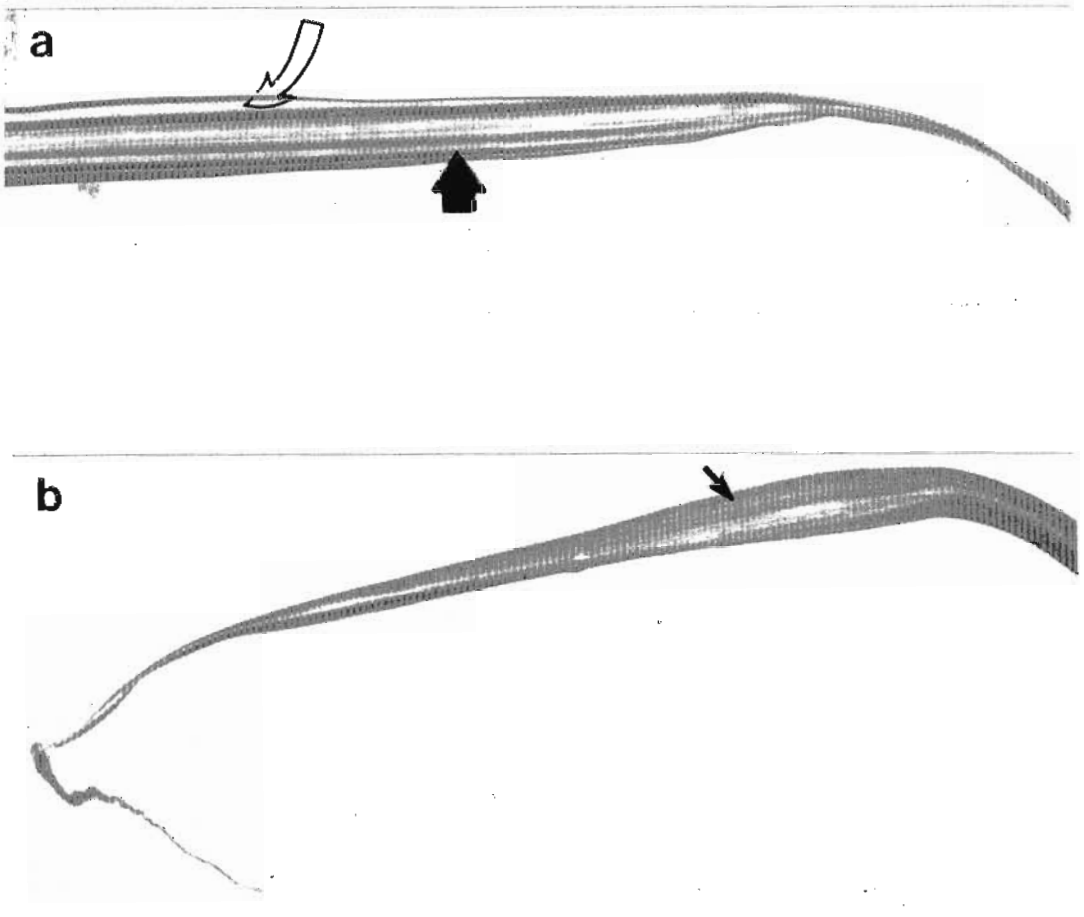

Fig. 1. Chronic phase symptoms shown on inoculated plants kept under greenhouse conditions:
(a) Pencil-line symptoms (indicated by arrows)
(b) Leaf-tip necrosis. Note the pencil-lines becoming diffuse (indicated by arrows).

together with the earlier water stressing, probably resulted in a multiple stress syndrome experienced by the plants. This caused the death of the highly susceptible varieties from the acute form of the disease (side shoots were

produced at the base of these stalks), while pencil-lines developed on varieties CB 36/14 (of intermediate resistance) and NCo 310 (highly resistant). Again, successful isolations were made from all of these plants.

2.3.4) Whole cell characteristics

(a) Colony form, cell morphology and staining reactions

Colonies produced on Wilbrink's and YSP agar were translucent and of a pale yellow to buff colour. All had circular forms with entire margins, smooth surfaces and convex elevations. Colony diameters ranged from 1,5 mm after 7 d to 8 mm after 28 d growth on Wilbrink's and YSP agar plates. The appearance of cells within colonies is shown in Fig. 2.

All cells were gram-negative, non-sporulating, non-encapsulated and rod-shaped. Motility was shown in both the hanging-drop preparation and in motility test medium, and confirmed by electron microscopy which revealed the presence of single polar flagella in all strains (Fig. 3).

Cell lengths varied between the strains, although cell widths were fairly constant (Table 3, Fig. 4). Strains LS155 and 2905 comprised cells of consistent lengths of, respectively, 1,28 and 1,33 micrometres. However, strains

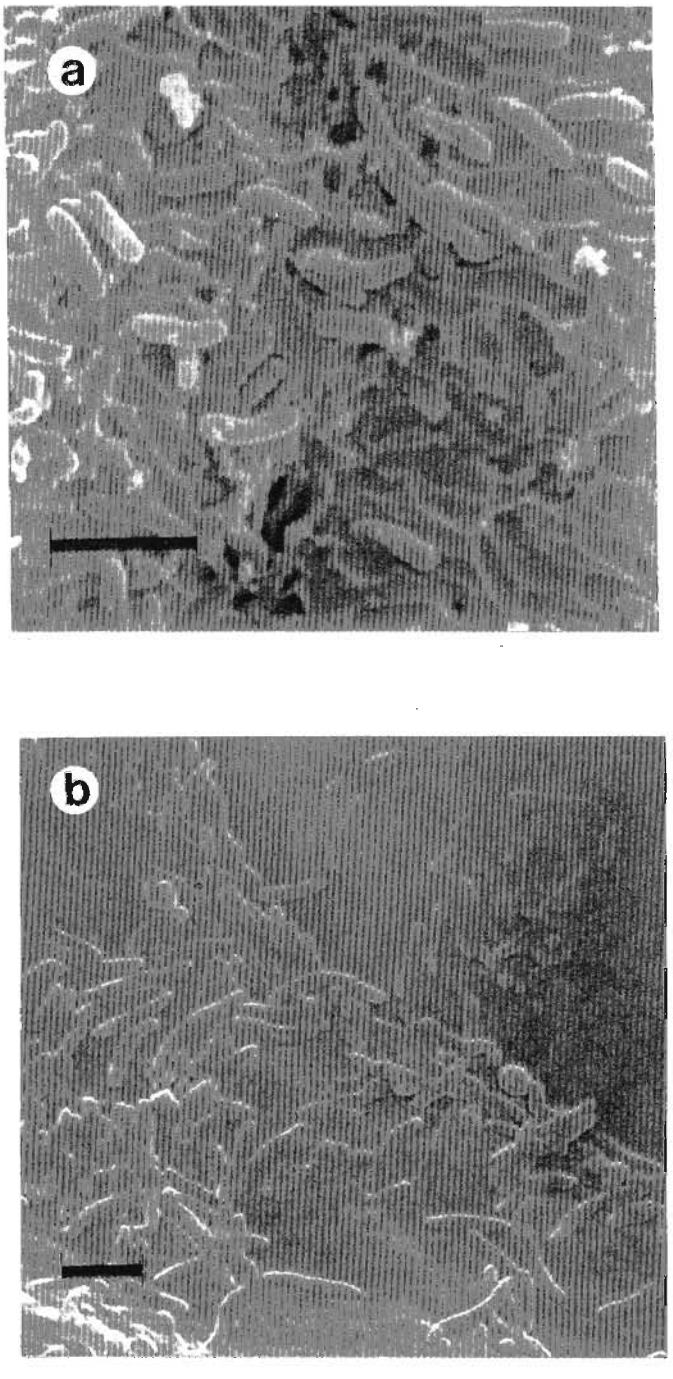

Fig. 2. Scanning electron micrographs of cells within colonies on agar. (a) strain LS155 and (b) strain 2901. Bar represents 1 micrometre.

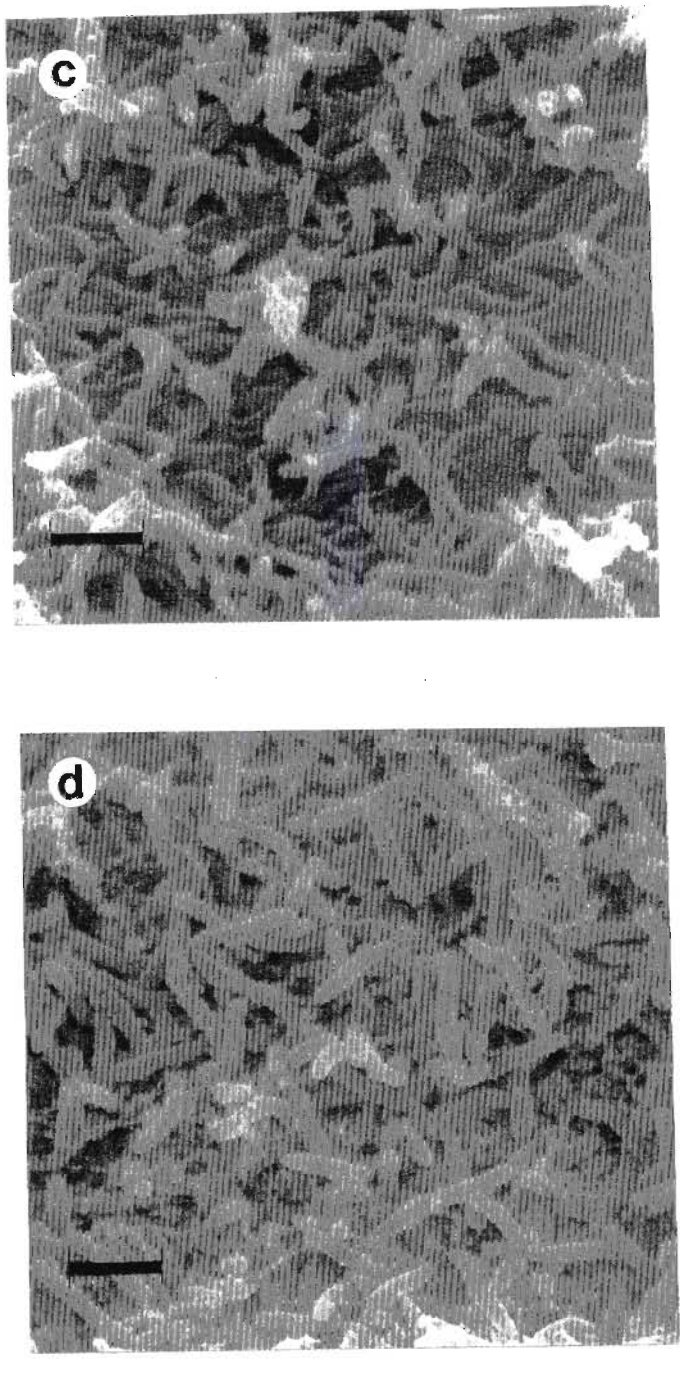

Fig. 2. Scanning electron micrographs of cells taken from colonies grown on agar and placed onto polycarbonate filters. Strains (c) PDDCC 196 and (d) 2905. Bar represents 1 micrometre.

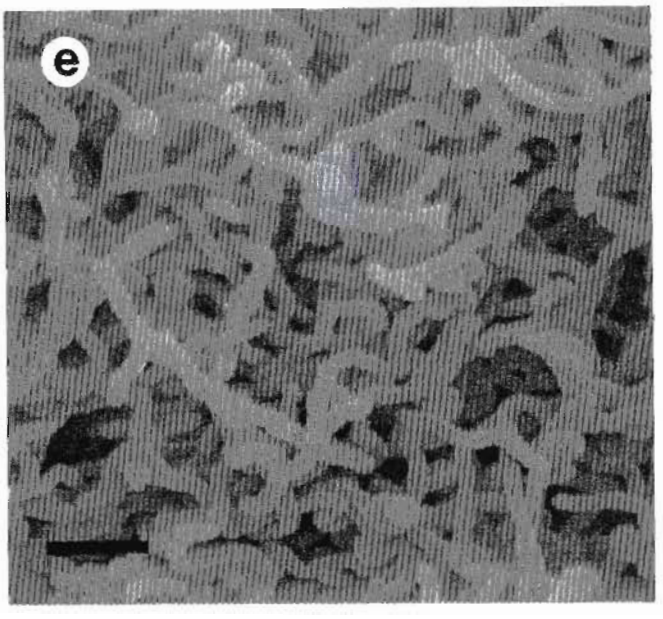

Fig. 2.e. Scanning electron micrograph of cells of strain XA 86-1 taken from colonies grown on agar and placed onto a polycarbonate filter. Bar represents 1 micrometre.

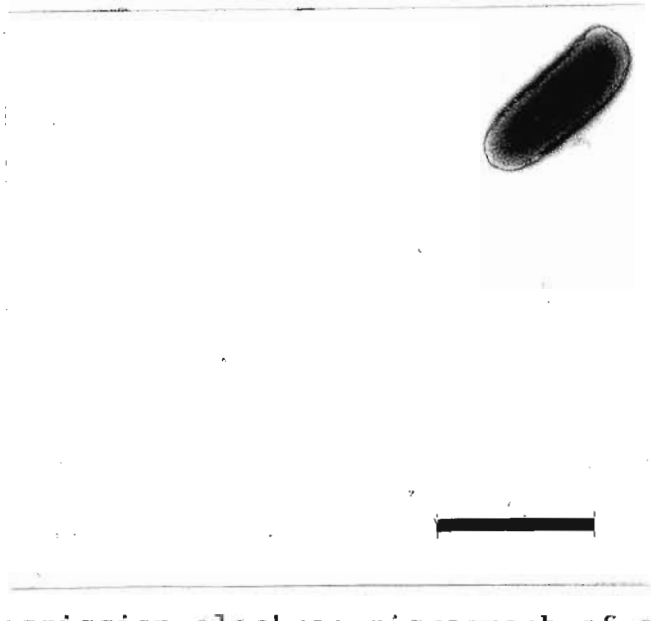

Fig. 3.a. Transmission electron micrograph of an individual cell of strain PDDCC 196, stained with 1% phosphotungstic acid (PTA)(pH 6,8), showing attachment of the single polar flagellum. Bar represents 1 micrometre.

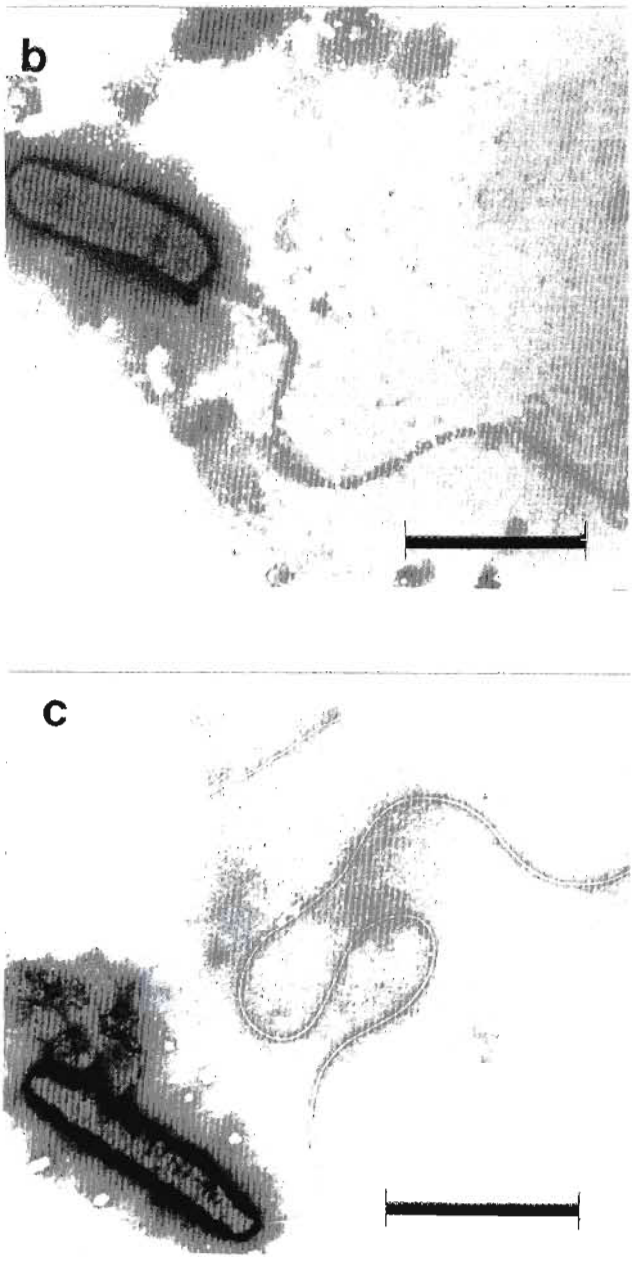

Fig. 3. Transmission electron micrographs of individual bacteria, stained with 1% PTA (pH 6,8), showing attachment of the single polar flagellum. Strains (b) LS155 and (c) 2901. Bar represents 1 micrometre.

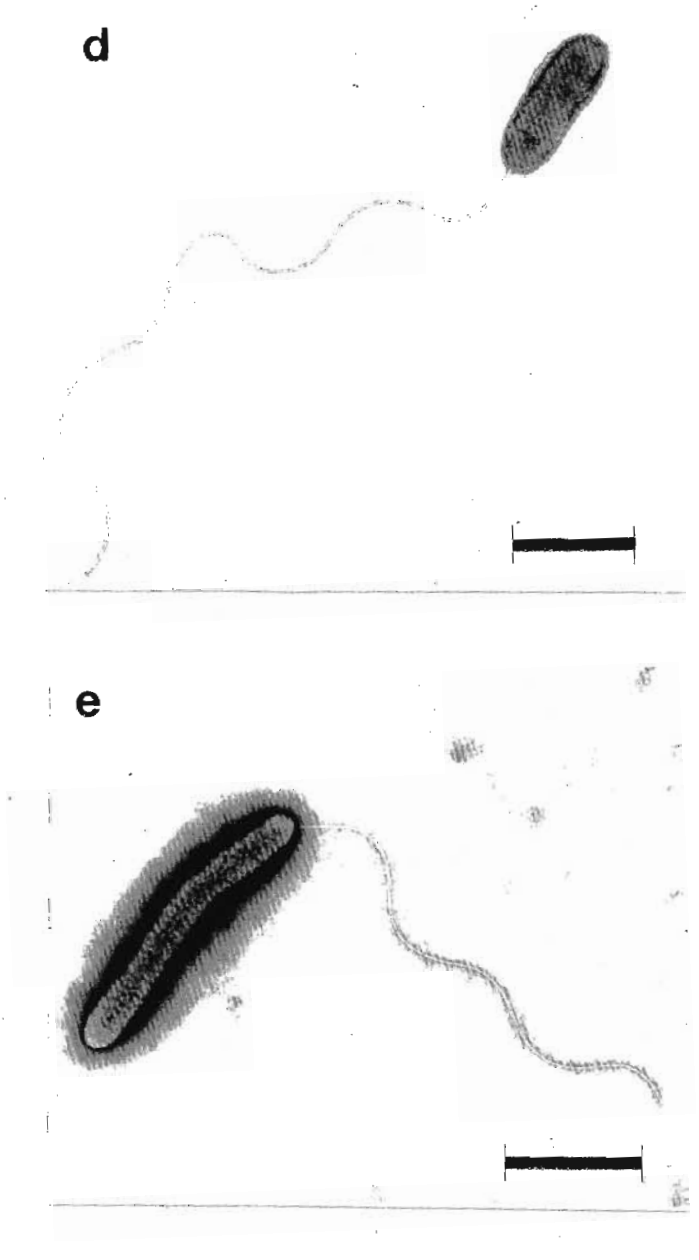

Fig. 3. Transmission electron micrographs of individual bacteria, stained with 1% PTA (pH 6,8), showing attachment of the single polar flagellum. Strains (d) 2905 and (e) XA 86-1. Bar represents 1 micrometre.

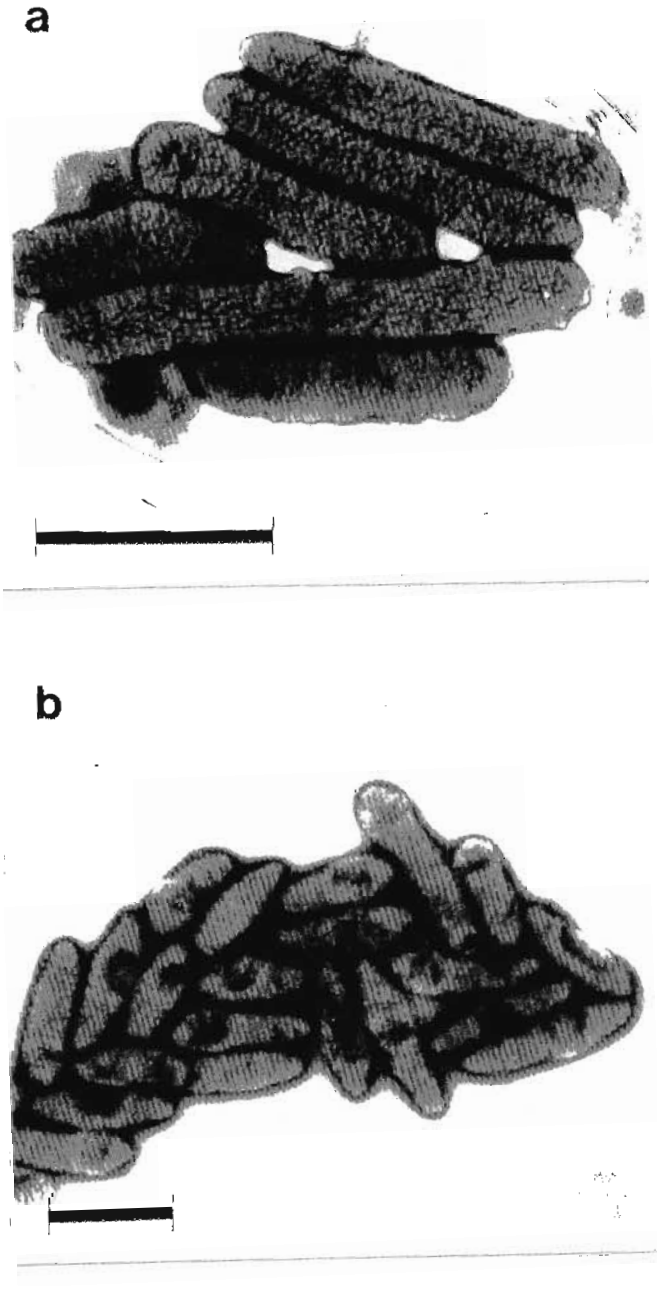

Fig. 4. Transmission electron micrographs of negatively stained cell groups (1% PTA, pH 6,8), showing size variations among cells of strains (a) PDDCC 196 and (b) LS155. Bar represents 1 micrometre.

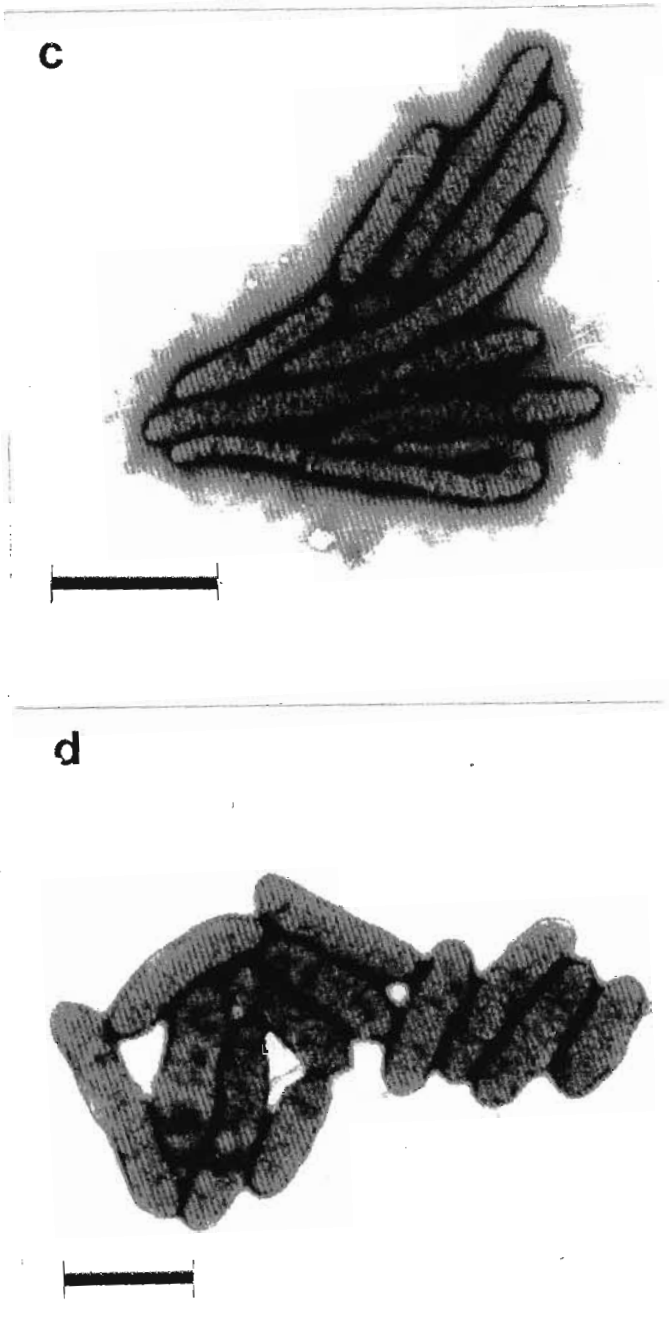

Fig. 4. Transmission electron micrographs of negatively stained cell groups (1% PTA, pH 6,8), showing size variations among cells of strains (c) 2901 and (d) 2905. Bar represents 1 micrometre.

e

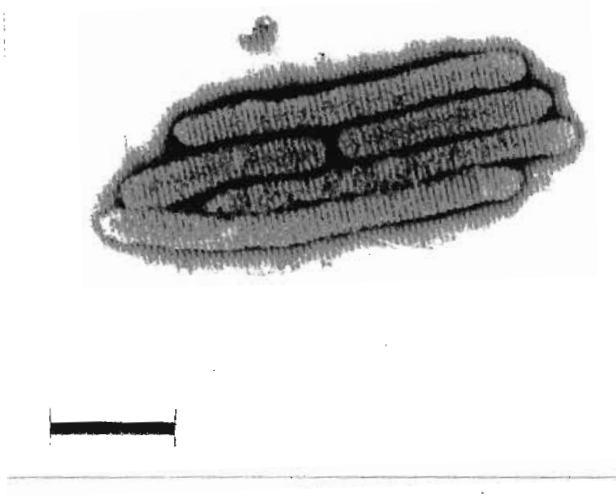

Fig. 4.e. Transmission electron micrograph of a group of cells of strain XA 86-1, negatively stained with 1% PTA (pH 6,8), showing size variations among cells, which were probably dividing. Bar represents 1 micrometre.

2901 and XA 86-1 which comprised longer and more slender cells (respectively, 1,57 and 1,88 micrometres long and 0,21

Table 3. Cell dimensions, in micrometres, of *X. albilineans* strains PDDCC 196, LS155, 2901, 2905 and XA 86-1

Strain	Length	Width
PDDCC 196	1,47 +/- 0,32	0,33 +/- 0,01
LS155	1,28 +/- 0,26	0,33 +/- 0,02
2901	1,57 +/- 0,58	0,21 +/- 0,01
2905	1,33 +/- 0,25	0,34 +/- 0,01
XA 86-1	1,88 +/- 0,99	0,24 +/- 0,01

and 0,24 micrometres wide) showed greater variation in cell lengths. This was especially evident for XA 86-1 which had a length variance factor of 0,99 micrometres. The reference strain, PDDCC 196, was of intermediate cell length (1,47 micrometres) with a cell width (0,33 micrometres) similar to that of strains LS155 (0,33 micrometres) and 2905 (0,34 micrometres).

(b) Pigments

From analyses of the broth culture media and attempts to isolate pigments in aqueous solution, the pigments of *X. albilineans* were found to be non-fluorescent, non-diffusible and water-insoluble. In addition, during preparation in non-aqueous solvents, the pigments could only be extracted in acetone and methanol. Benzene, chloroform, diethyl ether and petroleum ether (b.p. 40-60) proved to be unsuitable as solvents for pigment extraction.

Figure 5 shows the absorbance profiles of pigment extracts from each strain in acetone (Fig. 5b) and methanol (Fig. 5a) on a wavelength scan from 700 to 200 nm. Pigments from all strains showed identical absorption maxima in both the acetone and methanol extracts. Except for strains PDDCC 196 and 2901 which produced equivalent absorbance peaks at 417 and 441 nm and a shoulder at 468 nm, there was generally a

broad peak at 441 nm and shoulders at 417 and 468 nm in the methanol extracts. The pigments in acetone showed the following pattern: strains PDDCC 196, LS 155 and 2901 each produced an absorbance peak at 412 nm while strains 2905 and XA 86-1 absorbed weakly at this wavelength; each strain also had two identical small peaks at 588 and 635 nm and shoulders at 441 and 470 nm. None of the pigment extracts absorbed in the ultraviolet range from 200 to 400 nm.

(c) Biochemistry and physiology

Of the 38 substrates tested, only 11 were capable of being utilized by one or more of the strains of *X. albilineans* studied (Table 4). Although several carbohydrates were metabolized resulting in acid production, there was a complete lack of any concomitant gas production - a characteristic feature of the genus *Xanthomonas*. Another prominent characteristic was the ability of all strains to produce acid from several of the monosaccharides and disaccharides tested; six out of eight for the former and three out of six for the latter. Four of the six monosaccharides, viz., arabinose, glucose, mannose and xylose, were utilized by all strains, some weakly. The only disaccharide metabolized by all strains was sucrose. Other substrates which gave positive reactions were aesculin, positive for all strains; and starch, weak alpha-amylase activity in strains LS155 and 2901.

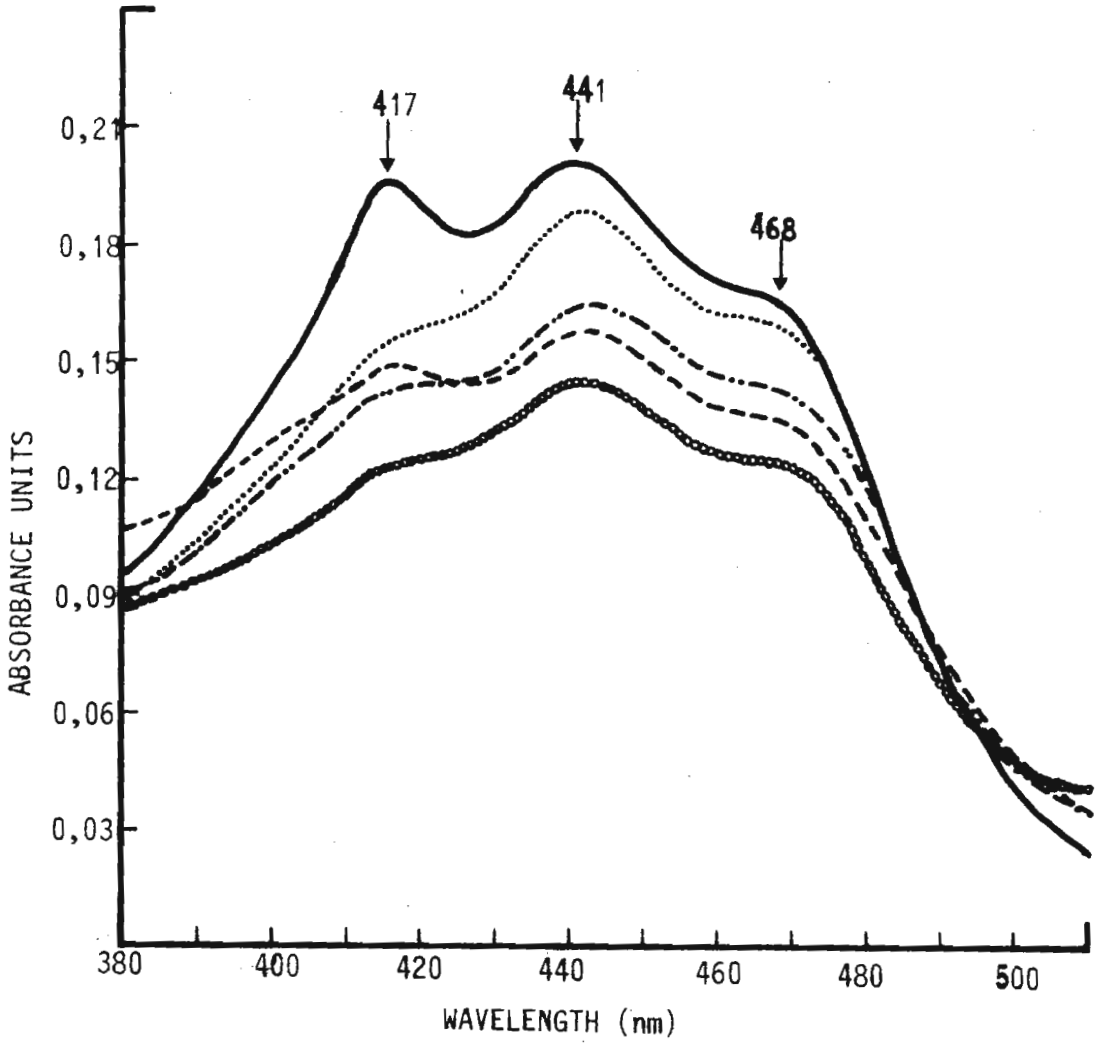

Fig. 5.a. Wavelength scans of pigment extracts in methanol. Arrows indicate absorbance maxima at the various wavelengths. PDDCC 196 (——), LS155 (-·-·-), 2901 (----), 2905 (·····) and XA 86-1 (○○○○○).

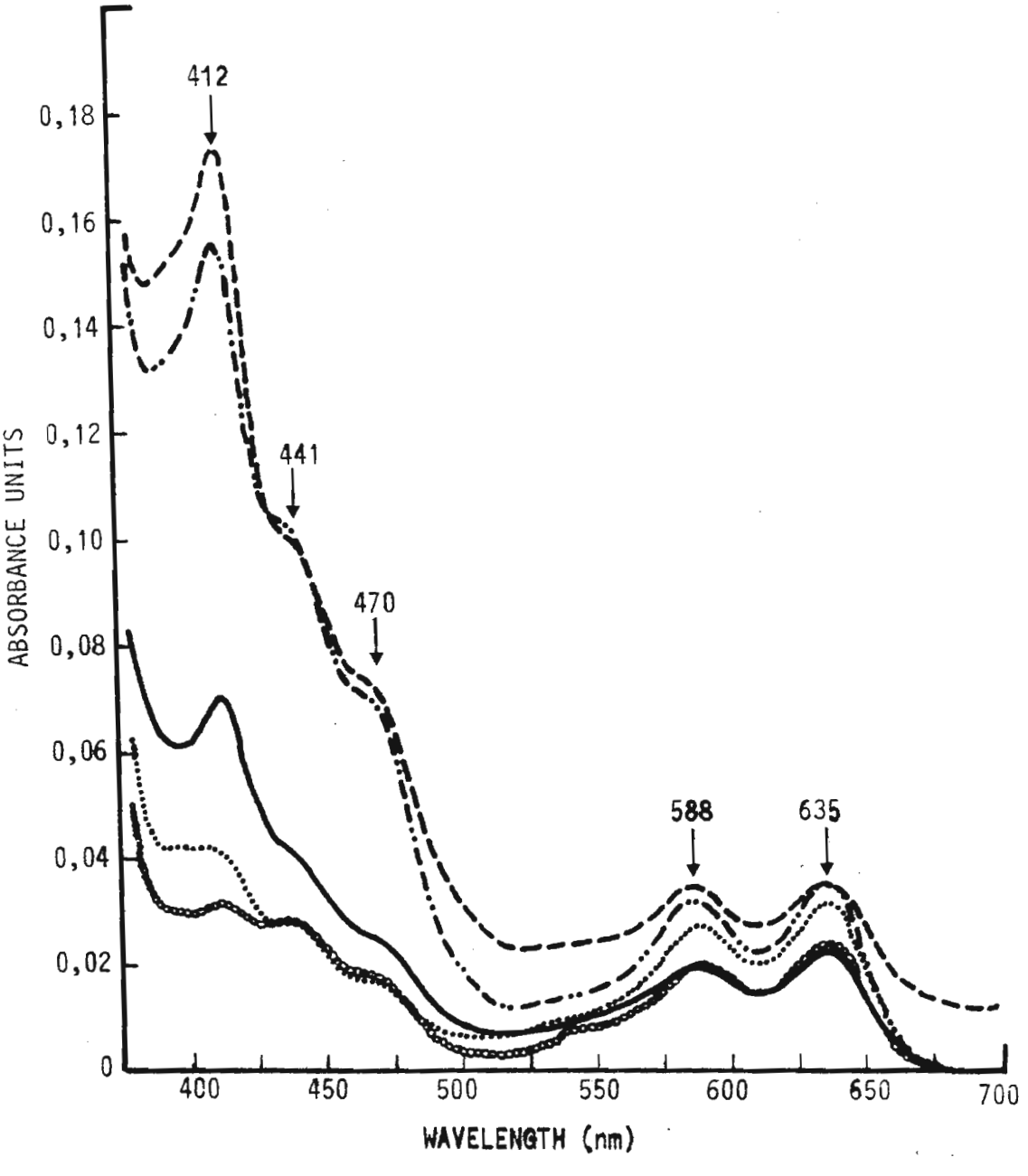

Fig. 5.b. Wavelength scans of pigment extracts in acetone. Arrows indicate absorbance maxima at the various wavelengths. PDDCC 196 (——), LS155 (-·-·-), 2901 (----), 2905 (·····) and XA 86-1 (ooooo).

Table 4. The utilization of carbohydrates, organic acids and other substrates within 28 d on the carbohydrate and organic acid basal medium

Substrate	Strain				
	PDDCC 196	LS155	2901	2905	XA 86-1
MONOSACCHARIDES					
Arabinose	+	+	+	+	+
Fructose	+	–	+	+	–
Galactose	+	+	–	+	+
Glucose	+	w	+	+	+
Mannose	+	+	+	+	+
Rhamnose	–	–	–	–	–
Ribose	–	–	–	–	–
Xylose	w	+	+	+	v
DISACCHARIDES					
Cellobiose	–	–	w	v	v
Lactose	–	–	–	–	–
Maltose	–	–	–	–	–
Melibiose	–	–	–	–	–
Sucrose	+	+	+	+	+
Trehalose	v	w	v	–	v
POLYSACCHARIDES					
Dextrin	–	–	–	–	–
Inulin	–	–	–	–	–
Raffinose	–	–	–	–	–
Starch	–	$	$	–	–
ALCOHOLS					
Adonitol	–	–	–	–	–
Dulcitol	–	–	–	–	–
Erythritol	–	–	–	–	–
Inositol	–	–	–	–	–
Mannitol	–	–	–	–	–
Sorbitol	–	–	–	–	–
GLUCOSIDES					
Aesculin	+	+	+	+	+
Salicin	–	–	–	–	–
ORGANIC ACIDS					
Acetate*	–	–	–	–	–
Benzoate*	–	–	–	–	–
Citrate	–	–	–	–	–
Formate*	–	–	–@	–	–
Fumarate	–	–	–	–	–
Lactate	–	–	–	–	–

Malate	-	-	-	-	-
Propionate*	-	-	-	-	-
Succinate	-	-	-	-	-
Tartrate	-	-	-	-	-
PROTEINS					
Casein	-	-	-	-	-
Gelatin	-	-	-	-	-

Key: - no acid production/no substrate utilization
+ acid production/substrate utilization
v variable reaction
w weak acid production
@ strain 2901 was not inhibited by formate
$ partial degradation to dextrins
* inhibition of growth

Table 5. Reactions of *X. albilineans* strains for various physiological tests

	strain				
Test	PDDCC 196	LS155	2901	2905	XA 86-1
Arginine dihydrolase	w	w	-	-	w
Lysine decarboxylase	-	w	w	w	-
Ornithine decarboxylase	w	w	w	w	w
Urease	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Indole production	-	-	-	-	-
Methyl red	-	-	-	-	-
Voges Proskauer	-	+	-	-	-
Simmon's citrate	-	-	-	-	-
Triple sugar-iron agar	-	-	-	-	-
Nitrate reduction	-	-	-	+	-
H_2S from cysteine	+	+	+	-	+

Dye's asparagine	-	-	-	-	-
Litmus milk	R/P	R/P	R/P	LF	R/P
Oxidation-fermentation:					
glucose	O	O	O	O/WF	WO
lactose	-	-	-	O/WF	-
sucrose	O	O/WF	O	WO/WF	O/WF

Key: - negative reaction
+ positive reaction
w weak reaction
P partial proteolysis
R litmus reduction
LF lactose fermentation
O oxidative metabolism
WO weak oxidative metabolism
WF weak fermentative metabolism

N.B.: WO and WF results, from the oxidation-fermentation tests, were noted following comparisons between control tubes (showing no indicator colour change) and unequivocal positive reactions (where the indicator colour changed to a bright yellow). Any indicator colour change between these two extremes was recorded as weak substrate metabolism for the particular set of conditions.

Additional physiological tests revealed few reactions for which the strains gave definite positive responses, although several weak reactions were observed (Table 5). Unequivocally positive and negative reactions were obtained for the following tests: hydrogen sulphide from cysteine, Voges-Proskauer, nitrate reduction and catalase. The metabolism of glucose and sucrose was oxidative, with evidence also of weak fermentation of sucrose by strains

LS155, 2905 and XA 86-1. In addition to weakly fermenting glucose, strain 2905 was the only organism capable of oxidative and weak fermentative metabolism of lactose. This was confirmed by its reaction in litmus milk. Other reactions in litmus milk included partial proteolysis and litmus reduction for the other four strains. The partial proteolytic activity contradicts the negative results obtained for casein and gelatin hydrolysis using agar plates (Table 4), and is discussed below.

(d) Oxygen relations

None of the strains studied was able to grow under anaerobic conditions although anaerobiosis did not result in cell death. Following exposure to aerobic conditions after 14 d anaerobic incubation, all cultures grew within 5 d.

(e) pH requirements

The optimal pH requirements for growth were consistently in the range of 6,8 to 7,2, especially in Wilbrink's medium (Fig. 6). The alkaline pH ranges (7 to 7,6) tended to favour the growth of strains LS155, 2901 and 2905 in YSP broth only.

(f) Sodium chloride requirements

Growth in different concentrations of sodium chloride added to Wilbrink's and YSP broth revealed two very clear cut-off points, above which less than 20% of the cell population survived (Fig. 7). These points at 0,4% and 0,8% applied respectively to strain groups PDDCC 196, LS155 and XA 86-1, and the Mauritian group of 2901 and 2905. Cell survival always exceeded 100% in Wilbrink's broth supplemented with sodium chloride concentrations ranging from 0,01 to 0,1%, as compared to growth in the control medium which lacked sodium chloride. This trend was not shown for YSP broth.

(g) Temperature relations

The temperature limits for active growth (not thermal death-points) were 20 C and 40 C, a general range within which all strains grew vigorously (Table 6;Fig. 8). Temperature growth optima varied within a 7 C range from 26,35 C (strain PDDCC 196) to 33 C (strain LS155). The optimum for strains 2901 and XA 86-1 was identical (31,1 C), for strain 2905 it was 32 C. In addition to these specific growth optima, four strains showed a sub-optimum growth temperature either lower than the optimum (strains LS155 and XA 86-1, respectively, 26,35 C and 27,25 C), or higher (strains PDDCC 196 and 2901, respectively, 28,35 C and 33 C). This was exaggerated for strain 2905 where two sub-optima were evident at 28,35 C and 35 C. A unique feature of strain LS155 was the presence of two growth optima at 28,35 C and 33 C.

Table 6. Individual tube temperatures measured on the temperature gradient incubator over the range 10 C to 45 C

Tube No.	Temp. (C)	Tube No.	Temp. (C)	Tube No.	Temp. (C)
1	10,00	11	23,50	21	33,00
2	11,70	12	24,40	22	34,00
3	13,15	13	25,30	23	35,00
4	14,75	14	26,35	24	36,00
5	16,25	15	27,25	25	37,00
6	17,50	16	28,35	26	38,70
7	18,75	17	29,20	27	40,35
8	20,00	18	30,20	28	41,80
9	21,25	19	31,10	29	43,40
10	22,50	20	32,00	30	45,00

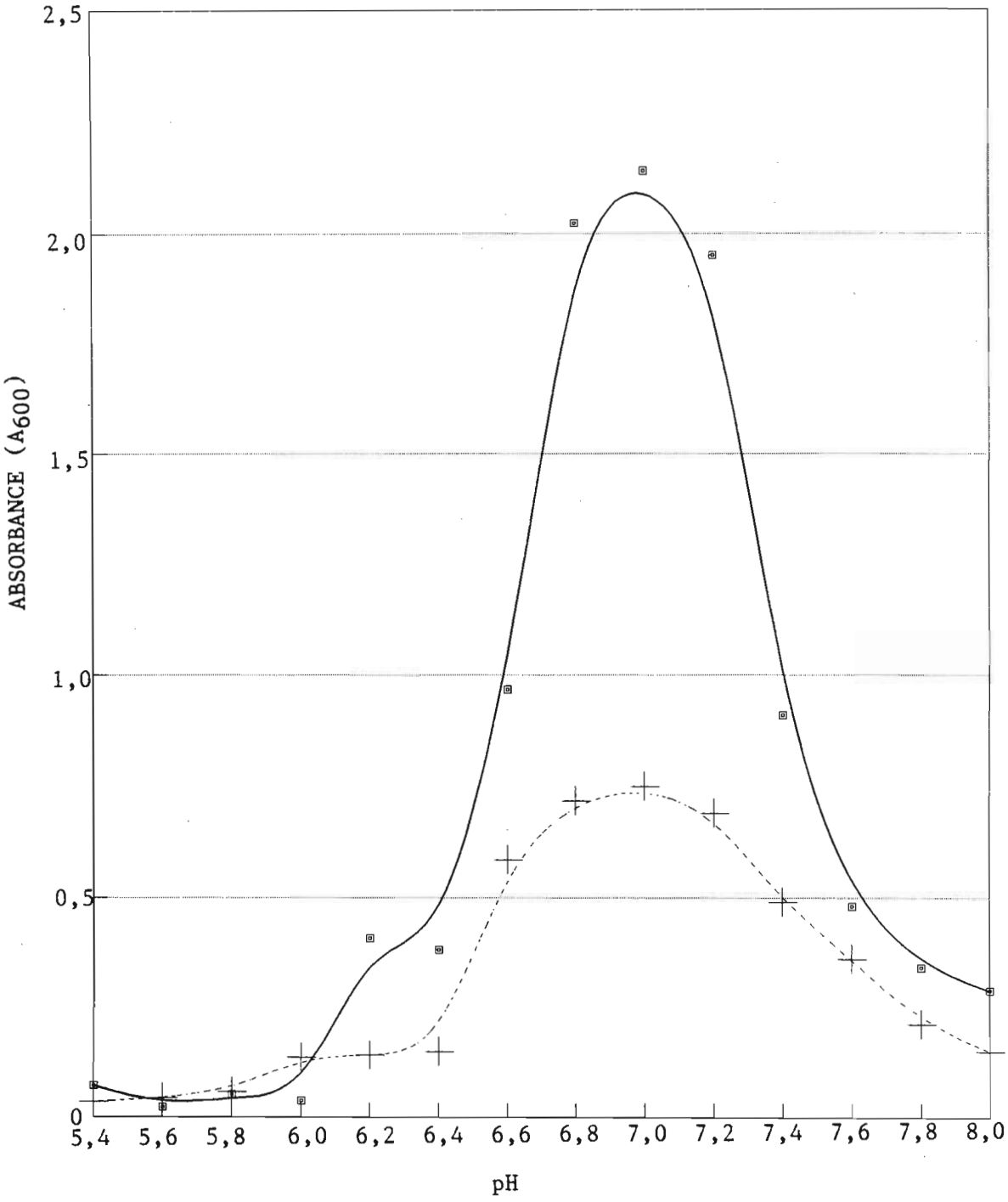

Fig. 6.a. Growth response of strain PDDCC 196 at varying pH levels in Wilbrink's (——) and YSP (---) broth.

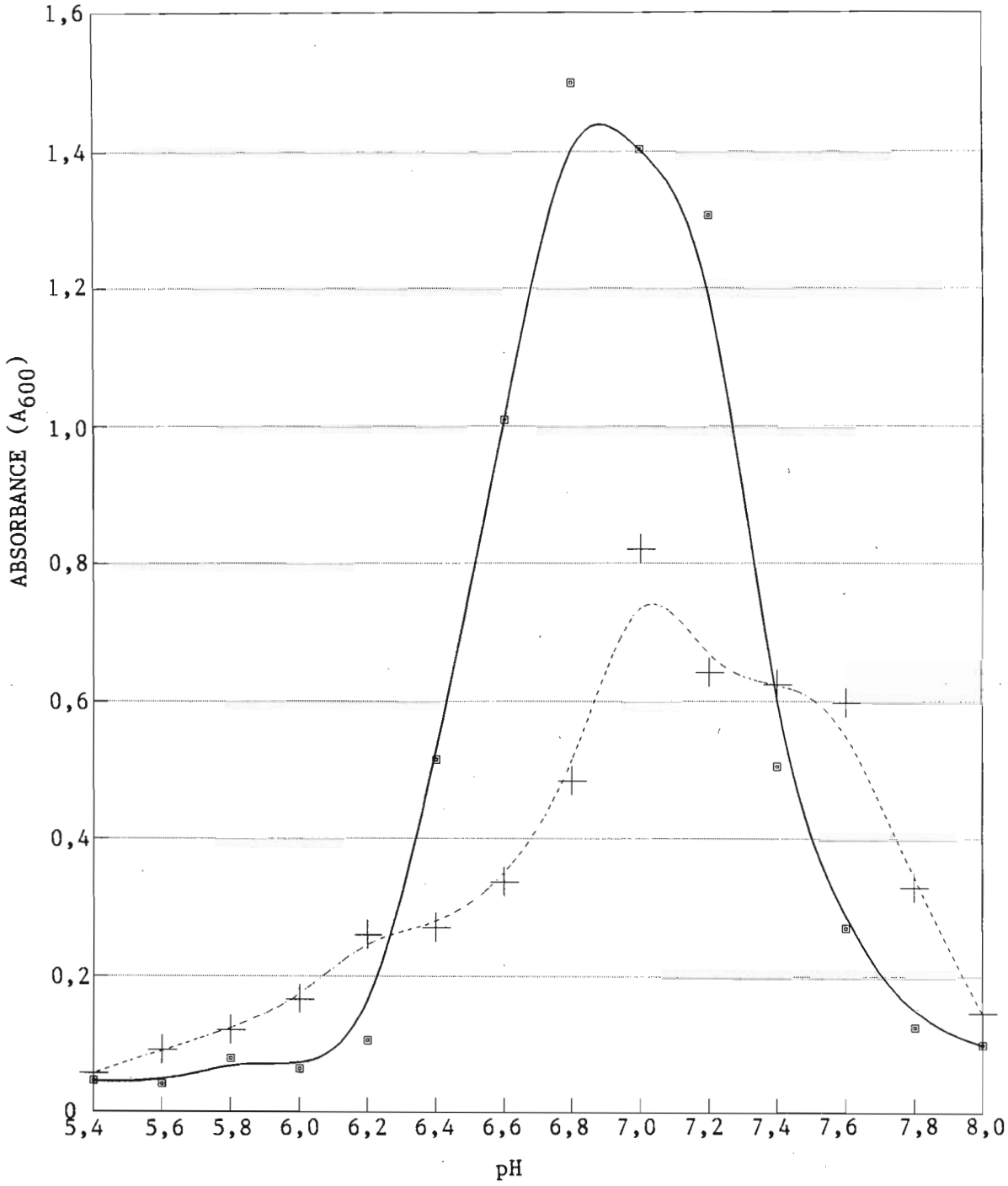

Fig. 6.b. Growth response of strain LS155 at varying pH levels in Wilbrink's (——) and YSP (----) broth.

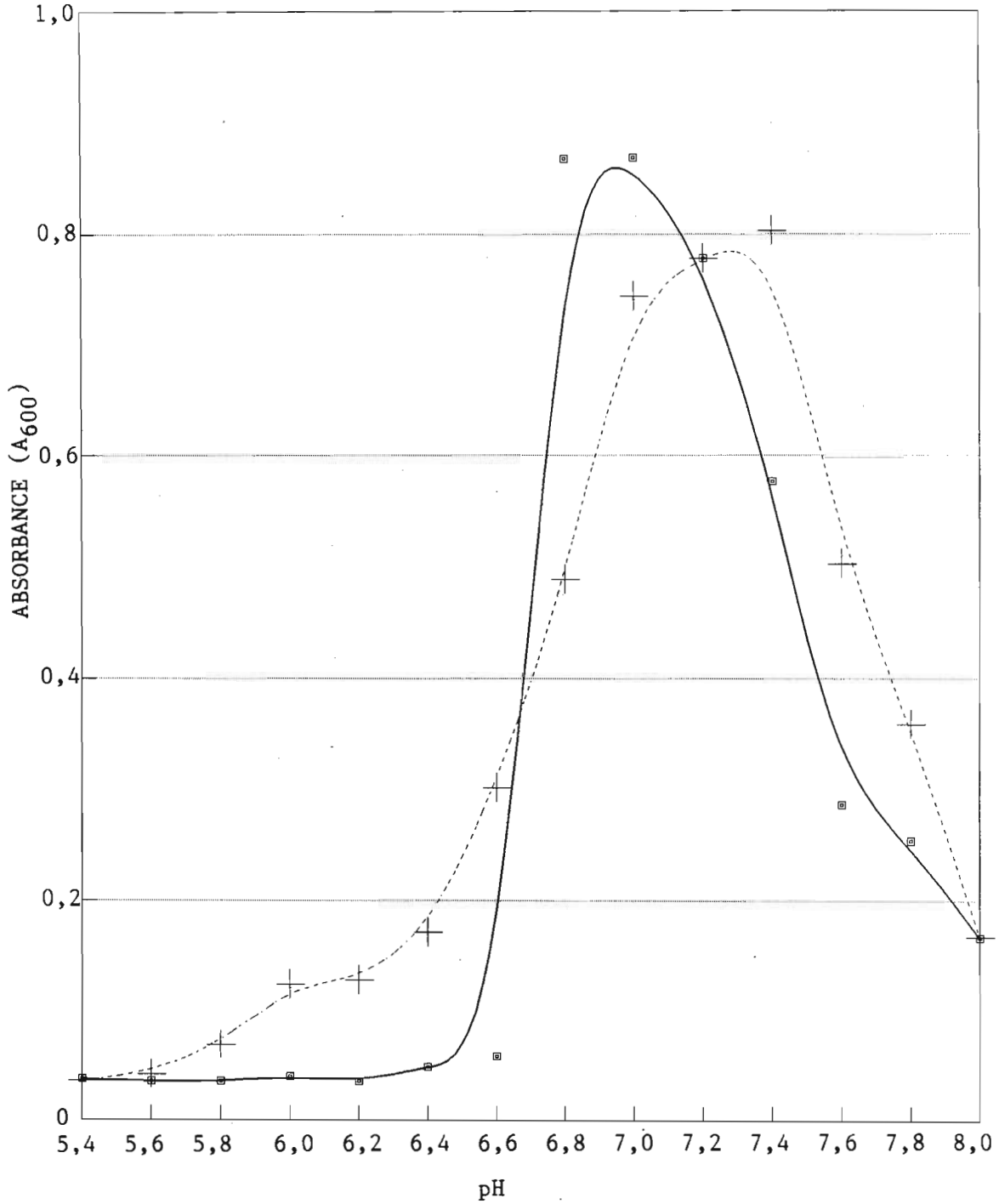

Fig. 6.c. Growth response of strain 2901 at varying pH levels in Wilbrink's (——) and YSP (---) broth.

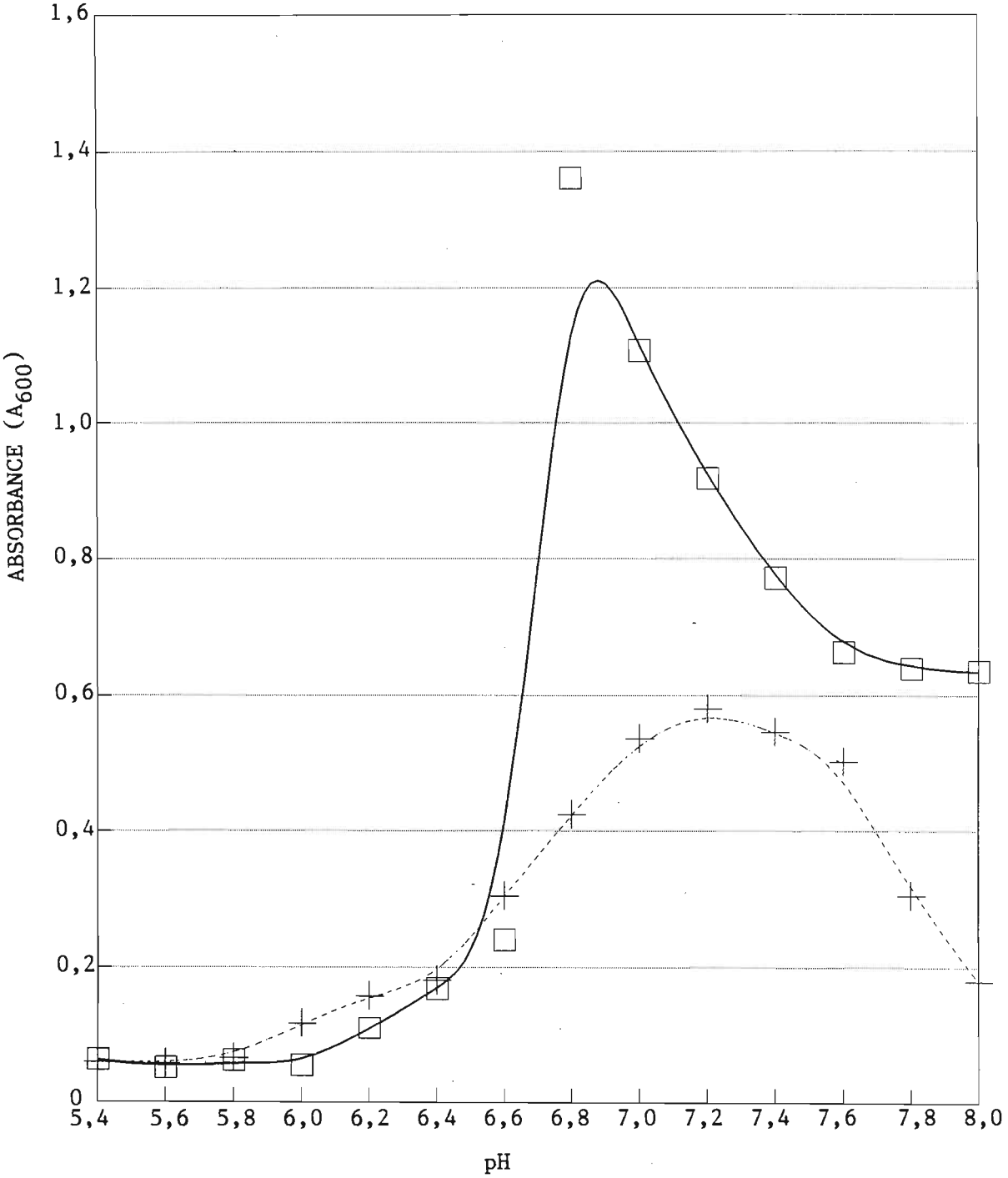

Fig. 6.d. Growth response of strain 2905 at varying pH levels in Wilbrink's (——) and YSP (---) broth.

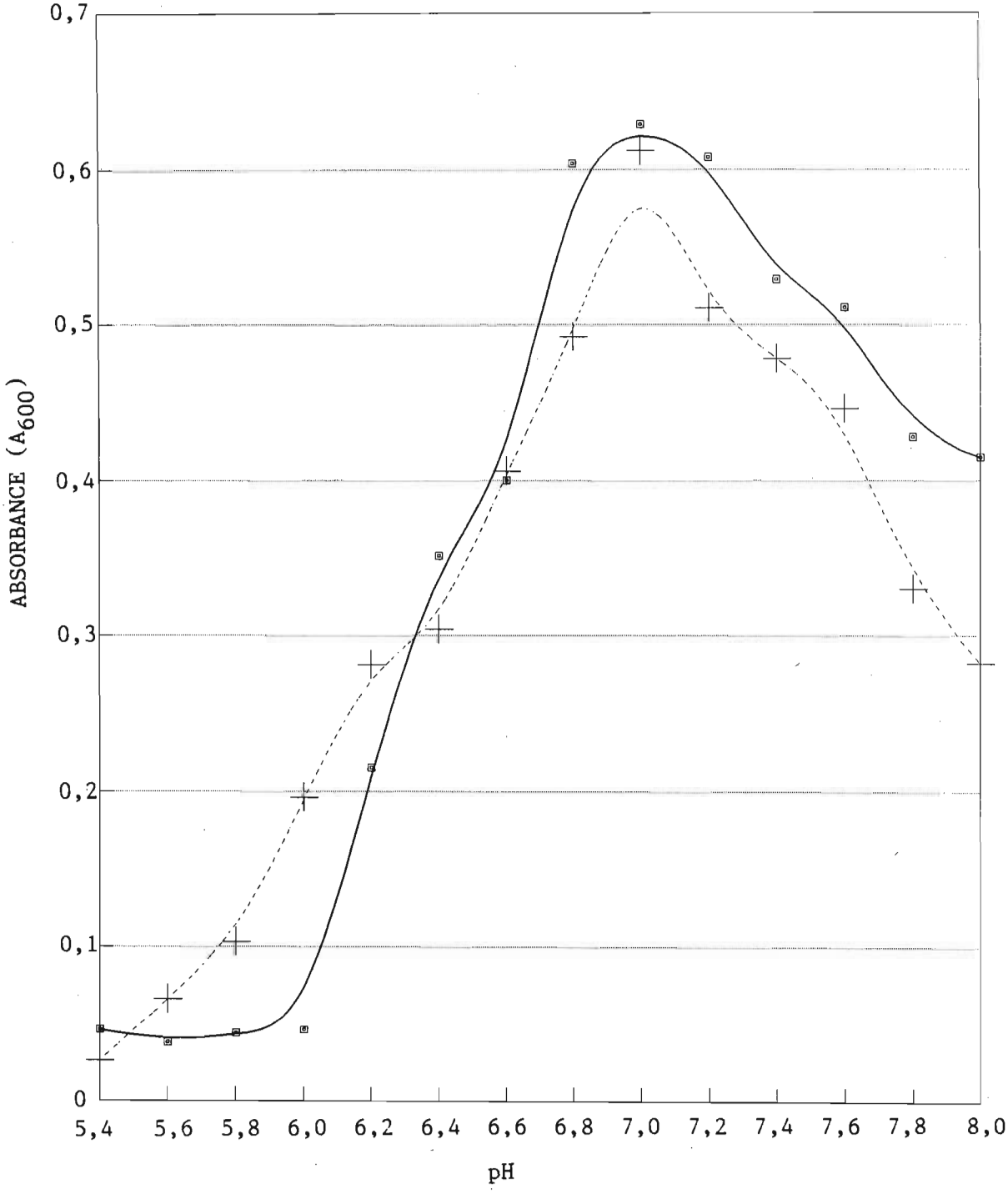

Fig. 6.e. Growth response of strain XA 86-1 at varying pH levels in Wilbrink's (——) and YSP (---) broth.

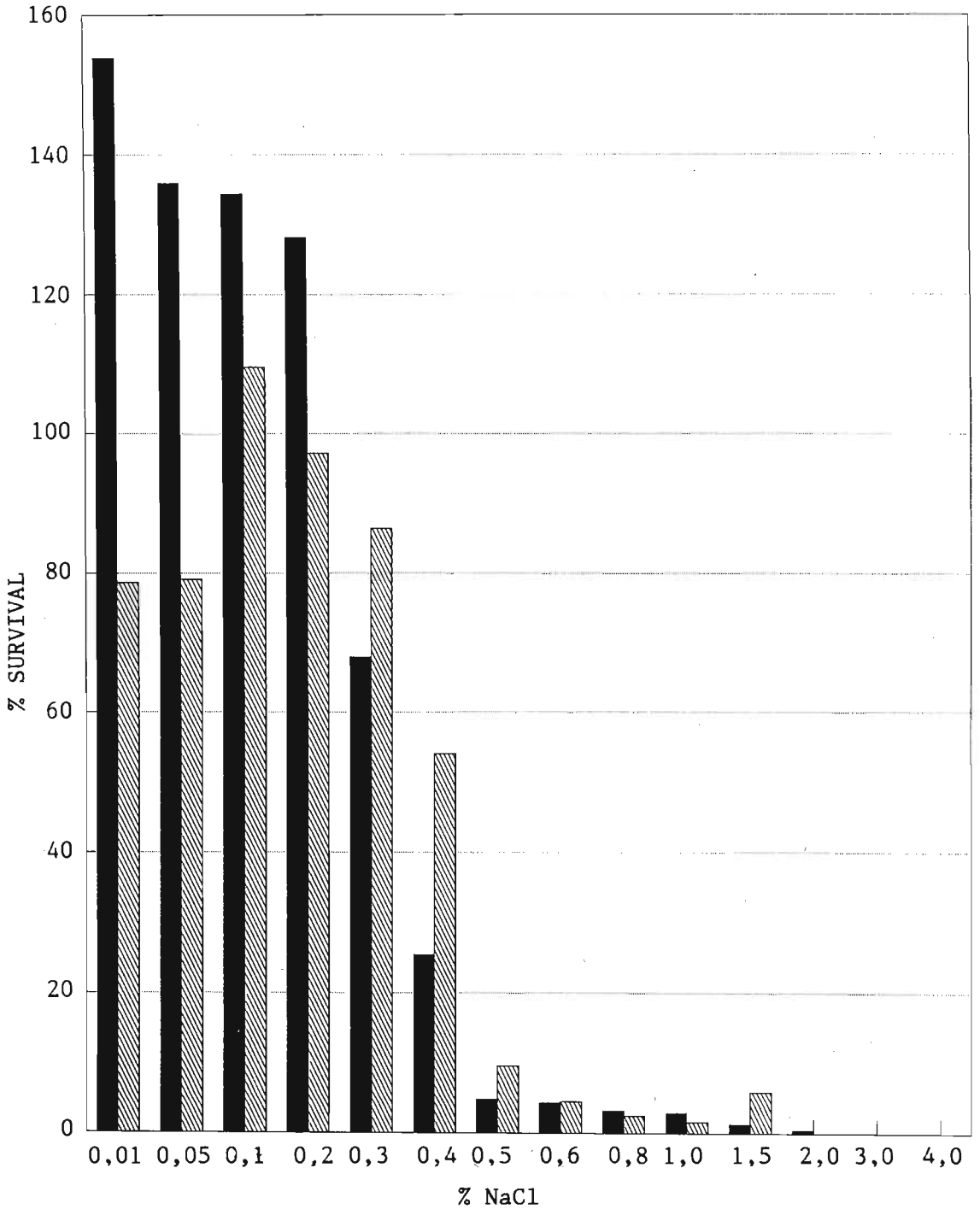

Fig. 7.a. Tolerance of strain PDDCC 196 to a range of sodium chloride concentrations added to Wilbrink's broth (solid bars) and YSP broth (striped bars).

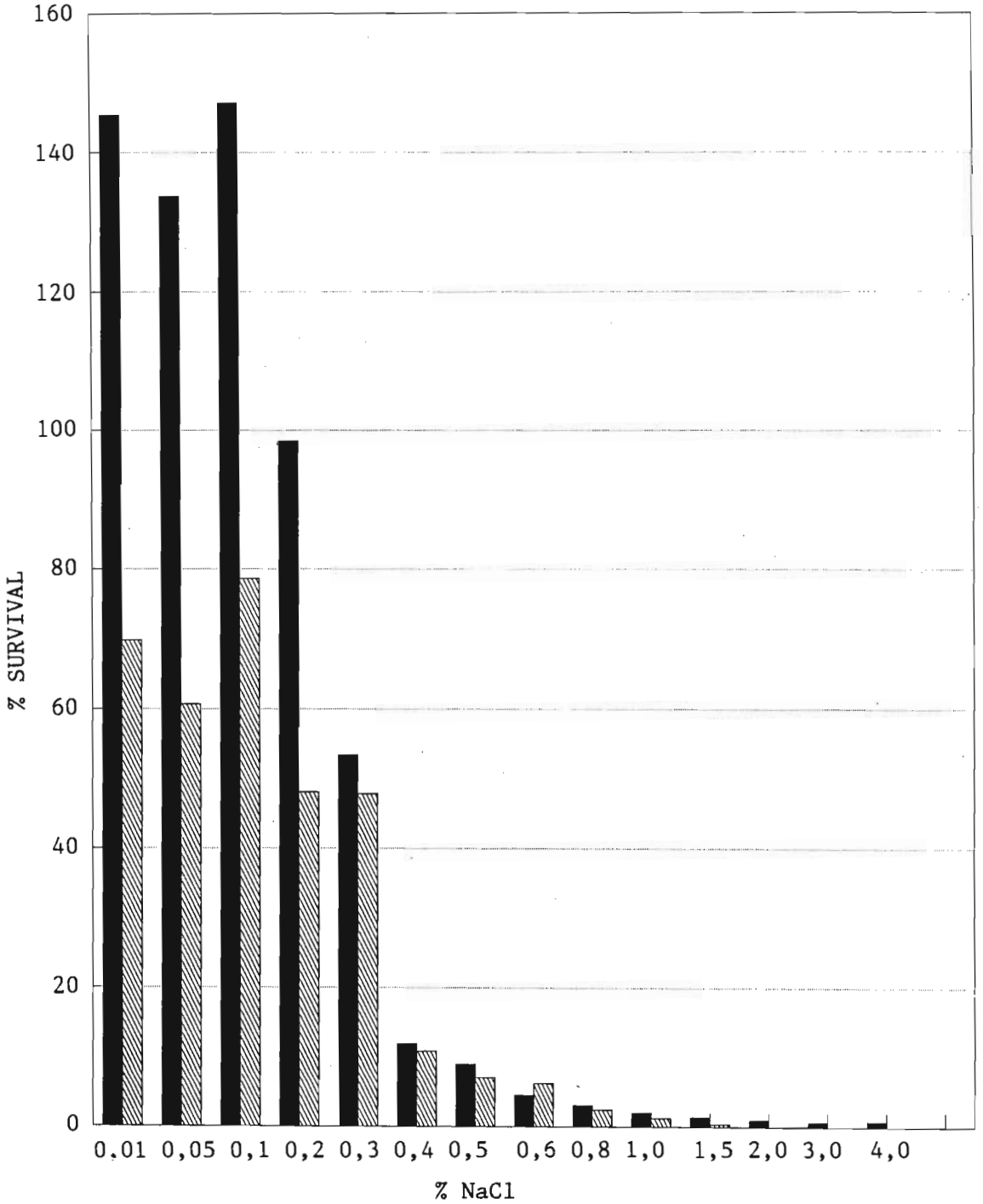

Fig. 7.b. Tolerance of strain LS155 to a range of sodium chloride concentrations added to Wilbrink's broth (solid bars) and YSP broth (striped bars).

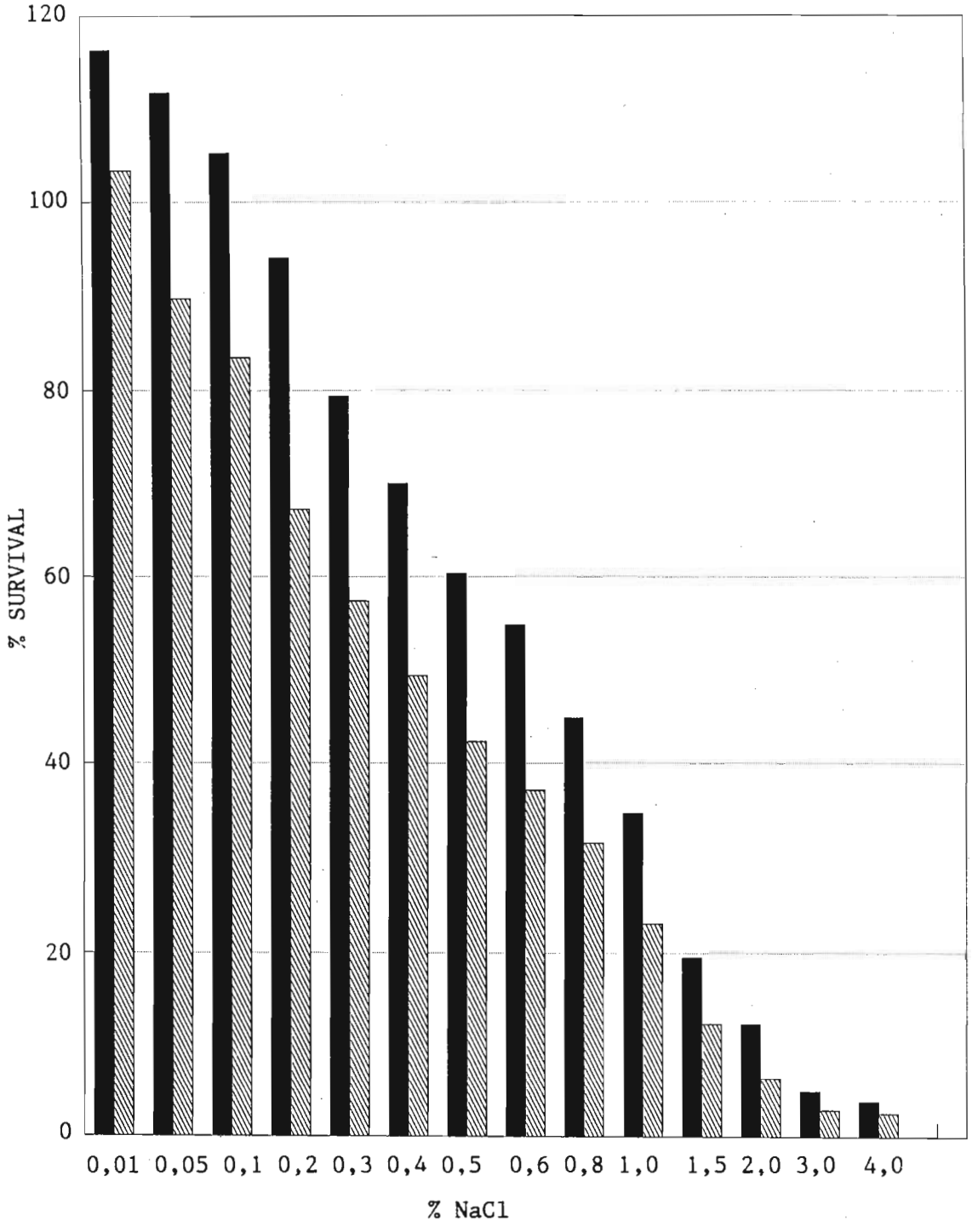

Fig. 7.c. Tolerance of strain 2901 to a range of sodium chloride concentrations added to Wilbrink's broth (solid bars) and YSP broth (striped bars).

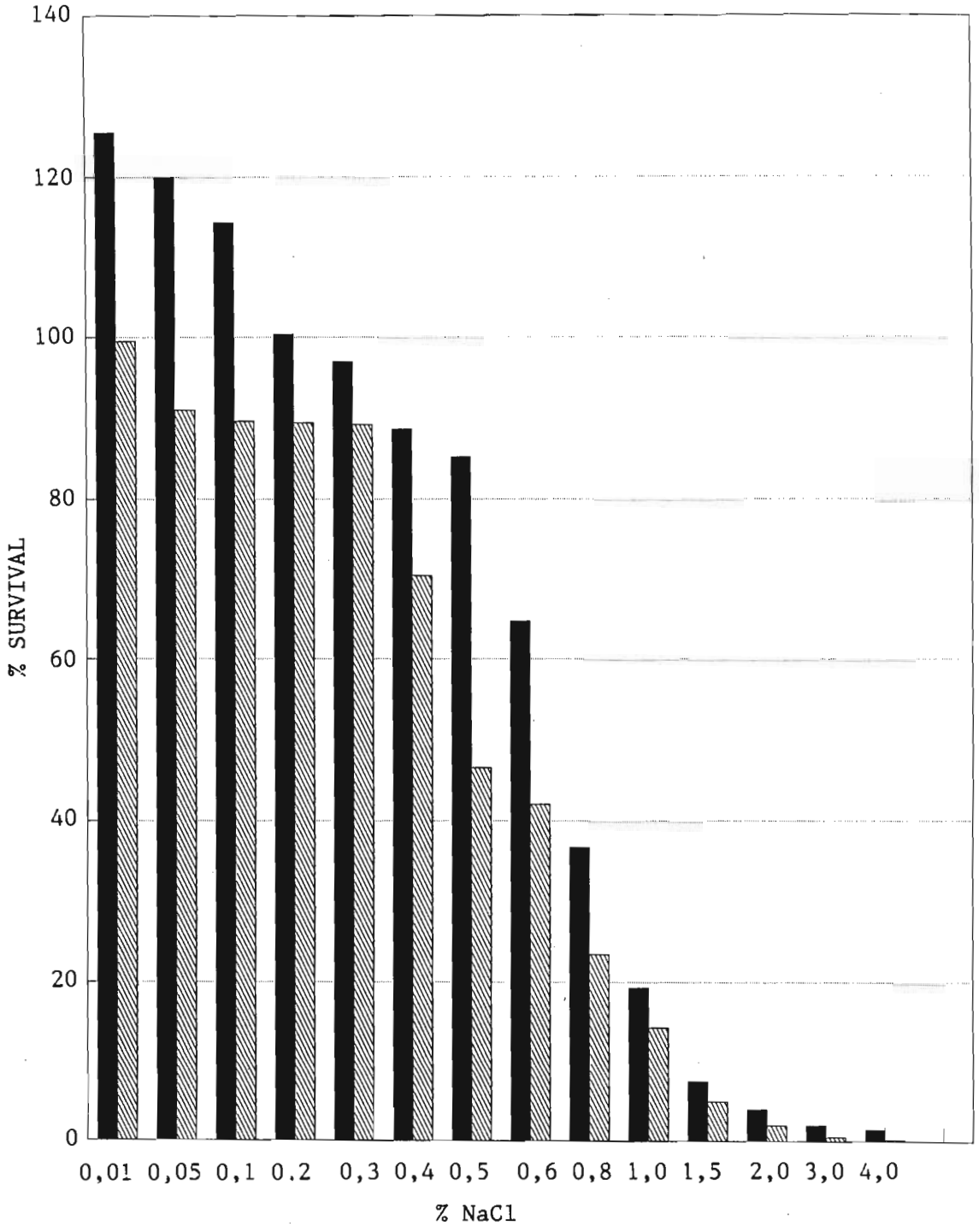

Fig. 7.d. Tolerance of strain 2905 to a range of sodium chloride concentrations added to Wilbrink's broth (solid bars) and YSP broth (striped bars).

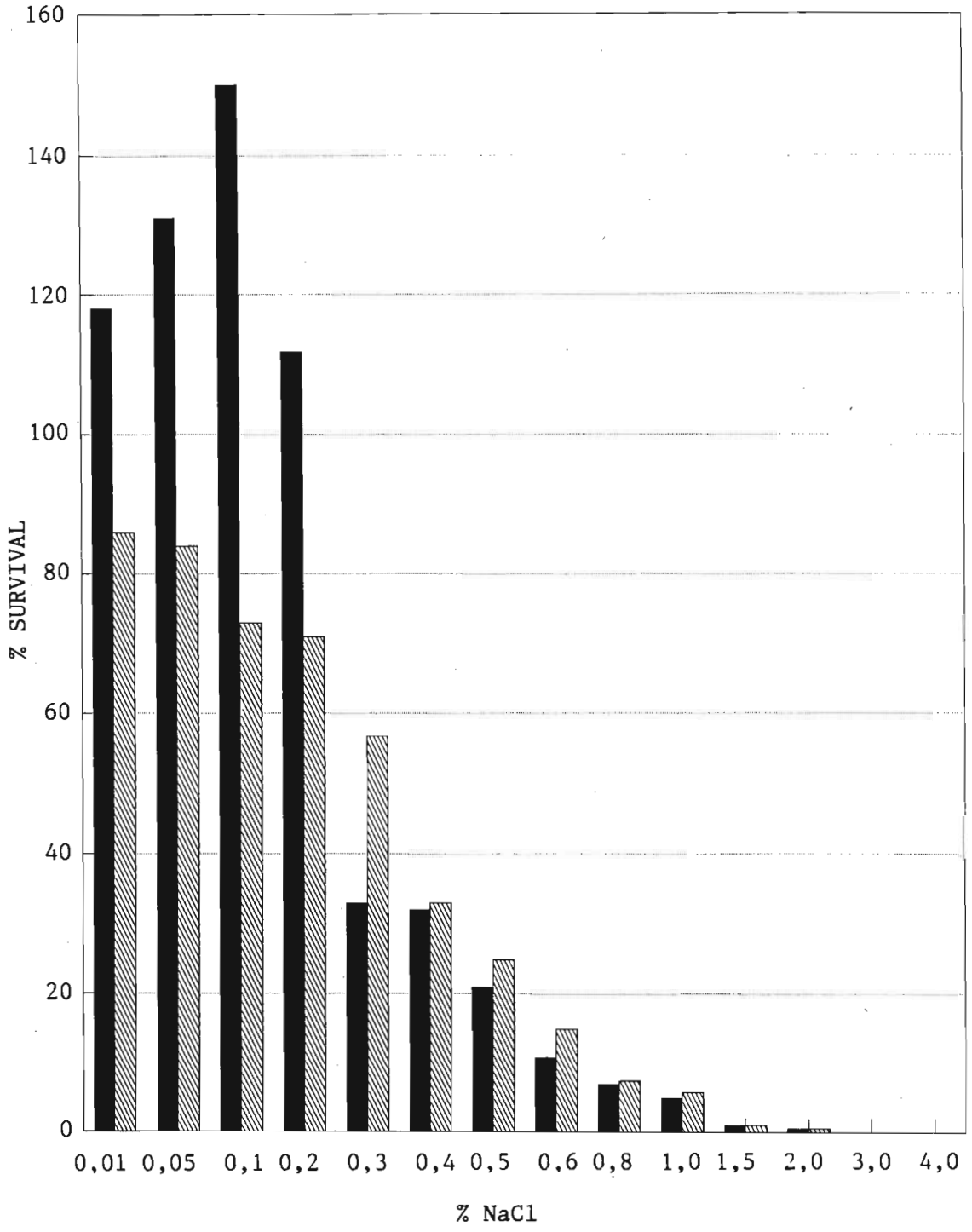

Fig. 7.e. Tolerance of strain XA 86-1 to a range of sodium chloride concentrations added to Wilbrink's broth (solid bars) and YSP broth (striped bars).

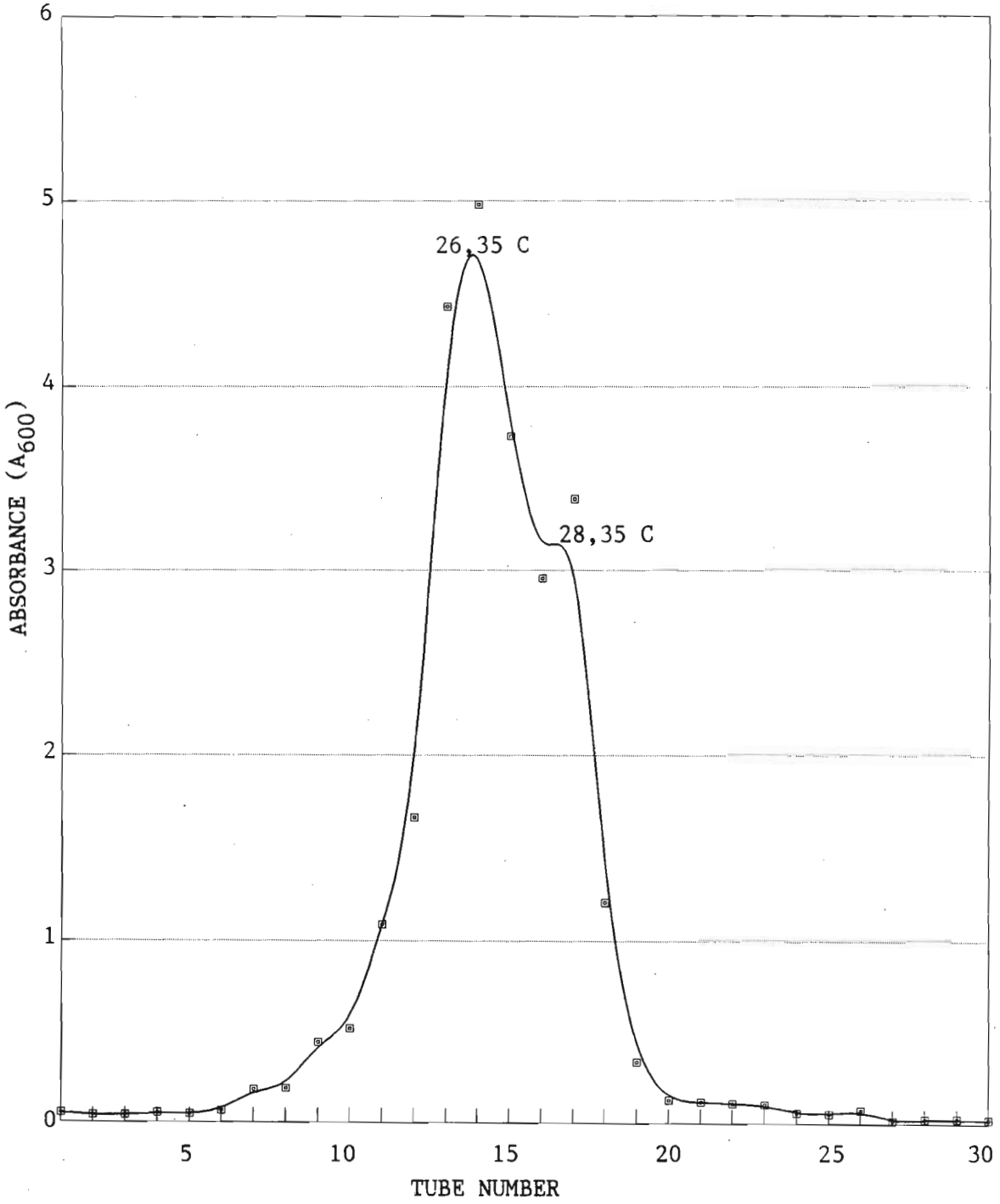

Fig. 8.a. Temperature growth optimum of strain PDDCC 196 in Wilbrink's broth.

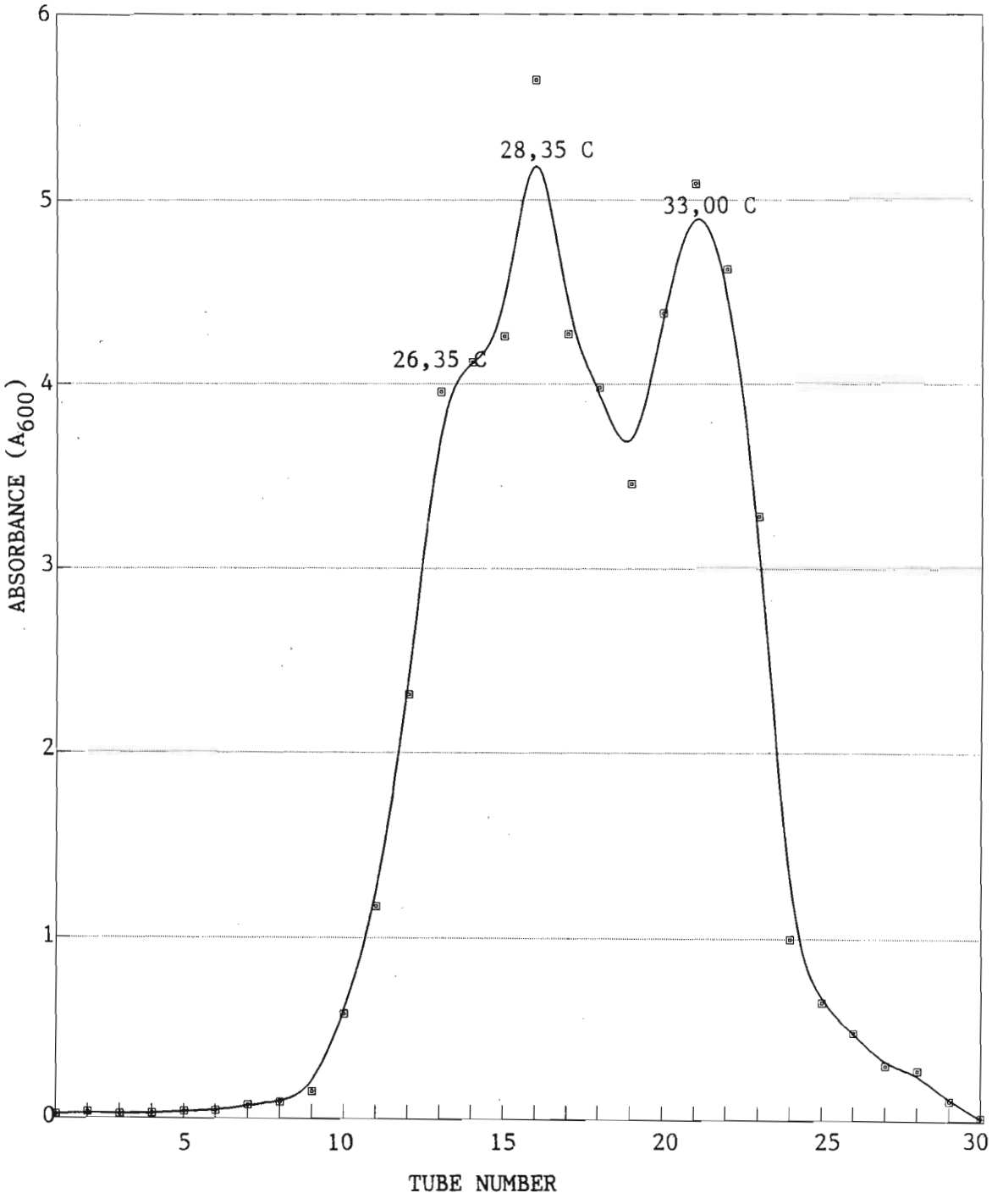

Fig. 8.b. Temperature growth optima of strain LS155 in Wilbrink's broth.

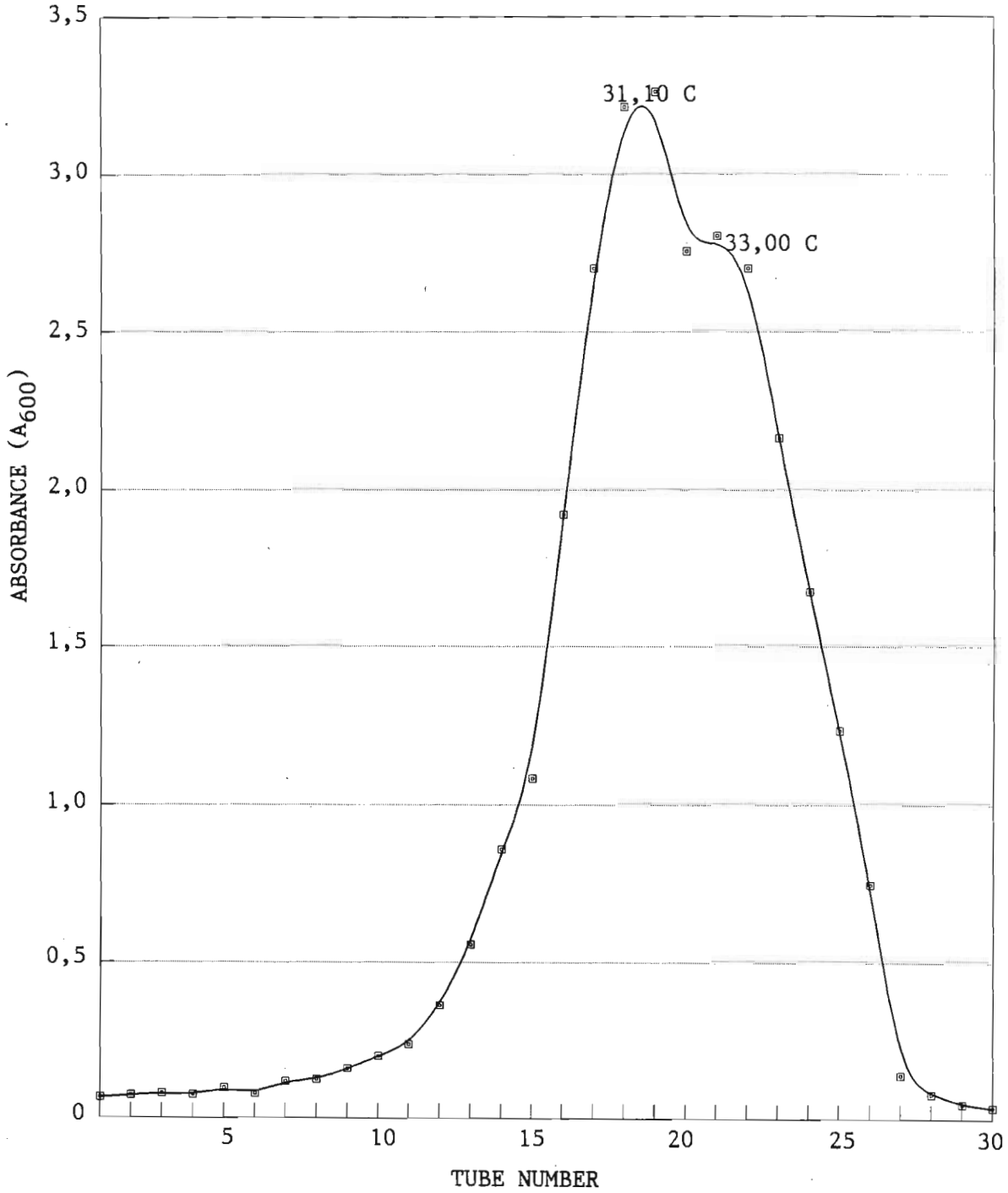

Fig. 8.c. Temperature growth optimum of strain 2901 in Wilbrink's broth.

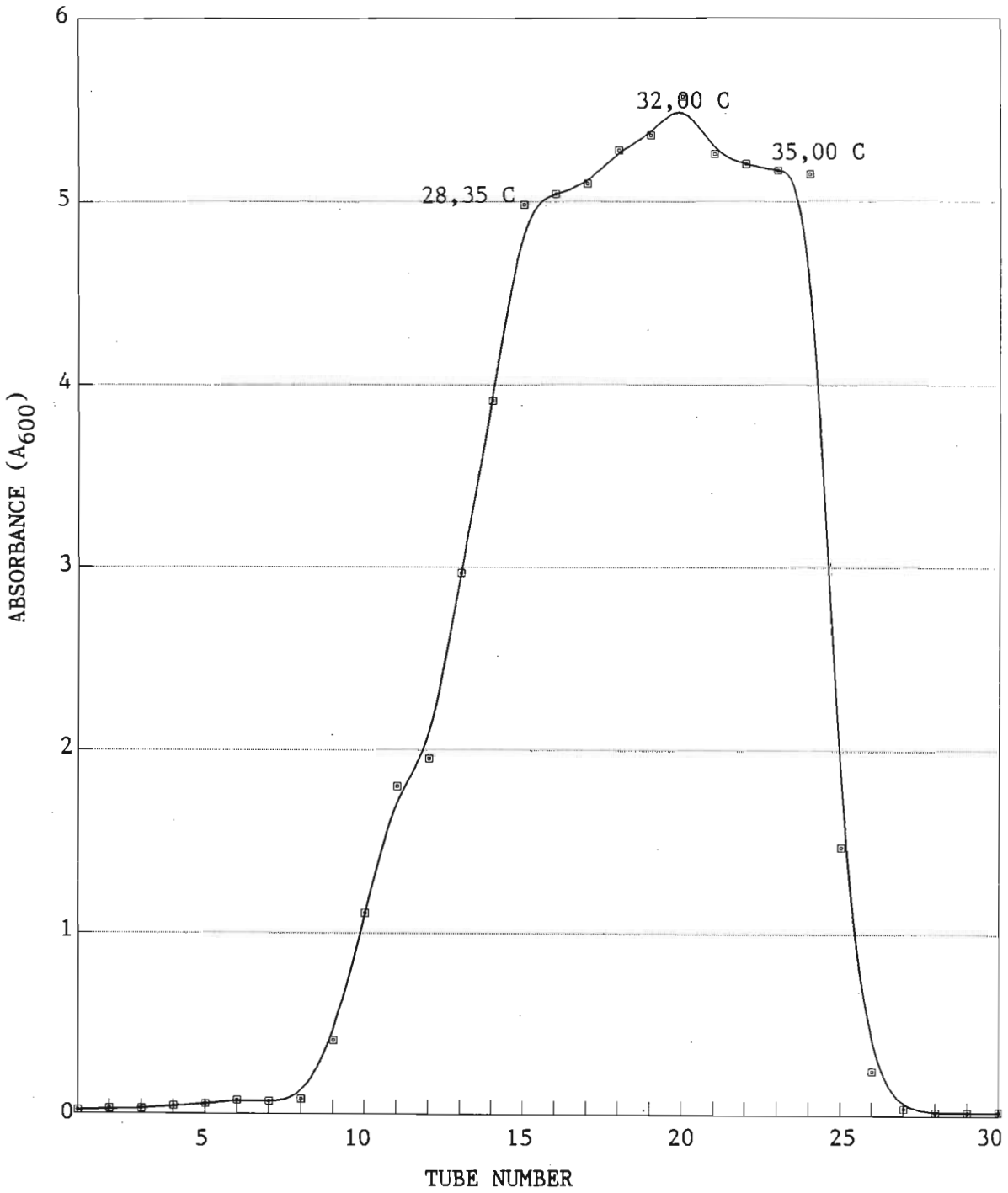

Fig. 8.d. Temperature growth optimum of strain 2905 in Wilbrink's broth.

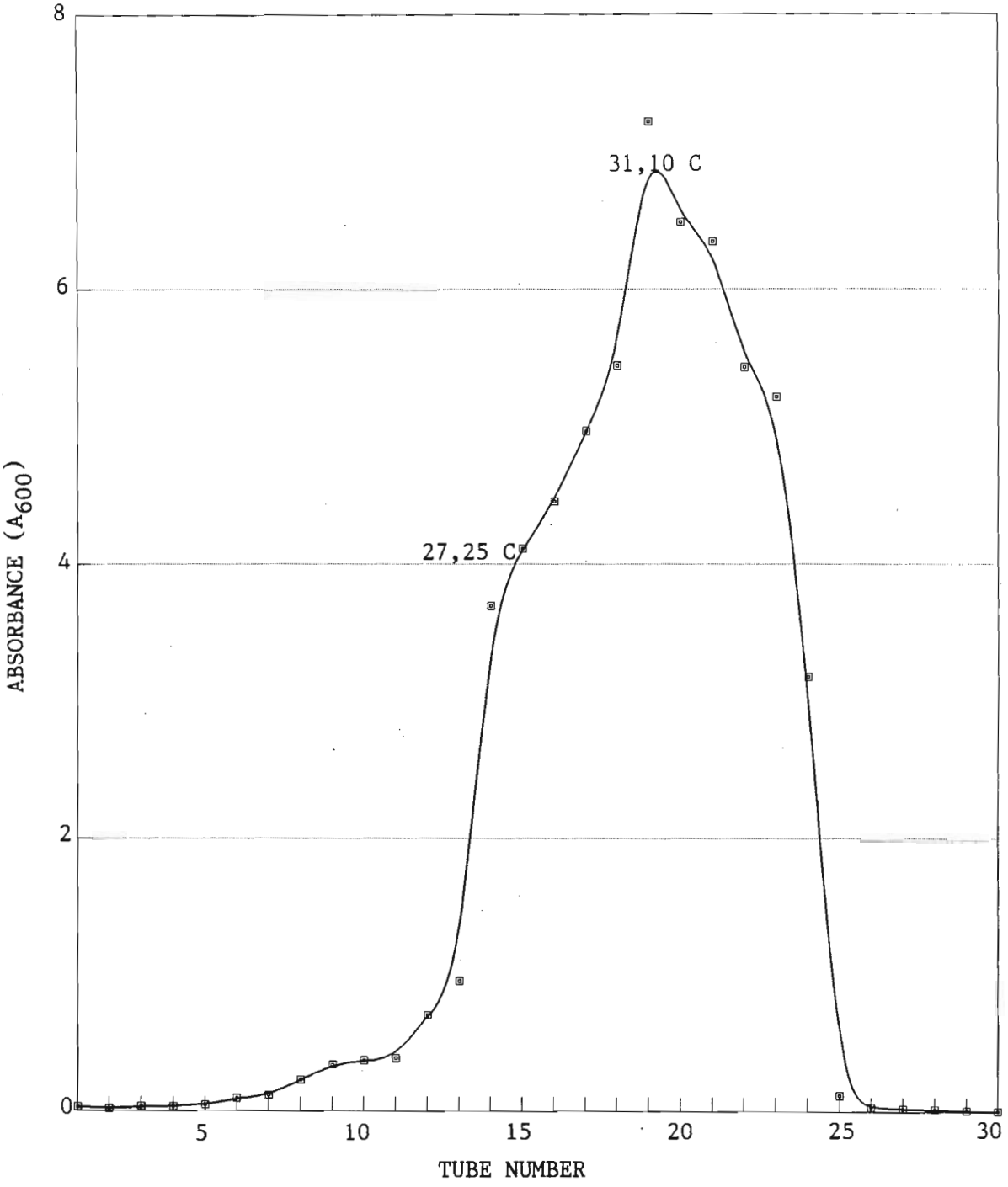

Fig. 8.e. Temperature growth optimum of strain XA 86-1 in Wilbrink's broth.

2.3.5) Cell membrane characteristics

(a) Isolation, preparation and morphology

Successful and reproducible isolations of cell membranes were made following French pressure cell treatment of cells. The use of sucrose density gradient ultracentrifugation and fractionation of these crude preparations produced four distinct peaks for each strain of *X. albilineans* (Fig. 9). The peaks were designated soluble (S), light (L), intermediate (I) and heavy (H). During further analyses of these bands, by polyacrylamide gel electrophoresis and immunodiffusion, the S and L bands were pooled because of their lower respective yields.

Table 7. Buoyant densities, in $g.cm^{-3}$, of membrane bands of the five strains of *X. albilineans* extrapolated from a standard sucrose density curve at 20 C

STRAIN	MEMBRANE BAND			
	S	L	I	H
PDDCC 196	1,114	1,162	1,213	1,253
LS155	1,119	1,162	1,199	1,236
2901	1,114	1,165	1,198	1,235
2905	1,122	1,168	1,210	1,247
XA 86-1	1,122	1,163	1,201	1,235

Except for differences in the buoyant densities of the I and H bands of two strains, the buoyant densities were otherwise similar for all five strains (Table 7). The buoyant densities of the I and H bands of strains PDDCC 196 and 2905 were slightly higher at 1,213 and 1,210 $g.cm^{-3}$ for the I bands, and 1,253 and 1,247 $g.cm^{-3}$ for the H bands, respectively. Densities for the I and H bands of the remaining strains were, respectively, in the region of 1,200 and 1,235 $g.cm^{-3}$. The density patterns among strains for the S band was as follows: PDDCC 196 and 2901 showed the lowest densities at 1,114 $g.cm^{-3}$ while 2905 and XA 86-1 had the highest densities at 1,122; LS155 was the intermediate of these two groups at 1,119 $g.cm^{-3}$. L band buoyant densities were in the range 1,162 to 1,168 $g.cm^{-3}$.

Preparations of crude membranes negatively stained for transmission electron microscopy, and prepared on polycarbonate filters for scanning electron microscopy revealed typical membrane vesicle structures in each of the five samples (Figs. 10 and 11). However, variations in the nature of the vesicles present were evident.

Strain XA 86-1 showed a large proportion of slender, tubular vesicles (diameter 0,02 micrometres) in addition to round and ellipsoidal ones (diameters ranging from 0,1 - 0,15 micrometres)(Figs. 10e and 11d).

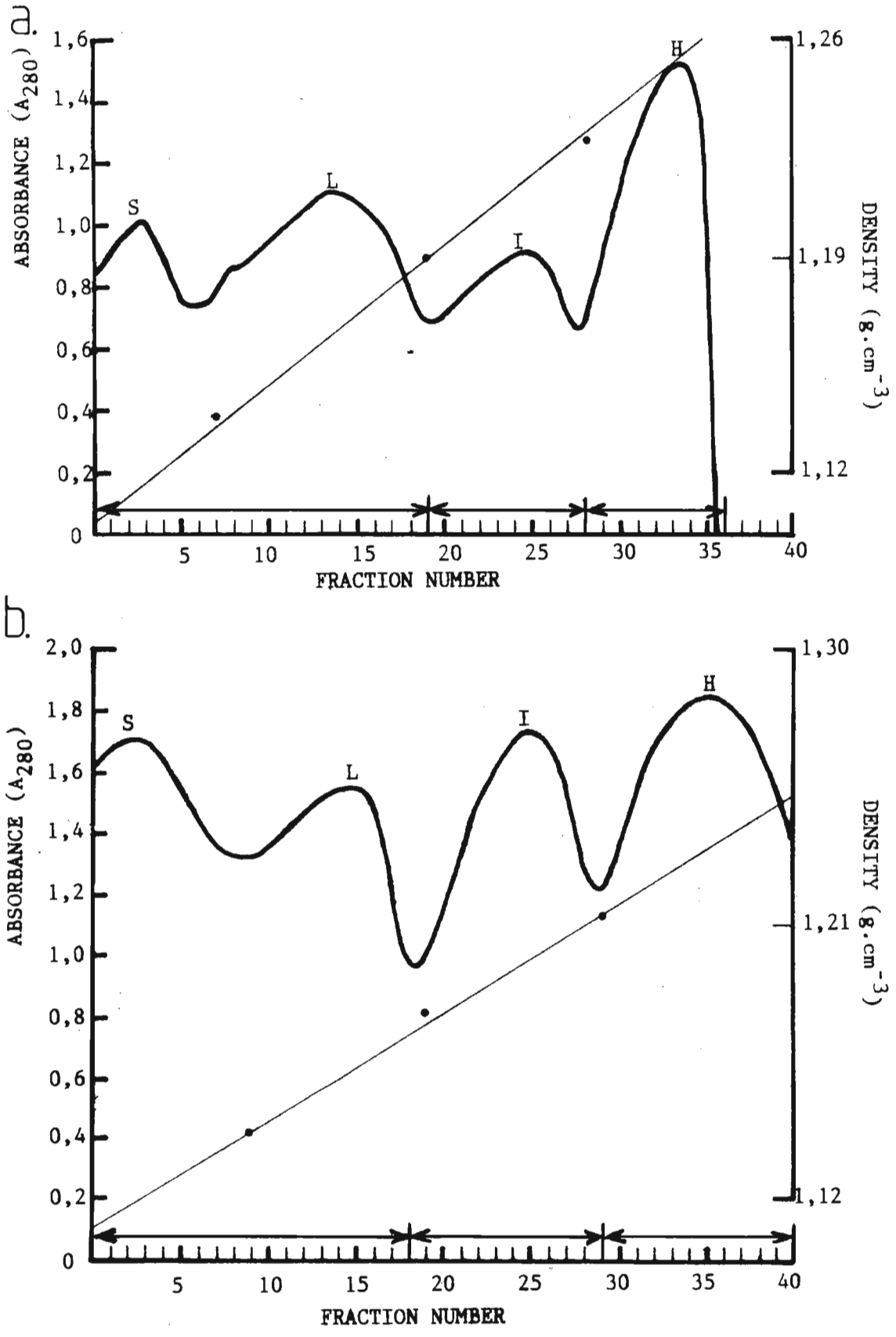

Fig. 9. Fractionation profiles (A_{280}) of crude membranes of strains (a) PDDCC 196 and (b) LS155 following sucrose density gradient centrifugation.

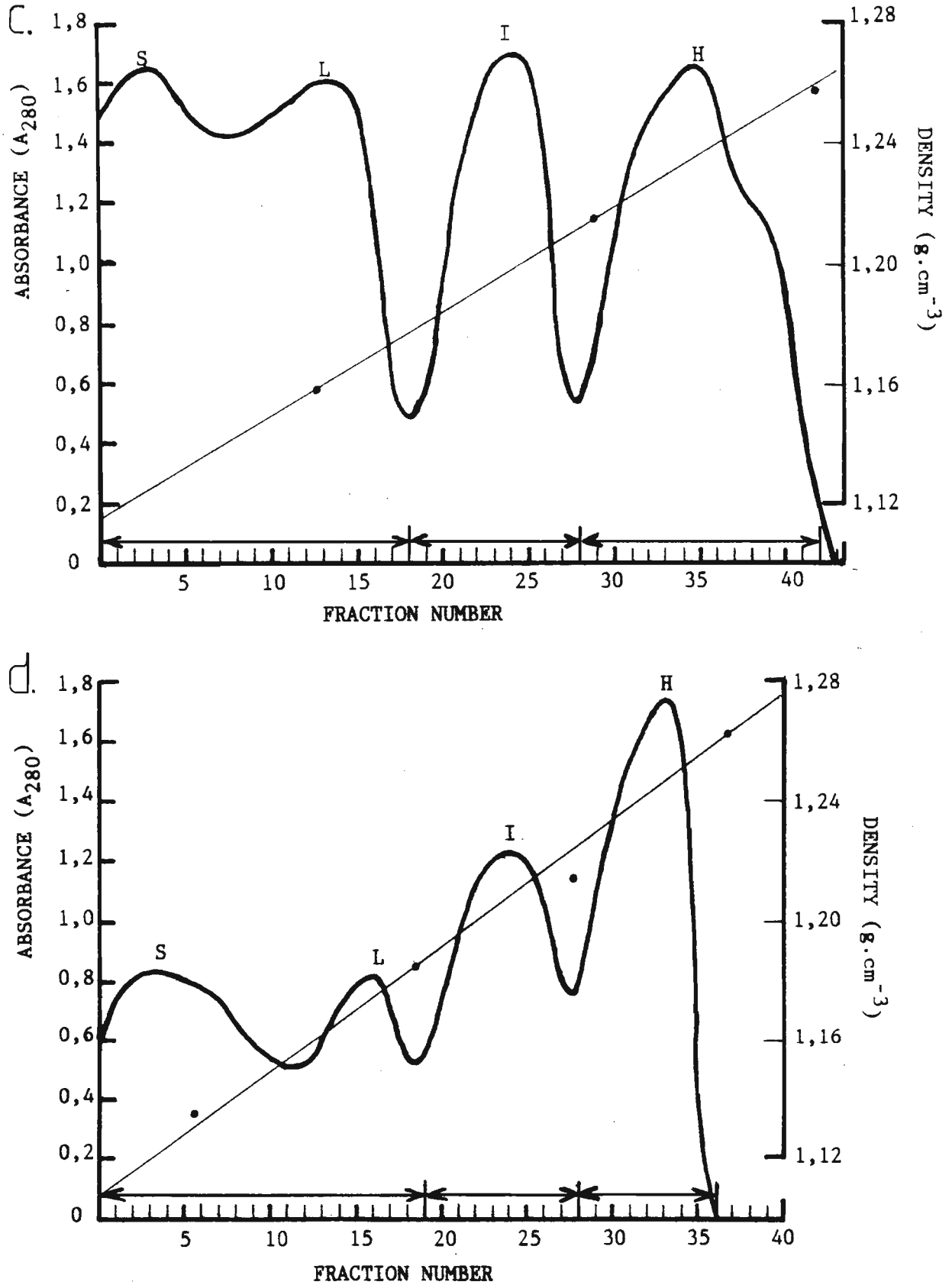

Fig. 9. Fractionation profiles (A_{280}) of crude membranes of strains (c) 2901 and (d) 2905 following sucrose density gradient centrifugation.

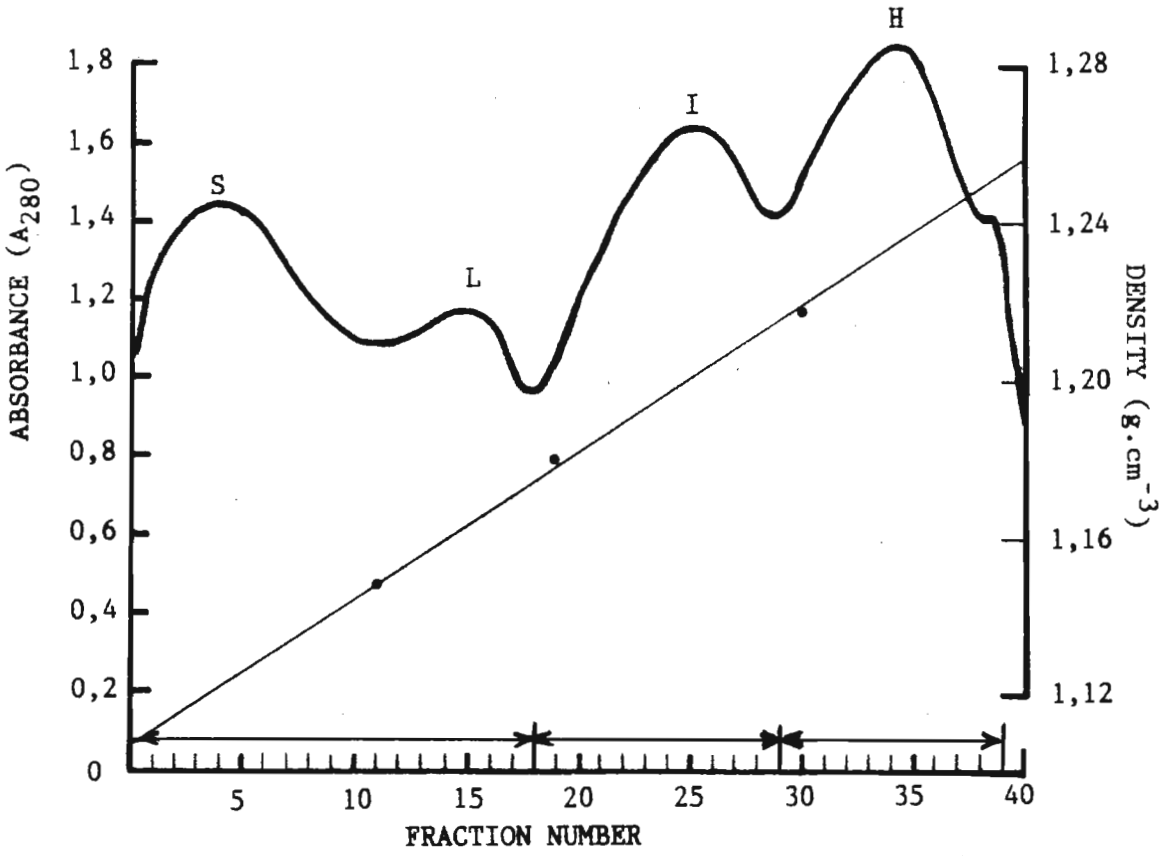

Fig. 9.e. Fractionation profile (A_{280}) of crude membranes of strain XA 86-1 following sucrose density gradient centrifugation.

Smaller numbers of tubular vesicles were present for strain 2901, which by comparison were broader (average diameter 0,04 micrometres) than those of strain XA 86-1; the rounded vesicles also showed a greater variation in size, from 0,03 to 0,14 micrometres in diameter, clearly shown in the transmission (Fig. 11c) and scanning electron micrographs (Fig. 10c). Tubular vesicles were occasionally present in PDDCC 196 preparations, with rounded vesicles in abundance.

These were usually of a uniform size (average 0,1 micrometres in diameter)(Fig. 10a) with few vesicles enclosing one another (Fig. 11a). The membrane vesicles of strains LS155 and 2905 were essentially of the rounded type, with no evidence at all of tubular ones (Figs. 10b,d and 11b). Areas around the vesicles of strain 2905 were electron dense, probably as a result of remaining polysaccharide material still adhering to vesicles. Strain LS155 membrane vesicles showed a wide range in sizes (Fig. 11b), from very large (0,39 micrometres) to very small (0,04 micrometres), when compared to the membranes of other strains.

Size variations among the rounded vesicles were clearly revealed by both transmission and scanning electron microscopy. However, scanning electron microscopy failed to clearly depict tubular vesicles and could not show vesicles enclosing one another.

(b) Polypeptide composition

Irrespective of whether the bacteria were cultured in Wilbrink's or YSP media (broth or agar) the polypeptide compositions of the membranes of all five strains, when examined by PAGE, were essentially similar (Fig. 12).

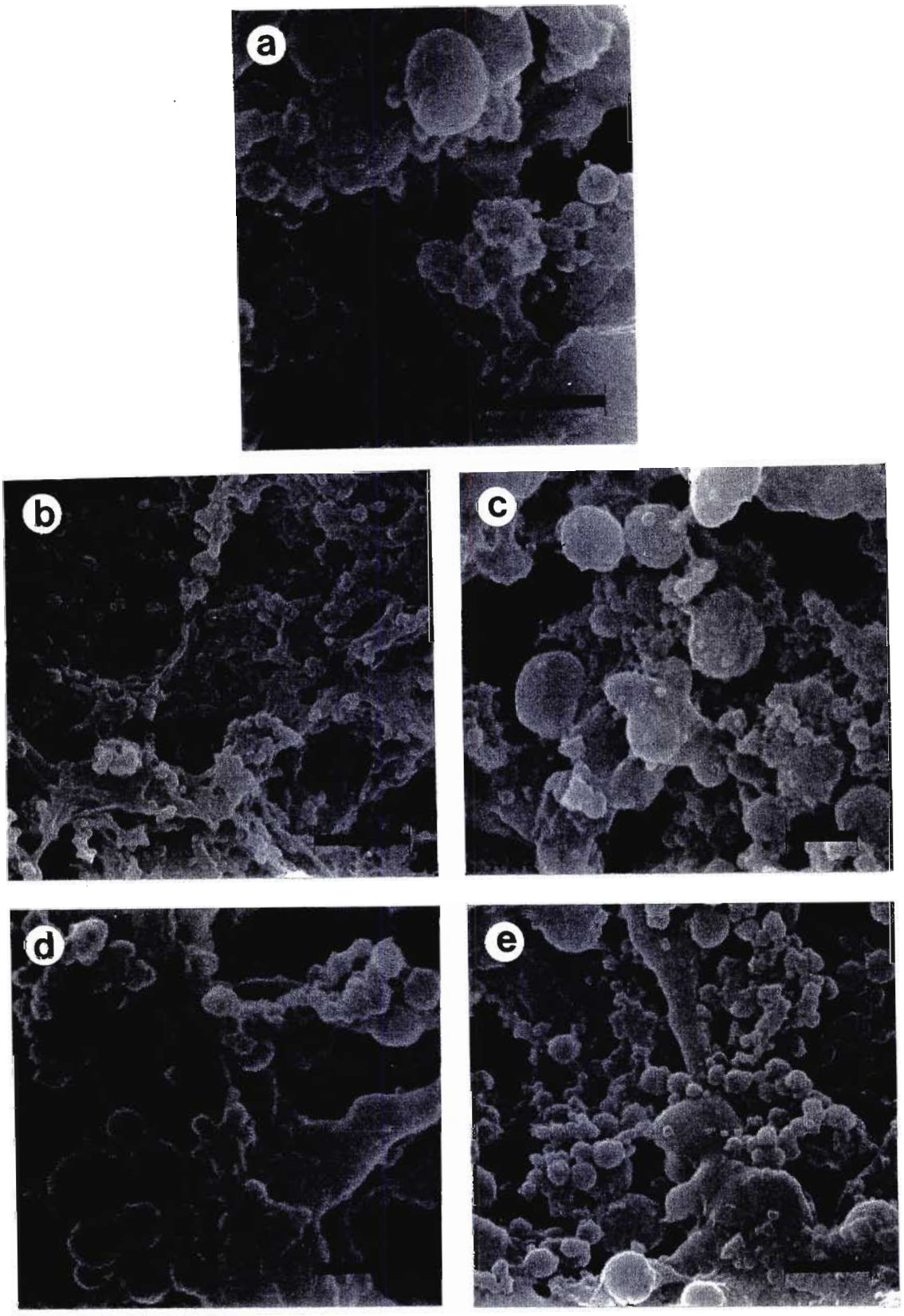

Fig. 10. Scanning electron micrographs of crude membrane preparations on polycarbonate filters. (a) PDDCC 196, (b) LS155, (c) 2901, (d) 2905 and (e) XA 86-1. Bar represents 1 μm.

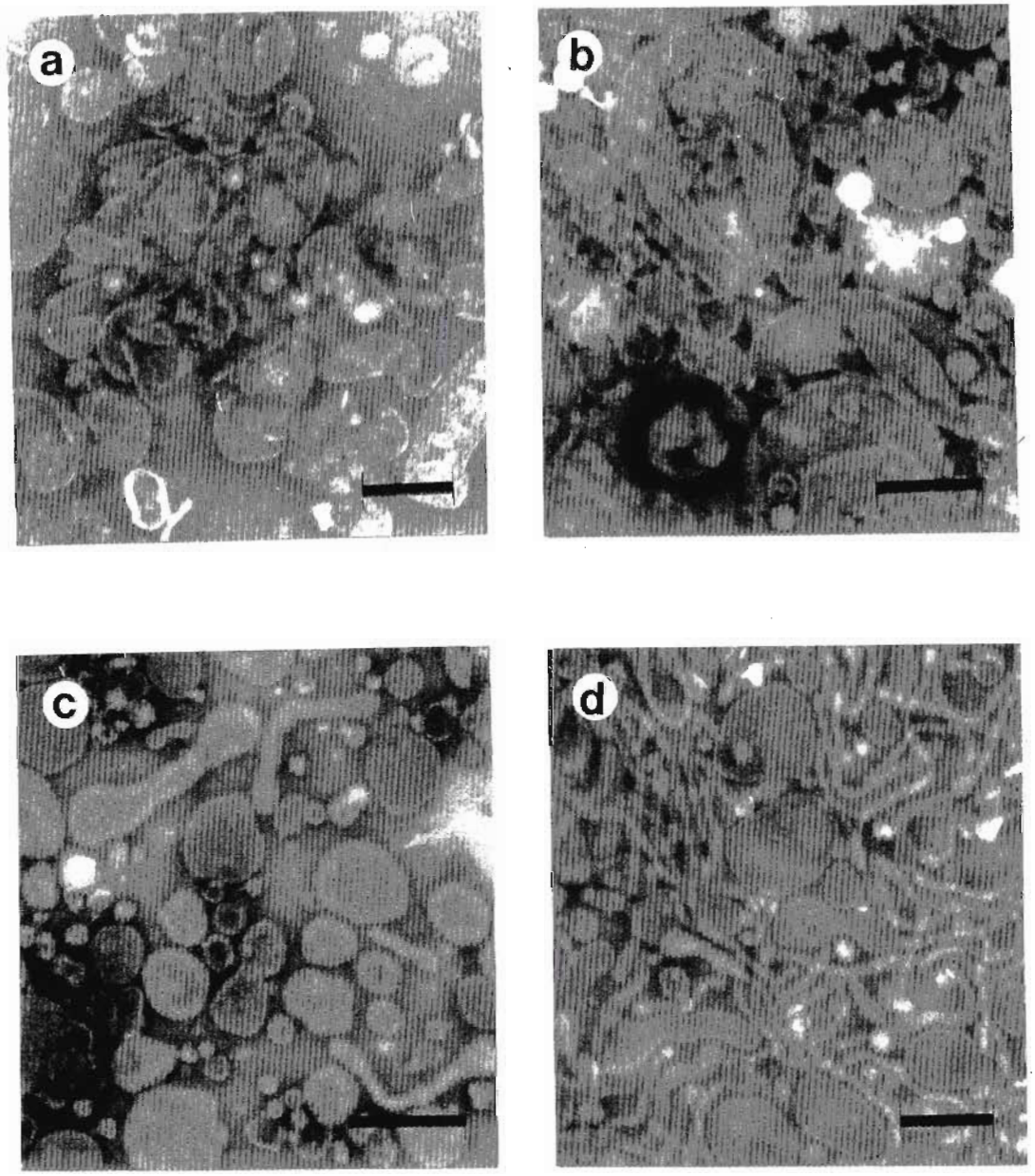

Fig. 11. Transmission electron micrographs of crude membrane preparations negatively stained with 1% PTA (pH 6,8). (a) PDDCC 196, (b) LS155, (c) 2901 and (d) XA 86-1. Bar represents 0,1 µm in (a) and 0,2 µm in (b), (c) and (d).

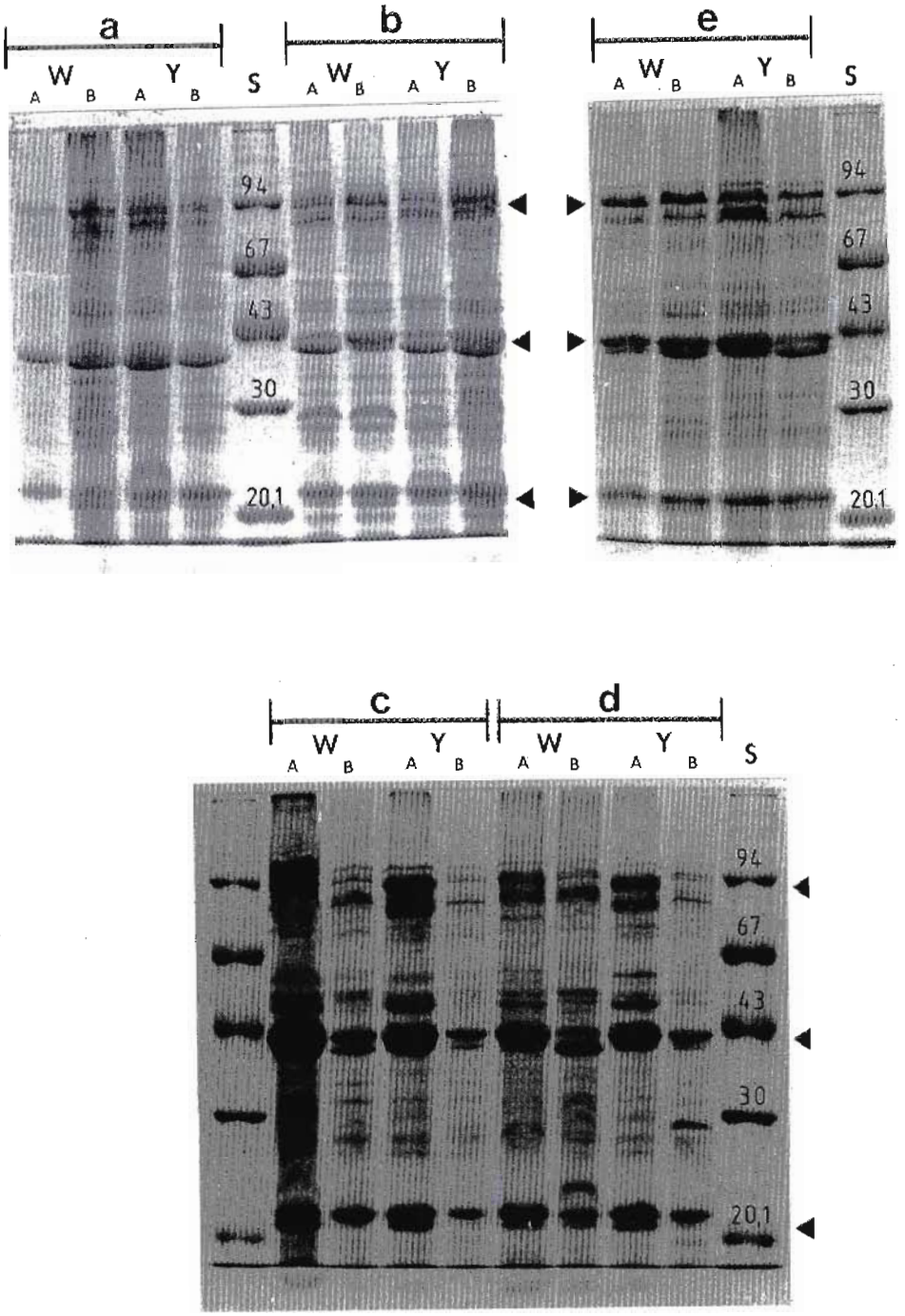

Fig. 12. Polyacrylamide gel electrophoregrams of crude membranes prepared from cells grown in Wilbrink's and YSP broth and agar. The letters identifying each lane are as follows: a=PDDCC 196, b=LS155, c=2901, d=2905, e=XA 86-1, W=Wilbrink's, Y=YSP, A=agar, B=broth and S=standards. Molecular masses (in kdal) of the standards are included in the figure. Arrows indicate the major polypeptide bands.

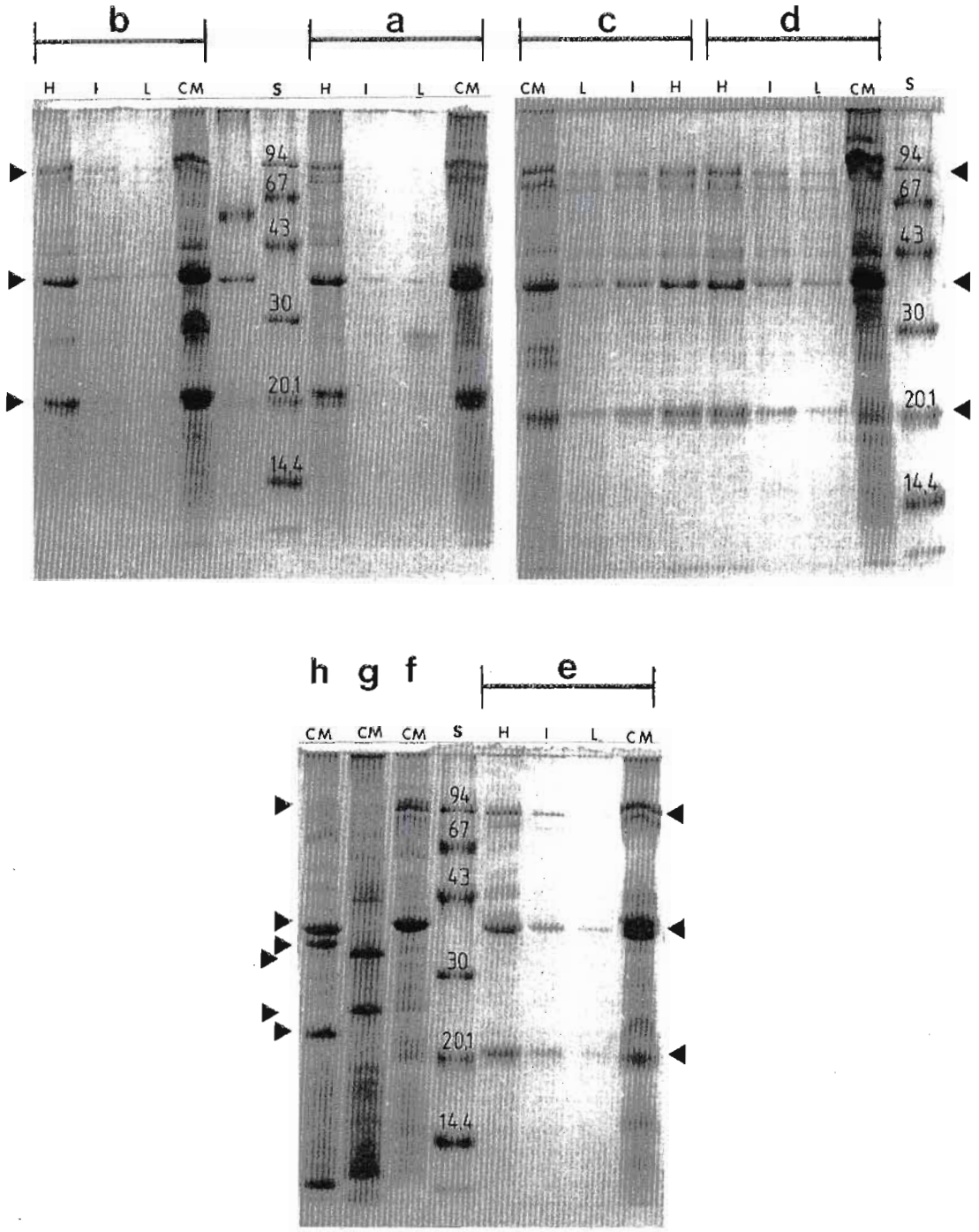

Fig. 13. Polyacrylamide gel electrophoregrams of crude membranes (CM) and the membrane bands (L=light, I=intermediate and H=heavy). Each bacterium is identified as follows: a=PDDCC 196, b=LS155, c=2901, d=2905, e=XA 86-1, f=*X. campestris* pv. *zeae*, g=*P. aeruginosa* and h=*E. coli*. Arrows indicate the major bands. Bacteria a-e were cultured in Wilbrink's broth and f-h in Nutrient broth. The molecular masses (in kdal) of the standards (S) are indicated on the figure.

However, minor differences in the relative concentrations of the various polypeptides did exist. A similar trend was also evident when the membrane bands (L, I and H) were analysed by PAGE (Fig. 13).

A distinguishing feature of all strains was the occurrence of major proteins in the 20, 35 and 90 kdal size range. Many minor proteins tended to cluster at the 27 and 52 kdal regions. These patterns were distinct from those of other gram-negative bacteria showing the following major proteins, viz., *X. campestris* pathovar *zeae* (101,5 and 35 kdal), *E. coli* (36,5 and 22,5 kdal) and *P. aeruginosa* (43, 33 and 26 kdal)(Fig. 13). Whether or not the 35 kdal protein is characteristic for the genus *Xanthomonas* cannot be stated with any certainty from these results, since a wide range of representative species would have to be analysed to make comparisons.

2.4 DISCUSSION

The pathologically important nature of leaf scald disease and its impact on the economy of the world's sugarcane industry are well established. It is the significance of this that has prompted and maintained ongoing research of the disease and its etiological agent since the beginning of the century. Currently, with the implementation of more rigid control measures, incidence of the disease has been

contained to the extent that its severity ranks secondary to other sugarcane diseases, e.g. ratoon stunting, sugarcane smut and sugarcane mosaic (Anon., 1980). However, the lingering threat of leaf scald disease has established its status as one of the most destructive diseases, even in countries with effective control programmes.

2.4.1) Pathogenicity

By convention, the successful diagnosis of leaf scald disease involves observing appropriate disease symptoms and isolating the causative agent from infected plants. The problems experienced by many workers have been the inconsistent frequency (Fox, 1961; Persley, 1975; Thomson, 1969a; Walker, 1968), or lack (Coleman, 1968; Sheffield, 1969) of symptom expression under field and laboratory conditions, and varying success rates of isolation (Khan, 1971; Ogier & Goberdhan, 1970). Isolations have been attempted following the treatment of samples with chemical disinfectants (Khan, 1971; Thompson, 1982a), or adding specific microbicides and antibiotics to the isolation media (Persley, 1972). Khan (1971), using two chemical disinfectants, achieved some success but noted high mortality rates for the pathogen, which usually failed to grow when mercuric chloride was used as a disinfectant. Disinfection with sodium hypochlorite has always been successful (Khan, 1971; Persley, 1972; Thompson, 1982a) with

pure colonies appearing within 4 to 7 d (Persley, 1972; Thompson, 1982a). However, methods for extracting the pathogen from diseased tissue differed, involving tissue homogenisation (Khan, 1971) and sap exudation (Thompson, 1982a). Results from these two studies were similar to those obtained for the first and third isolation techniques in this study, respectively.

From the extensive contamination obtained by the first technique, a similar problem experienced by Khan (1971), it was obvious that ordinary washing procedures and short surface disinfection times were ineffective in removing much of the epiphytic microflora present on the surfaces of leaves and stalks. Even the more stringent disinfection protocol of the second technique resulted in the growth of contaminating microorganisms. It was therefore apparent, before adopting the third technique, that several factors had to be considered for the successful isolation of *X. albilineans* from diseased tissue. In order of priority, these factors included:

(i) The critical selection of sampling points on infected plants. No matter how severe the disinfection process, indiscriminate sampling of entire infected plants, by the first and second techniques, did not yield successful isolations in proportion to the effort put into the procedure. However, the third technique has shown the likeliest sampling sites to be leaves exhibiting pencil-line

symptoms or complete etiolation, and the growing points of young shoots on infected plants. Clean, successful isolates were always obtained from these points, and similar successes have been reported by Thompson (1982a) and Walker (1968).

(ii) The isolation technique. An efficient technique should provide conditions restricting the growth of contaminating microbes, at the same time not affecting and allowing for the extraction and culture of the pathogen. Tissue maceration and the placing of pieces of infected material in contact with the isolation media were ineffective in this regard because equal growth opportunities were provided for all microorganisms present in these samples, hence the large degree of contamination obtained using these methods. Again, the third technique was most successful in this regard. The rigorous surface disinfection programme eliminated much of the epiphytic microflora (the source of most contamination) and the successful exudation of bacteria from the small fragments of tissue soaked in distilled water resulted in a high concentration of the pathogen relative to any remaining contaminants. Persley's (1972) method of incorporating specific microbicides and antibiotics into the isolation media, which discouraged the growth of contaminating microorganisms, was discounted in this study, since the effects of such chemical agents on the pathogen itself would have been difficult to assess.

(iii) The disinfection programme. The reports of Khan (1971), Persley (1972) and Thompson (1982a) indicated that short surface sterilizing times of 1 min in 1% sodium hypochlorite proved adequate in obtaining pure isolates of *X. albilineans*. However, methods of isolation differed, making comparisons between these difficult. A comparison of the work of Thompson (1982a) and the results of the present study, where sodium hypochlorite concentrations of 10%, 5% and 1% and a longer total sterilizing time of 15 min was used, shows that the disinfection routine described above, together with the critical choice of sampling positions of infected plants, always yielded successful, clean isolates. In essence then, the most important prerequisites for the successful isolation of *X. albilineans* in the present work were not only the isolation techniques, nor the disinfection routines, but also the sites on the plant used as a source of the pathogen.

Another area of controversy is a description of the precise conditions, and understanding what effects these have on the manifestation of disease symptoms, under both natural and experimental conditions. It is known that healthy, susceptible plants in the field, not subjected to any form of environmental stress, can harbour the pathogen without any outward sign of disease (Egan, 1970). This situation may persist for several seasons, while the disease spreads through a crop undetected. Should conditions then become

unfavourable to the plant, disease symptoms are expressed, and widespread crop devastation usually occurs (Martin & Robinson, 1961). Various environmental conditions precipitating the effects of the disease have been reported. Included among these were the effects of a wet season followed by a hot, dry one. It was in the latter season that symptoms were usually evident (Bisessar, 1970; Zummo, 1976). Persley (1973b), in a conflicting report, indicated a dry autumn and an early cold winter as favouring acute-phase symptoms, which showed up during the ensuing winter and spring. In addition to adverse climatic conditions, poor soil drainage or low soil fertility also accelerated disease expression (Martin & Robinson, 1961).

Attempts to simulate similar unfavourable situations experimentally have resulted in varying success (Egan, 1969b; Thompson, 1982a; Walker, 1968), or no success (Ricaud & Ryan, 1989), making correlations between the experimental and field situations difficult (Ricaud, 1969a; Wismer, 1969). The problem is further complicated by differing varietal reactions to the disease, since even so-called resistant varieties of sugarcane have been implicated in harbouring the pathogen and aiding its dissemination (Egan, 1970). Because of the economic implications regarding control of the disease, various researchers have proposed methods to expedite the testing of sugarcane varieties for susceptibility or resistance to leaf scald. A comprehensive

study in this regard by Thompson (1982a,b) who, after reviewing all the past methods and practices, provided the following recommendations:

(i) Inoculation by the spray-on technique, using a suspension of cultured bacteria in distilled water rather than sap extracted from diseased plants, and

(ii) Experimental material, comprising sugarcane setts from the middle third of a stalk, with one inch of internode tissue on either side of a node.

These guidelines were found to result in consistent and representative disease symptoms among sugarcane varieties, usually within 12 to 52 d. Varieties could then be evaluated on a rating system which ranged from highly susceptible to resistant. Earlier and subsequent modifications to the method of Thompson (1982a,b) by Birch & Patil (1983), Roth & Thomson (1970), Satyanarayana & Rao (1980) and Waraitch & Singh (1978), successfully resulted in the appearance of disease symptoms, usually within 25 d. Instead of using setts, these methods employed young growing stalks of susceptible and resistant sugarcane varieties, which were decapitated above the growing point and inoculated with a suspension of bacteria in distilled water. The use of juice from infected plants as inoculum (Egan, 1969b; Pinto, 1972; Ricaud, 1969a,b, 1971; Wismer, 1969), either on setts or decapitated plants, resulted in variable periods of up to 12 months before symptoms were observed.

This method also required cutting back the stalks and re-inoculating (Ricaud, 1969a).

Although the method of Thompson (1982a,b) has not been evaluated by other workers, its merits are obvious when large numbers of samples require analysis. For the smaller sample numbers used in this study, the decapitation technique together with pipette application of a standard volume of a predetermined inoculum concentration, proved adequate and disease symptoms were observed within the period of 14 to 42 d.

Whether or not the rapid expression of symptoms by the method used in this study was a true indication of establishment of the disease, as reported by Birch & Patil (1983), Satyanarayana & Rao (1980), Thompson (1982a,b) and Waraitch & Singh (1978), is a debatable issue. The rapid appearance of symptoms could be a reaction by the plant to a sudden large dose of inoculum. This is clearly evident in the context of the evidence provided by Birch & Patil (1985, 1987a,b) on the production of a diffusible toxin produced by *X. albilineans*, and the fact that the studies by Thompson (1982a,b) were only conducted on varieties of sugarcane that were susceptible to leaf scald. Toxin, rapidly diffusing through the tissues of new and emerging leaves, would inhibit plastid differentiation, resulting in the characteristic pencil-line symptoms. This was clearly

demonstrated in this study when, even under favourable moisture, temperature and humidity regimes, symptoms appeared on all varieties, including the resistant ones, after 14 d. Later, the symptoms became indistinct and eventually disappeared from the intermediate and resistant varieties and from one of the susceptible varieties tested (Table 2). The appearance of symptoms on variety NCo 376, a resistant variety, within two weeks following inoculation and the subsequent complete disappearance of symptoms, never to reappear, is evidence for support of the contention that symptom expression within 14 to 21 d following inoculation is no more than a reaction by the plant to toxin produced by a large inoculum dose, and is not necessarily an indication of successful disease establishment.

Under satisfactory plant growth conditions (a moist and well-drained soil, constant and controlled temperatures, and adequate humidity) the pathogen appeared to assume a state of latency, with no visible evidence of disease. But, after 6 months, and following severe moisture and temperature stressing, disease symptoms reappeared. Whether it is the weakened condition of the plants that results in a rapid proliferation of the pathogen through them; or, whether some stress-induced factor in the plants induces high levels of toxin production (and possibly the formation of lethal substances) by the pathogen, causing the chronic and acute phase symptoms, remains to be ascertained. Ricaud & Ryan

(1989) briefly mention the unpublished and unsuccessful experiments of Ryan & Birch, where artificially imposed moisture and temperature stresses failed to induce symptom expression. In the absence of any detail regarding experimental protocol, comment on these results is not possible.

Finally, unsuccessful attempts to correlate the regulation of toxin production with the presence of plasmids, which were not found in *X. albilineans*, have led to the proposal that toxin regulation was mediated by chromosomal genes (Birch & Patil, 1987a). According to Birch & Patil (1987a), the occurrence of the latent infection in plants therefore is probably the result of an instability in these genes, which cause high rates of spontaneous variation in toxin production among isolates of *X. albilineans*. With the lack of toxin production, is the concomitant lack of chronic and/or acute phase symptoms. Recently, however, plasmids have been detected in the strains of *X. albilineans* used in this study (Singh, 1989). Two plasmids occurred in both strains 2901 and XA 86-1, while only one was present in each of the other strains, PDDCC 196, LS155 and 2905. These plasmids have not been characterized and their role is as yet unknown.

2.4.2) The causal agent

Apart from several mechanisms of the host-pathogen relationship which require clarification, the status of the pathogen itself is also uncertain. Attempts to shed more light on the host-pathogen association has therefore resulted in work being focused on *X. albilineans* itself (Birch & Patil, 1985,1987a,1987b), trying to establish whether strains of the pathogen exist (Baudin & Chatenet, 1981). Proof of the existence of strains would help determine reasons for the occurrence of the different forms of symptoms, and may provide a basis for research into varietal responses to infection by the same strain of the pathogen.

The first step in strain identification would be to establish the effects of infecting specific sugarcane varieties with different geographical isolates of *X. albilineans*, and to note any variations in symptom expression which may result. To this end, Egan (1970) proposed standardizing a set of sugarcane varieties for differentiating strains from various geographical localities. Owing to legislation, a comparative assessment of symptom expression by local sugarcane varieties to strains PDDCC 196, LS155, 2901 and 2905 could not be undertaken as part of this study and only the phenotypic characters of each strain were analysed.

The five strains studied were a homogeneous group of bacteria on the basis of colony characteristics, cell morphology, staining and general requirements for growth (Table 8). These characteristics agree with descriptions for the genus (Bradbury, 1984; Dye, 1966,1980; Skerman, 1967). All strains were gram-negative, rod-shaped, motile, possessed single polar flagella, were strictly aerobic, lacked capsules and did not produce spores. Cell lengths ranged from 1,28 to 1,88 micrometres, and diameters from 0,20 to 0,35 micrometres. The temperature range for active growth was broad, from 20 C to 40 C, while the pH growth range was narrow, tending towards neutrality, from 6,8 to 7,2. Tolerance to sodium chloride was weak with less than 20% of the cell populations surviving in concentrations greater than 0,5% to 1%. Pigments of all strains extracted in acetone and methanol showed identical absorbance profiles. Minor variations among the strains were evident and the characteristics outlined in Table 8 can be used as preliminary distinguishing criteria for the species. However, no single feature should be used to distinguish between strains with any certainty.

Biochemical and physiological criteria for distinguishing among the strains are listed in Table 9, where reactions to all the biochemical and physiological tests are noted. These criteria should be used to supplement those listed in Table 8. Cognisance should also be taken of the fact that

acid production from the utilization of carbohydrates did not result in simultaneous gas evolution, a characteristic of the genus *Xanthomonas*.

Table 8. Summary of general features describing *X. albilineans* strains PDDCC 196, LS155, 2901, 2905 and XA 86-1

FEATURE	PDDCC 196	LS155	2901	2905	XA 86-1
Gram reaction	-	-	-	-	-
Shape	rod	rod	rod	rod	rod
Single polar flagellum	+	+	+	+	+
Capsule	-	-	-	-	-
Spore	-	-	-	-	-
Strict aerobe	+	+	+	+	+
Motile	+	+	+	+	+
Yellow pigmentation	+	+	+	+	+
Cell length (micrometres) (mean)	1,47	1,28	1,57	1,33	1,88
Cell diameter (micrometres)	0,33	0,33	0,21	0,34	0,24
Optimum growth temperature (C)	26,35	28,35	31,10	32,00	31,10
Optimum growth pH (Wilbrink's & YSP broth)	7,0	6,8-7,0	6,8-7,4	6,8-7,2	7,0
%NaCl cut-off point for 20% population survival	0,5	0,5	1,0	1,0	0,5

Table 9. Biochemical and physiological characteristics which are diagnostic for the species and which may be used to distinguish between strains

BIOCHEMICAL TEST	PDDCC 196	LS155	2901	2905	XA 86-1
Acid production from the following carbohydrates:					
arabinose	+	+	+	+	+
cellobiose	-	-	w	v	v
fructose	+	-	+	+	-
galactose	+	+	-	+	+
glucose	+	w	+	+	+
mannose	+	+	+	+	+
sucrose	+	+	+	+	+
trehalose	v	w	v	-	v
xylose	w	+	+	+	v
Aesculin	+	+	+	+	+
Starch hydrolysis	-	w	w	-	-
Arginine dihydrolase	w	w	-	-	w
Lysine decarboxylase	-	w	w	w	-
Ornithine decarboxylase	w	w	w	w	w
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
H_2S from cysteine	+	+	+	-	+
Voges Proskauer	-	+	-	-	-
Nitrate reduction	-	-	-	+	-
Litmus milk	R/P	R/P	R/P	LF	R/P

Key: + acid production/substrate utilization
- no acid production/no substrate utilization
w weak reaction
v variable reaction
R litmus reduction
P partial proteolysis
LF lactose fermentation

Several tests, for which unequivocal positive or negative responses were obtained, have been designated as species

diagnostic. Other tests, also giving unequivocal results, but varying for the strains, have been designated as strain diagnostic. These tests, together with other criteria, have been compiled as characteristic attributes of the species and of individual strains (Table 10). Features outlined for the species are not repeated for the strains.

Table 10. Characteristic features of the species and of individual strains, viz., PDDCC 196, LS155, 2901, 2905 and XA 86-1

Xanthomonas albilineans

All organisms yellow pigmented; gram-negative; rod-shaped; motile; non-sporulating; not producing capsules; having a single polar flagellum; catalase positive; oxidase negative; hydrolysing aesculin completely; producing acid but no gas from arabinose, mannose and sucrose.

BIOCHEMICAL TEST	STRAIN PDDCC 196	LS155	2901	2905	XA 86-1
1. Acid production from:					
fructose	+	-	+	+	-
galactose	+	+	-	+	+
2. H_2S from cysteine	+	+	+	-	+
3. Nitrate reduction	-	-	-	+	-
4. Starch hydrolysis	-	w	w	-	-
5. Voges Proskauer	-	+	-	-	-
6. Litmus milk	R/P	R/P	R/P	LF	R/P

Key: + positive; - negative; w weak reaction; R litmus reduction; P partial proteolysis; LF lactose fermentation

The results of biochemical and physiological tests from this study and from other reports vary in certain respects. For example, the weak proteolytic activity and indicator reduction of four strains in litmus milk have been reported as negative by Bradbury (1984), Dye (1966,1980) and Ricaud & Ryan (1989), although the third report indicates growth of the organism in milk. These reports may be justified by the evidence from this study where all strains showed growth on milk agar plates, with no evidence of casein breakdown. Slow gelatin liquefaction was reported by Bradbury (1984) and Dye (1966,1980). However, the 28 d plate method and the 42 d tube methods of this study, while showing growth of the organisms, produced no evidence of any gelatin liquefaction. Other tests for which there have been conflicting reports include:

(i) Starch hydrolysis - reported as negative for the species (Bradbury, 1984), when in fact, alpha-amylase activity (the partial breakdown of starch to dextrins) was detected in strains LS155 and 2901;

(ii) No acid production from arabinose and fructose (Bradbury,1984; Dye, 1980), yet these two tests have been deemed diagnostic features for the species and strains, respectively, from this study. Dye (1966) reported that arabinose was inhibitory for *X. albilineans*.

(iii) H_2S from peptone was reported as a negative attribute by Bradbury (1984). Dye (1966), in agreement with results obtained here (except for strain 2905), noted the production

of H_2S from cysteine. Other sources of sulphur, e.g. thiosulphate in the lysine decarboxylase sulphydrase and triple sugar-iron tests, could not be metabolized to produce H_2S.

(iv) Only one report indicates the utilization of the organic acid succinate (Dye, 1966).

(v) All reports, including the present one, agree on the catabolism of glucose by the species resulting in acid production. However, strain LS155 differs by producing a weak acid reaction from glucose utilization, confirmed by the positive result for the Voges Proskauer test. This weak acid reaction is the result of the conversion of the acid end-products from glucose metabolism to non-acidic or neutral end-products, especially acetylmethylcarbinol, which is detected by the Voges Proskauer test (Anon., 1953). Pyruvate, from glucose metabolism, is converted to 2-acetolactate by the enzyme acetolactate synthase. Acetoin is produced by the decarboxylation of 2-acetolactate - the reaction being catalysed by the enzyme acetolactate decarboxylase.

Apart from variations in results for these biochemical tests, there is consensus regarding the results for other biochemical and physiological reactions.

One important fact to note in studies on *X. albilineans*, and for most xanthomonads, is that these organisms do not grow on relatively acidic media and will die in unbuffered,

sugar-containing, protein free media in which they produce acids. On the other hand, xanthomonads produce alkaline end-products as a result of strong protein degradation and will not survive for long if the resulting high pH's are not controlled (Starr, 1981). The use of strong buffers to control pH shifts is also not recommended because the organisms do not tolerate high salt concentrations (Starr, 1981). Thus, a compromise becomes necessary when constituting a medium containing carbohydrates, proteins and a buffer for biochemical and physiological tests. The carbohydrate and organic acid basal medium used in this study, modified from Dye's Medium C (Dye, 1962) and YS Broth Base (Dye, 1962,1980), was adequate in fulfilling these needs. Yeast extract provided a complex protein while the immediate nitrogen requirements were met by the incorporation of $NH_4H_2PO_4$, which also provided the acidic buffer salt. The basic buffer salt was K_2HPO_4. Magnesium, in the form $MgSO_4$, provided the divalent cation required for enzyme function and membrane stability. The pH of the medium was adjusted to 6 for two reasons:

(i) Being the mid-point of the pH range for bromocresol purple, minor shifts in pH would be easily detected as colour changes; and

(ii) Initial experiments with media at pH 7 showed rapid, strong, alkaline changes in the media, with subsequent inhibition of growth in some substrates, e.g. arabinose (also noted by Dye, 1966), cellobiose and dextrin. As a

result, any weak acid production from carbohydrate utilization, was not detected. The weak acid environment, at pH 6, in uninoculated media was quickly offset, following inoculation, by alkalinization of the medium from peptone degradation, so that the medium reverted to neutrality. The buffer salts played a role in this regard in preventing any dramatic pH fluctuations.

Reasons for variations in the results from biochemical and physiological tests among the various researchers, may be attributed to the fact that little consideration was given to the basal medium composition, especially when the medium contained an additional salt in the form of sodium chloride, as in YS broth base and Dye's Medium C (Bradbury, 1984; Dye, 1962,1980).

The use of sodium chloride in media for culturing *X. albilineans* has not been recommended by Dye (1966) because of its inhibitory effect on the bacterium. Bradbury (1984) indicated that the maximum sodium chloride tolerance level for the organism was 0,5%. This study verifies this finding, but has also provided evidence that *X. albilineans* could tolerate higher concentrations of sodium chloride, showing weak growth (Fig. 7). On the basis of the beneficial effects of low concentrations of sodium chloride (0,01 to 0,1%) on cell survival, the results of this study favour a modification of Wilbrink's medium by including 0,1%

sodium chloride. This concentration has been found to enhance population growth rates above that normally attained in medium lacking sodium chloride. No such effect was obtained in YSP medium. On the contrary, the incorporation of sodium chloride into YSP medium resulted in reduced growth when compared to controls from which it was omitted. The buffering capacity of each of these media is thought to have some effect in this regard.

Figures 6 and 7 show the relative growth, at varying pH's and sodium chloride concentrations, respectively, of each of the strains in Wilbrink's and YSP broth. Whereas equivalent amounts of growth were obtained for strains 2901 and XA 86-1 in these two media, large differences were noted for the other three strains. Population densities in Wilbrink's broth were usually two-fold higher when compared to YSP broth. In addition, during the course of this study, cultures grown on Wilbrink's agar were consistently observed to remain viable over longer periods than those grown on YSP agar. These two media differ in that YSP medium provides a highly enriched nutritional substrate with no buffering capacity, while Wilbrink's medium has a lower nutritional status and a weak buffering capacity. Although the use of YSP medium is popularly advocated by several workers (Bradbury, 1984; Dye, 1980; Ricaud & Ryan, 1989), especially for the isolation of *X. albilineans*, attention should be drawn to the fact that Wilbrink's medium is a superior medium on

the basis of its buffering capacity which encourages better cell growth over a wider pH range (Fig. 6) and sustains cell viability over longer periods.

Media effects on the protein composition of cell envelopes of the strains of *X. albilineans* studied here were negligible. Although minor variations in the relative concentrations of different proteins were observed, the overall protein profiles remained the same in Wilbrink's and YSP broth and agar (Fig. 12). Comparisons of the protein profiles of the different strains also showed a consistent pattern. These profiles were unique for *X. albilineans* when compared to those of *X. campestris* pv. *zeae*, *E. coli* and *P. aeruginosa* (Fig. 13), and could be regarded as an additional taxonomic characteristic for the species, although the protein profiles of a wider range of *Xanthomonas* species would need to be examined beforehand. The use of membrane protein profiles for strain differentiation appears limited at this stage and requires further investigation. The one major protein (in the 35 kdal region) which was common to the four species did not necessarily imply homogeneity of the protein (Minsavage & Schaad, 1983). Such proteins could be differentiated into two, three and four distinct bands of different molecular weights by varying the electrophoresis systems and conditions.

Minsavage & Schaad (1983) found that the membrane protein profiles of pathovars of *X. campestris* varied according to the types of symptom produced, and virulence on their respective hosts. Similar analyses did not form part of the present study, but the results of such research have important connotations for any future work on the membranes of *X. albilineans*. Pathovars of *X. campestris* that produced typically similar symptoms had similar profiles, which were distinct from other pathovars that produced different symptoms and virulence reactions. Thus, a strong correlation existed between virulence and protein profiles, and not with differences in pigmentation and colony morphologies. These unique protein profiles for the virulent strains of *X. campestris* were distinguishable from those of other xanthomonads and were unaffected by culture age, although differences in incubation temperature and heat treatment for electrophoresis (dos Santos & Dianese, 1985) caused changes in some polypeptides. As in this study, the use of different culture media did not affect protein profiles (Minsavage & Schaad, 1983), although such effects have been observed during studies with *P. aeruginosa* (Mizuno & Kageyama, 1979).

The number of bands and the relative distribution of proteins among bands varies depending on the treatment of the crude membrane extract prior to density gradient centrifugation. Solubilization with different detergents

(e.g. Triton X-100, sodium-dodecyl-sulphate, sodium desoxycholate, Tween-20 and Tween-80), before electrophoresis, resulted in different polypeptide profiles and different degrees of solubility of the crude membranes in these detergents; however, for a particular method, the profiles for related species remained unchanged (dos Santos & Dianese, 1985; Minsavage & Schaad, 1983), although functions of proteins could be impaired as a result of denaturation (Hancock *et al.*, 1981). Usually two membrane bands would be produced upon solubilization of crude extracts, while four resulted if extracts were not solubilized (Dianese & Schaad, 1982), as shown in Fig. 9. The relative densities of the light and heavy bands, upon solubilization and non-solubilization, would remain unchanged, and for *X. campestris* these were 1,142 $g.cm^{-3}$ and 1,255 $g.cm^{-3}$, respectively (Dianese & Schaad, 1982; dos Santos & Dianese, 1985). For the *X. albilineans* strains studied here the buoyant densities were higher for the light band, in the range 1,162 to 1,168 $g.cm^{-3}$, and lower for the heavy band with a wider range, between 1,235 to 1,253 $g.cm^{-3}$. The membrane vesicle morphologies of the two species, *X. albilineans* and *X. campestris* were comparable in that both the tubular and rounded types were formed during preparation (Dianese & Schaad, 1982).

Although the four isolates of *X. albilineans* and the type species presented a relatively uniform phenotypic profile,

certain key criteria, shown in Table 10, could be used to distinguish between them. Most of the other criteria examined, including the optimum growth temperatures, many of the biochemical and physiological reactions, and the previously undescribed membrane protein profiles and isolated cell envelope morphology, provided few, or no, distinguishing features which could be used for strain designation.

Chapter 3. SEROLOGICAL RELATIONSHIPS

3.1 INTRODUCTION

The hypothesis of Egan (1969a,1970), that strains of *X. albilineans* occur, has been tested serologically only in relatively recent times (Baudin & Chatenet, 1981; Ricaud & Ryan, 1989). However, in the past, serological methods were used for detecting the disease, e.g., by immunofluorescence (Leoville & Coleno, 1976) and microagglutination (Ricaud *et al.*, 1978). Though sensitive, these methods have proven uneconomical and sometimes inconsistent, with successful diagnoses ranging between 66% and 93% of known infected material. The objective of this part of the study was to examine the serological relationships of intact cells and isolated cell envelopes, and to establish whether characteristic cell surface antigens were expressed by individual isolates.

3.2 MATERIALS AND METHODS

3.2.1) Antigen preparation

All five strains of *X. albilineans* were grown on Wilbrink's and YSP agar. Plates were incubated at 26 C for 5 to 7 d. Following purity checks, cells were harvested aseptically, washed twice by centrifugation (10000 x *g*; 10 min; 4 C) in

sterile distilled water, and finally resuspended in sterile physiological saline (0,85% NaCl) to a density of 2 x 10^9 cells.ml^{-1} using a Thoma bacterial counting chamber (Weber Scientific International Ltd., Sussex, England). Equal volumes of cell suspension and Freund's incomplete adjuvant (Difco Laboratories, USA) were emulsified, giving a final antigen concentration of 1 x 10^9 cells.ml^{-1} for immunization. Sterile 1 ml plastic, disposable tuberculin syringes were loaded with the emulsion and refrigerated at 4 C until used.

3.2.2) Antiserum production

Control sera (undiluted and diluted 1:10) from disease-free conventional rabbits (mature adult male and female) were screened for non-specific agglutination of cells of all the test strains of *X. albilineans* prior to immunization. Rabbits giving positive agglutination at the 1:10 dilution were discarded from the experiment. The remaining animals were immunized according to the schedule outlined in Table 11. Three animals were used per test strain antigen.

Following the last scheduled immunization on day 29, all rabbits were bled (primary bleed) from the marginal ear vein 7 and 14 d later, collecting approximately 5 ml blood per animal per bleed. Sera obtained from the processed blood (Kolmer & Boerner, 1945) was titrated against homologous bacteria (antigen) before being dispensed into sterile tubes

and stored at -20 C. Antiserum titres determined by the tube agglutination test ranged between 1:320 to 1:5120 (using a final antigen concentration of 1 x 10^{10} bacteria.ml^{-1}) per individual rabbit. These animals were given 1 ml intramuscular booster injections with the same antigen as before, 30 d following the primary bleed, and killed by exsanguination (secondary/final bleed) 14 d later. Sera were recovered from the processed blood as described above.

Table 11. Rabbit immunization schedule using whole cells of *X. albilineans*, emulsified 1:1 with Freund's incomplete adjuvant, at a final concentration of 1 x 10^9 cells.ml^{-1}

DAY	ANTIGEN DOSE (ml)	IMMUNIZATION ROUTE*
1	1,0	IM
15	1,0	IM
21	1,0	IM
22	0,5	IM
24	0,5	IM
26	0,5	SC
29	1,0	IM

*IM = intramuscular
SC = subcutaneous

3.2.3) Agglutination tests using whole cells

The reaction of each antiserum with its homologous bacterium, and with the other heterologous bacteria, was quantified using the slide agglutination test with a final cell concentration of 1 x 10^6 cells.ml^{-1}. The antiserum

dilution range assayed was from 1:5 to 1:20480, employing serial two-fold dilutions in physiological saline.

In homologous and heterologous antigen testing, thirty microlitres each of the antisera dilutions and bacterial suspensions were mixed on depression slides and incubated at room temperature for 30 min. Controls included pre-immunization sera (diluted 1:10) and homologous and heterologous bacteria, and bacterial suspensions in saline.

Following incubation, all slides were examined for agglutination. No agglutination was observed on the control slides, and these were used as non-agglutination references.

3.2.4) Precipitin reactions of the membrane preparations

Crude membrane antigenicity was studied using Ouchterlony double diffusion tests, by independently diffusing each of the previously mentioned five *X. albilineans* antisera (obtained by whole cell injection, described in Section 3.2.2)) against a set of eight crude membrane antigens, including the three additional ones of *Xanthomonas campestris* pv. *zeae*, *Escherichia coli* and *Pseudomonas aeruginosa*, prepared as described in Section 2.2.4)(a)(i)). In addition, in order to examine in more detail whole cell antigenicity within each of the five

X. albilineans isolates, each strain antiserum was diffused simultaneously against homologous strain crude membrane extract and the membrane fractions derived therefrom by density gradient centrifugation as described in Section 2.2.4)(a)(ii).

Tests were conducted in 65 mm diameter petri plates containing 10 ml of medium comprising 0,8% agarose, 1% trypan blue (Schaad, 1974; Thaveechai & Schaad, 1986), 3% polyethyleneglycol (4000) and 0,025% thiomersal in physiological saline (0,85% NaCl). Five millimeter diameter wells were punched out in a pattern that allowed either four or eight equidistantly placed wells to surround a central well, the distance between the edges of the central and peripheral wells being 8 mm. The central well was filled with 50 microlitres of a 1:5 dilution of antiserum, and the peripheral wells with 30 microlitres of the appropriate antigens. Plates were placed in a humidity chamber at 28 C and observed and photographed after 5 d. Results were recorded as reactions of identity (complete fusion), partial identity (partial fusion or spur formation) and non-identity (non-interaction)(Chaparas *et al.*, 1983).

3.3 RESULTS

3.3.1) Agglutination tests

Table 12 shows the slide agglutination titres of the prepared antisera, reacted with their respective homologous and heterologous whole bacteria.

Table 12. Slide agglutination titres of antisera against homologous and heterologous bacteria at a final concentration of 1 x 10^6 cells.ml^{-1}. Results are expressed as a reciprocal of the antiserum dilution

ANTISERUM TO	ANTIGEN PDDCC 196	LS155	2901	2905	XA 86-1
PDDCC 196	640	40	80	160	80
LS155	40	320	80	80	160
2901	320	320	2560	160	20
2905	40	160	320	1280	640
XA 86-1	320	640	160	1280	5120

Antisera homologous titres ranged from a low 1:320 (LS155) to a high 1:5120 (XA 86-1). Antisera to strains PDDCC 196, 2901 and 2905 showed titres of 1:640, 1:2560 and 1:1280, respectively. Cross-reactivity between antisera and heterologous antigens was evident in all cases, the majority at dilutions of 1:320 and less. The only exceptions were the anti-XA 86-1 and anti-2905 antisera. Anti-2905 diluted

1:640 was able to agglutinate cells of XA 86-1, while anti-XA 86-1 at dilutions of 1:1280 and 1:640, respectively, agglutinated cells of strains 2905 and LS155.

Relationships between strains are not evident from the results presented in Table 12. However, extrapolation of the data from Table 12 using serological difference indices (SDI's) shows clustering of the five strains (Tables 13,14; Figs. 14,15).

Table 13. Index rating of the antisera dilutions used in the slide agglutination tests

Reciprocal of antiserum dilution	Index
5	1
10	2
20	3
40	4
80	5
160	6
320	7
640	8
1280	9
2560	10
5120	11

PDDCC 196	0				
LS155	3,5	0			
2901	3	2,5	0		
2905	3,5	2,5	3	0	
XA 86-1	3,5	2	6	1,5	0
	PDDCC 196	LS155	2901	2905	XA 86-1

Fig. 14. Data matrix of the serological difference indices.

Table 14. Average-linkage clustering using serological difference indices

SDI	Clusters
0	PDDCC 196 LS155 2901 2905 XA 86-1
1	PDDCC 196 LS155 2901 2905 XA 86-1
1,5	2905,XA 86-1 PDDCC 196 LS155 2901
2	2905,XA 86-1 PDDCC 196 LS155 2901
2,5	2905,XA 86-1,LS155 PDDCC 196 2901
3	2905,XA 86-1,LS155 PDDCC 196,2901
3,5	2905,XA 86-1,LS155 PDDCC 196,2901
4	2905,XA 86-1,LS155,PDDCC 196,2901

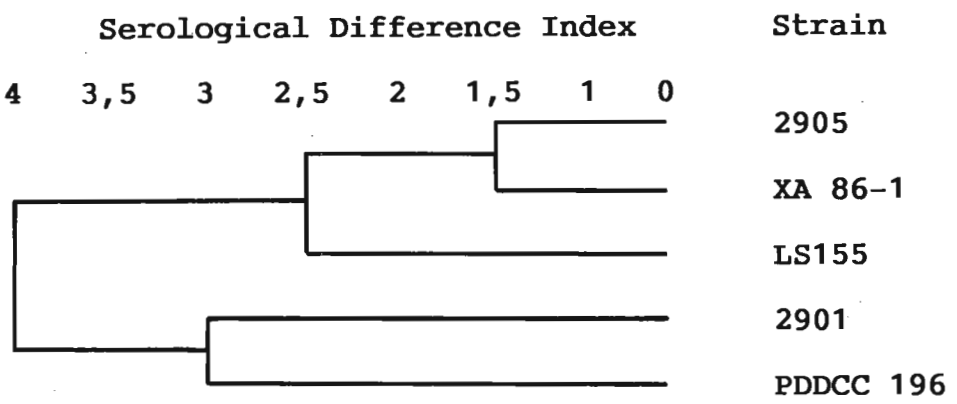

Fig. 15. Dendrogram of the average-linked clusters.

The greatest SDI difference (SDI 6) is shown between strains 2901 and XA 86-1, indicative of a weak serological relationship between them although both strains showed the strongest immunogenic properties (Table 12). On the other hand, the closest relationship is between strains XA 86-1 and 2905 (SDI 1,5), with LS155 slightly more distantly related to XA 86-1 (SDI 2). The fairly close relationship of strains XA 86-1, 2905 and LS155 makes possible their grouping, as shown from the data matrix of the SDI's, the average-linkage clustering and the dendrogram above. The

remaining two strains, 2901 and PDDCC 196, are both an average SDI 3 from any of the three grouped strains and from each other. This means that these two strains are distinguishable serotypes outside the suggested grouping. XA 86-1 and 2905 show identical SDI values (SDI 3,5) from the type species PDDCC 196, while the SDI distance of XA 86-1 and 2901 (SDI 6) is twice that between 2905 and 2901 (SDI 3). Thus, within the grouping, XA 86-1 shows a considerably stronger reaction with PDDCC 196 (SDI 3,5) than with 2901 (SDI 6).

3.3.2) Precipitin reactions

Using standard guidelines (Chaparas *et al.*, 1983), immunodiffusion analysis of the membrane preparations to establish surface antigen similarities and differences gave the following results:

In the challenge between the total (or crude) membrane preparations, one sharp line of precipitation (two for strain 2901) with a broad diffuse area around it, developed for all strains (Fig. 16). This line of identity among the strains was also present for *X. campestris* pv. *zeae* challenged against anti-2905 (Fig. 16d), anti-XA 86-1 (Fig. 16e) and anti-PDDCC 196 (Fig. 16a), the latter two reactions showing partial identity. What is not clearly seen in Fig. 16 is a very broad diffuse area of precipitation around the central

well in all tests. In addition, weakly apparent in Fig. 16e is the presence of several lines of precipitation. The two sharp lines closest to the central well are antigens of partial identity developing for all *Xanthomonas* species only. The arc furthest from the central well is one of identity for all strains of *X. albilineans* except for XA 86-1, and *X. campestris* and *P. aeruginosa*, where spur formation occurred, denoting partial identity. Two arcs of non-identity also developed between the central well and XA 86-1, beyond the other arcs and furthest from the central well.

Immunodiffusion tests using the crude membranes and membrane bands L, I and H resulted in at least one arc of identity among the bands of all strains (Fig. 17). However, a total of three arcs of identity appeared between the H band and the crude membrane preparation (C) of strain 2905 (Fig. 17d). Large areas of diffuse precipitation adjacent to the centre wells in all tests were probably the result of varying distances of migration by small membrane vesicles present within these preparations. The very large antigens within membrane preparations also resulted in arc formation close to the wells containing these membranes. These large antigens clearly were unable to diffuse sufficiently far into the medium to enable them to react with arcs formed by adjacent wells. Although only band H contained antigens showing identity to those present in the crude membrane preparations, it may not be overlooked that the larger

antigens detectable in each band could have been unable, if any identity existed, to show arc fusion at their ends.

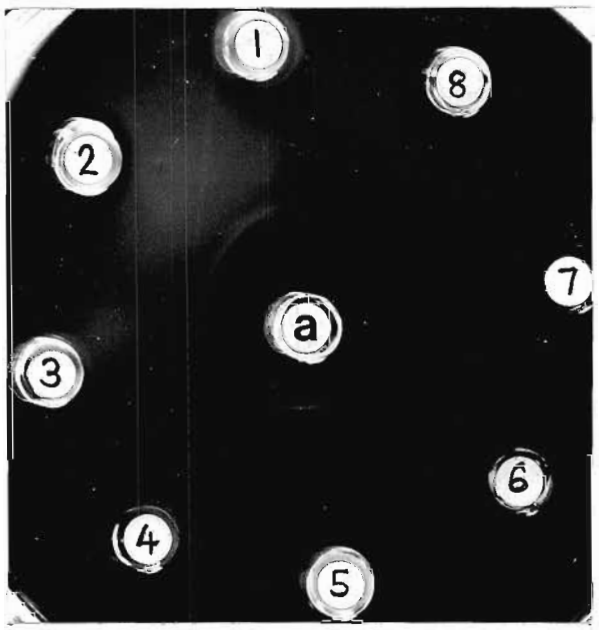

Fig. 16. Immunodiffusion precipitation patterns of crude membrane preparations (1=PDDCC 196, 2=LS155, 3=2901, 4=2905, 5=XA 86-1, 6=*E. coli*, 7=*P. aeruginosa* and 8=*X. campestris* pv. *zeae*) against antisera to strain (a) PDDCC 196.

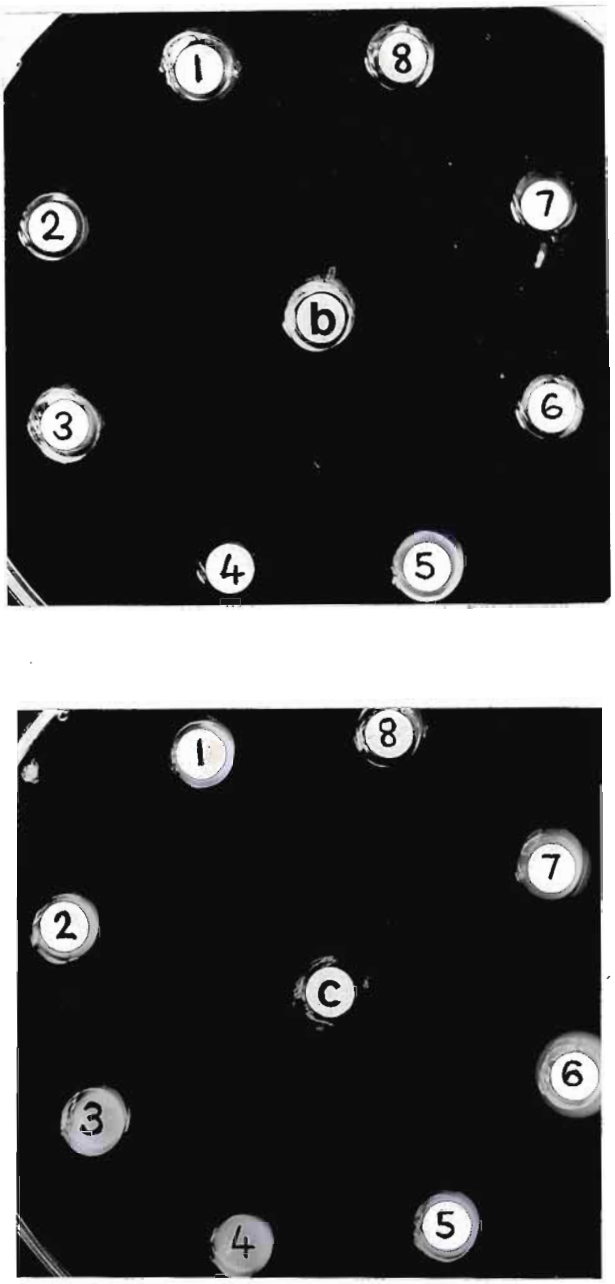

Fig. 16. Immunodiffusion precipitation patterns of crude membrane preparations (1=PDDCC 196, 2=LS155, 3=2901, 4=2905, 5=XA 86-1, 6=*E. coli*, 7=*P. aeruginosa* and 8=*X. campestris* pv. *zeae*) against antisera to strains (b) LS155 and (c) 2901.

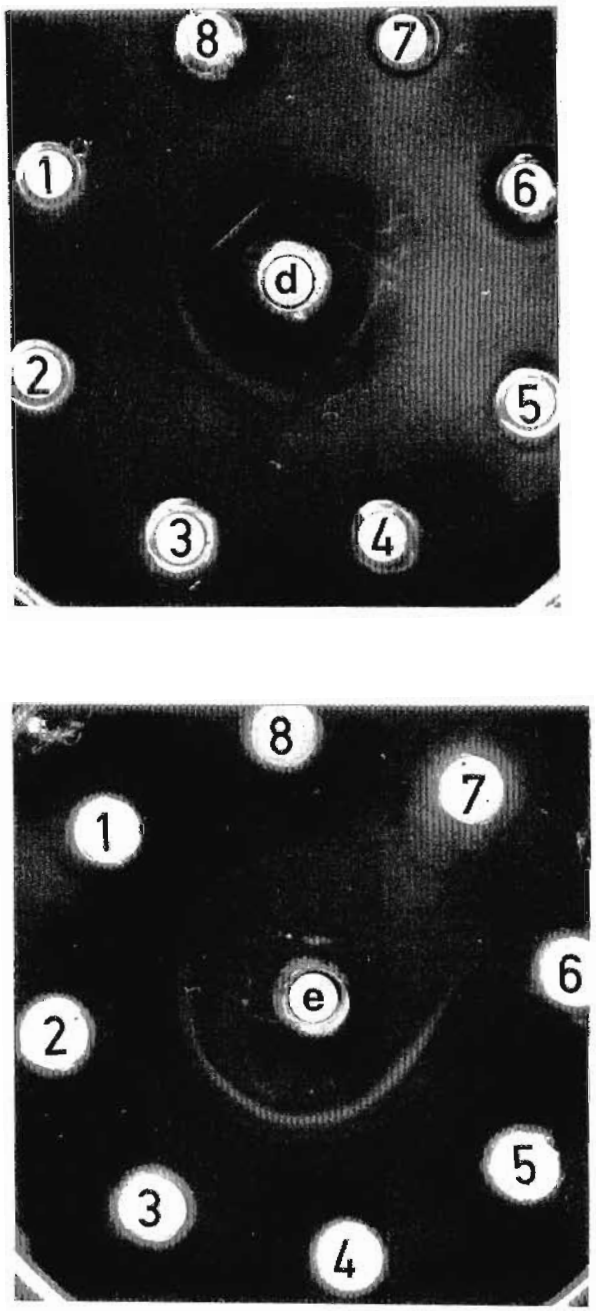

Fig. 16. Immunodiffusion precipitation patterns of crude membrane preparations (1=PDDCC 196, 2=LS155, 3=2901, 4=2905, 5=XA 86-1, 6=*E. coli*, 7=*P. aeruginosa* and 8=*X. campestris* pv. *zeae*) against antisera to strains (d) 2905 and (e) XA 86-1.

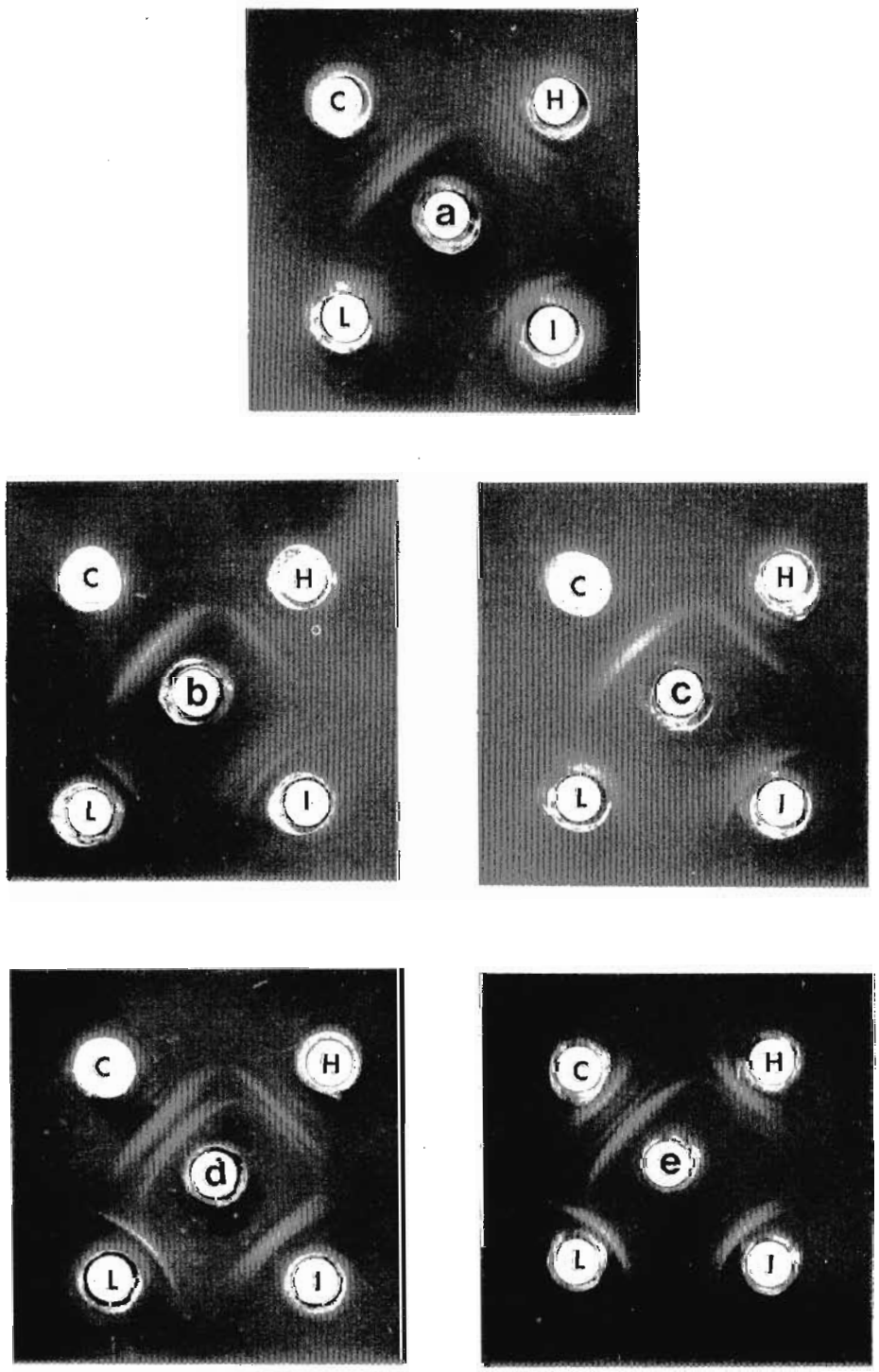

Fig. 17. Immunodiffusion precipitation patterns of membrane preparations (C=crude, L=light, I=intermediate and H=heavy) against their homologous antisera: (a) PDDCC 196, (b) LS155, (c) 2901, (d) 2905 and (e) XA 86-1.

3.4 DISCUSSION

The use of serology in identifying bacteria is, according to Schaad (1979), "almost as old as the science of plant pathology itself". The purpose of developing antisera to the five strains in the study was three-fold. Firstly, it was necessary to establish the strength of the immunogenic properties of each strain; secondly, to check the extent of cross-reactivity among strains; and thirdly, to locate possible cell surface antigenic sites on the membrane fragments obtained.

The immunogenicity of strains 2901 and XA 86-1 were found to be strongest, with antiserum titres against their respective homologous bacteria being 1:2560 and 1:5120. A good correlation was evident between cell size and the immunogenicity of strains from the cell agglutination tests, the shorter bacteria generally having weaker immunogenic properties (Table 3, Table 12). Ricaud & Ryan (1989) reported that the longer filamentous cells were usually more aggressive in pathogenicity testing, the significance of which could not be determined from this study for reasons mentioned earlier. Although strains 2901 and XA 86-1 showed the strongest immunogenic properties, from SDI analysis they proved to be the most distinct serologically from each other. This was interesting in view of their morphological similarity (Table 3, Figs. 3c and 3e) and that both these

strains have two plasmids rather than one each, as shown for the remaining three strains (Singh, 1989). This poses the question of the possible role of plasmids in antigenicity, and whether the presence of more than one plasmid increases the virulence of strains.

In a study on four strains of *X. albilineans*, Baudin & Chatenet (1981) found that interactions between antisera and their homologous bacteria were significantly stronger when compared to interactions between the same antisera and heterologous bacteria, and suggested that serotypes of the species existed. This contradicts the results of this study, where cross-agglutination between strains using antiserum dilutions of up to 1:1280 were noted (Table 12). Baudin & Chatenet (1981) showed that antisera titres against homologous bacteria exceeded 1:10000 whilst titres against heterologous bacteria did not exceed 1:320. Since antisera were raised against two of the four strains studied in that work, their results were not analysed using SDI's, which could possibly have shown serological relationships, if any, between strains. The results from the present study showed much lower antisera titres when compared to those of Baudin & Chatenet (1981), which is acceptable since their method of titration, by immunofluorescence, was more sensitive. On the basis of SDI's, serological relationships between strains not evident from the results of the agglutination tests were given greater clarity. The five strains were

grouped as follows: strains 2905, XA 86-1 and LS155 formed a cluster separate from strain 2901 and the type species PDDCC 196, which were also serologically distinct from each other (Fig. 15).

Methods used to generate antisera in this study and by Baudin & Chatenet (1981) were similar, with viable cells being used as antigens. Both Elrod & Braun (1947) and Wells *et al.* (1987), in studies on *Xanthomonas* and *Xylella* (a xanthomonad) species, respectively, reported cross-reactions between heterologous plant-pathogenic bacteria which made serotyping in this group of organisms difficult. This cross-reactivity was attributed to the existence of common epitopes, usually present in the lipopolysaccharides of the outer layers, in these bacteria. However, antisera prepared against cells from which the polysaccharide was removed usually eliminated these cross-agglutination reactions (Schaad, 1979). Such a procedure was not considered in this study, because antisera needed to be generated against bacteria where the surface structure was unchanged, to facilitate the identification of these organisms upon isolation, or *in situ*.

To avoid changes in the genetic and physical make-up and a loss of pathogenicity of *X. albilineans* cultures, satisfactory measures for their preservation were investigated. Methods

advocated for the successful preservation of plant pathogens include:

(i) Maintaining the pathogen in live plants under glasshouse conditions (Thompson, 1982a); and

(ii) Storing isolates in distilled water (Perez, 1970).

Storage by lyophilization and under mineral oil were disregarded because of the unsatisfactory recovery of cultures, as was maintaining the pathogen in live plants. Maintenance of cultures by lyophilization has also not been advised (Bradbury, 1984). Short- and long-term cultures were therefore maintained in distilled water (Perez, 1970) and ultra-frozen (Gibson & Khoury, 1986), respectively. The retardation of growth and cell metabolic activities by these methods was deemed adequate to prevent changes in the genetic and physical characters of cultures.

Finally, all membrane preparations of *X. albilineans* possessed at least one antigen in common. This antigen, obviously, was surface-located since all antisera were raised against intact bacteria. Evidence for its location in membrane band H is provided by the immunodiffusion patterns shown in Fig. 17, where the single major precipitin arcs of band H and the crude membrane preparation in strains PDDCC 196, LS155, 2901 and XA 86-1 (three in strain 2905) showed complete identity. The extent to which cross-reactions between the membrane preparations of the five strains and the other bacteria used in this study, except *E.coli*, against antiserum to XA 86-1 is

indicative of the common phylogenetic origin of these bacteria. The taxonomic proximity of *Xanthomonas* and *Pseudomonas* from nucleic acid homology studies (Palleroni *et al.*, 1973) is reflected in the antigenicity of their proteins. Collins *et al.* (1987) demonstrated that protein antigens would not cross-react unless they were homologous, isofunctional and of a common phylogenetic origin. The extent of cross-reaction was detectable until 30 to 40% of the amino acid sequence in the protein was substituted.

From an analysis of the immunological methods used in attempting to differentiate between these isolates of *X. albilineans*, it appears that serotyping, on the basis of the raw data from agglutination tests, is inadequate, although reported otherwise by Baudin & Chatenet (1981). Subjecting such data to SDI treatment makes more detailed serological comparisons between organisms possible. Problems of a similar nature were encountered in the immunological characterization of *Erwinia chrysanthemi* isolated from different hosts, when even single purified antigens (specific proteins) showed non-specific relationships among strains (Yakrus & Schaad, 1979). This subsequently led to the use of a membrane protein complex, extracted from whole cells, being used as an antigen. The antisera to this type of antigen, used in Ouchterlony double diffusion tests, was successful in determining relatedness among strains of the species. Of the immunological assays employed in this

study, Ouchterlony double diffusion showed some promise in resolving the issue of strain designation. However, for the development of an antigen against which antisera would be raised to allow unequivocal strain identification, careful consideration must be given to two criteria, viz., the feasibility for its use in taxonomic studies, and its employment in the economic sector to facilitate detection of the pathogen under field and experimental conditions.

Studies on leaf scald disease and its etiological agent have been addressed on two fronts. Detection, control and pathology of the disease are well established ongoing areas of research, while more intensive investigations into the mechanisms effecting the disease have only been undertaken within the last decade. The two most significant findings to date have been the isolation and identification of a group of toxins from *X. albilineans*, associated with leaf chlorosis in infected plants (Birch & Patil, 1985, 1987a,b), and a report on the occurrence of plasmids in the five strains of *X. albilineans* studied here (Singh, 1989). The relationship between these plasmids and virulence of the strains has not been ascertained. In addition, researchers world-wide are still grappling with the problem of identifying strains of the species, and trying to establish what criteria differentiate them. Although this study was restricted to an evaluation of the phenotypic characters of five strains, several debatable points concerning past

methodology have become apparent. In addition to highlighting some of these, recommendations have been made to encourage greater awareness of the factors affecting studies on *X. albilineans*. This study has also provided some positive evidence on previously ambiguous phenotypic criteria tentatively supporting the existence of pathotypes, or strains, of *X. albilineans*. Finally, there is an obvious need for the supplementation of these results with genetic analyses in order to complete the characterization of the bacteria examined.

SUMMARY

Leaf scald disease was first documented during the years between 1920 and 1926, and first recorded in South Africa in 1968. Since that time research of the disease has concentrated on finding means to prevent its spread to the major sugarcane growing countries of the world. Although this has not been possible, the development of successful control measures has contained the disease so that it now ranks only secondary to other sugarcane diseases. As a consequence of the potential of the disease to cause widespread destruction of crops because of its unpredictable nature, even in countries with effective control programmes, recent studies have concentrated on the etiological agent of leaf scald.

The major problems relate to varying and inconsistent symptom expression by sugarcane varieties, in response to the disease, in different countries. This resulted in the concept that strains of *Xanthomonas albilineans* existed. Very little comparative work among geographical isolates has been carried out to confirm this, although specific attributes, e.g. pectolytic activity, total protein profiles and serological identities of several isolates have been compared. This study presents a more complete profile of strains of *X. albilineans* originating from Australia, Mauritius, South Africa and the type species. Criteria examined

included: colony and cell morphology, staining reactions; growth response to a range of pH, sodium chloride concentrations and temperature; biochemical and physiological reactions; the protein profiles of isolated cell envelopes; and the antigenicity of whole bacteria and membrane components. In addition, conditions affecting the pathogenicity of the South African strain were examined, optimizing conditions for isolating and maintaining stocks; and comparing the use of growth and assay media for culturing and testing the reactions of the species.

All five strains presented a homogeneous group of bacteria, with similar responses to most criteria, e.g., variations in cell dimensions, optimum growth temperatures, weak and variable responses to biochemical and physiological tests, varying densities of membrane band H and the membrane protein profiles from PAGE. Several characteristic differences among the isolates were used as diagnostic criteria separating them, e.g., acid production from fructose and galactose, H_2S from cysteine, nitrate reduction to nitrite, starch hydrolysis, acetoin production and the litmus milk reactions. Two additional criteria have been added to the species diagnostic characteristics, viz., acid production from arabinose and mannose. Apart from separating strains, the serological difference indices have made possible the grouping of strains on the basis of their antigenic identities. Thus, in addition to distinguishing

between isolates, relationships between them, not evident from the phenotypic criteria, have have been established.

Of the two media, Wilbrink's and YSP, routinely used for isolation and culture of the species, Wilbrink's medium was found to be superior because its buffering capacity afforded a greater stability to the medium. On this medium, better growth and increased survival of cell populations were noted over a wider range of sodium chloride concentrations and pH, when compared to YSP medium. On the basis of results obtained, a recommended minor modification to Wilbrink's medium is the incorporation of 0,1% NaCl - which enhances and sustains cell growth to a greater extent than the medium lacking sodium chloride.

Whereas other criteria showed great uniformity, differences were evident in the biochemical and physiological responses of isolates of *X. albilineans*. With an increased awareness of growth conditions and the use of suitably constituted media for comparative biochemical studies, standardised analyses of geographical isolates would be possible using the criteria described here. However, a greater number of isolates would have to be studied before any recommendations for a sub-species designation would be acceptable. This study has provided evidence that strain differences do exist.

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