PSEUDOMONAS ON PEAS:
ICE NUCLEATION, IDENTIFICATION AND PATHOGENICITY

by

MITRA MAZAREI
B.Sc. (Agr.) Univ. of Shiraz, Iran

Department of Crop Protection
Waite Agricultural Research Institute
University of Adelaide
South Australia

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dedicated to my family : Reza, Amin and Iman

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ABBREVIATIONS

cfu	colony forming units
cv	cultivar
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbant assay
λ Hind III	lambda DNA cut with the restriction enzyme from Haemophilus influenzae
HR	hypersensitivity
INA	ice nucleation active
kb	kilobase
M	molar
OD	optical density
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDW	sterile distilled water
SSC	saline sodium citrate
TE	Tris-EDTA buffer
TEM	transmission electron microscope
TEMED	N, N, N`, N`-tetramethylethylenediamine
UV	ultraviolet

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SUMMARY

Ice nucleation active (INA) bacteria were detected in a pea field in South Australia. They were identified as strains of Pseudomonas syringae and Pseudomonas fluorescens biotype 1. A clear impression was obtained that P. fluorescens was the predominant INA organism in that pea field.

The probability of detecting ice nucleating activity in washings from pea leaves decreased with time and also with increasing crop maturity. Numbers of INA bacteria also decreased with time. There was no influence of rainfall on numbers of INA bacteria, but there was some effect of air temperature. With decreasing temperature numbers increased and with increasing temperature numbers decreased to an undetectable level.

Some chemical agents were tested as cryoprotectants on these two ice nucleating species of INA bacteria, both in vitro and in planta and, it was established that some specific metal cations such as Mn, Al, Zn, Ca, Ni, Co, and Fe have some effect on ice nucleating activity, either because of bactericidal properties (Al, Zn, Ni, Co, and Fe) or by interfering with the nucleating process (Ca and Mn). At low concentration (0.001M), Zn and Al were still active and no phytotoxicity of these metals on pea plants was evident.

One INA strain of P. syringae, isolated from South Australia was compared for its ice nucleation activity in vitro with one INA strain of P. syringae isolated from New Zealand. The NZ strain was more active, and the effect of Mn and Ca as cryoprotectants was lower on the NZ strain, but the effect of Zn and Al was the same on both strains.

Most published methods to distinguish Pseudomonas syringae pv. syringae from Pseudomonas syringae pv. pisi were evaluated. Inoculation of susceptible cultivars was the most reliable. Results were confirmed by inoculation of lemon fruit.

A much more rapid and convenient serological method was developed to distinguish the two pathovars. Antisera against glutaraldehyde-fixed cells had a high level of specificity in Ouchterlony gel double-diffusion tests and, after cross-absorption with heterologous antigens, in indirect ELISA.

Specificity of polyclonal antibodies to different antigenic preparations (sonicated and heat-killed) of cells of these two pathovars (P. syringae pv. syringae and P. syringae pv. pisi) was investigated. Antisera to sonicated cells gave a strong reaction against homologous antigen; with heterologous antigen, cross reaction occurred with whole untreated cells and sonicated cells but there was no cross reaction with heat-killed cells. Antisera to heat-killed cells of these two pathovars gave a weak reaction with whole untreated cells of homologous antigen; however, when heat-killed cells of both homologous and heterologous were used as antigen, a strong reaction was observed with both. Antiserum to heat-killed cells had the useful ability to distinguish Pseudomonas syringae from other species of Pseudomonas and other genera.

Of the bacterial surface antigens of each pathovar, only cell wall and flagella showed no cross reactivity with heterologous antisera. Antisera to flagella of these two pathovars showed the highest level of specificity. No cross reactivity with whole untreated, sonicated, or heat-killed cells of heterologous antigens was observed.

All strains tested which were pathogenic to peas and which were positively recognized by antiserum to P. syringae pv. pisi contained two to four plasmids; non-pathogens had none. Two plasmids from a pathogenic strain were transferred individually to a non-pathogenic recipient strain of P. syringae pv. syringae; both plasmids converted the recipient to a pathogen on peas and from hypersensitivity (HR) negative to HR positive on tobacco. Neither plasmid encoded homoserine catabolism.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text. The author consents to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Mitra Mazarei

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Introduction / Literature Review

Pseudomonas is a large genus of bacteria. Its cells are Gram-negative, straight rods, and are motile by polar flagella (Palleroni, 1984; Sands, *et al.*, 1980).

An important species of this genus is Pseudomonas syringae. It is widely distributed on plants and occurs as both pathogens and non-pathogens. Pathogenic forms of Pseudomonas syringae have been reported on many of plants (Freigoun and Crosse, 1975; Leben, 1965). They colonize plants and after infection elicit pathological symptoms whereas non-pathogenic strains, may colonize plant hosts but they do not elicit pathological conditions. However, many pathogenic strains of P. syringae have been reported to survive in large numbers as epiphytes on a variety of symptomless host plants, such as stone fruits(Cameron, 1970; Crosse, 1966; Dowler and Weaver, 1975; Garden *et al.*, 1972), olive (Ercolani, 1969), bean (Hirano *et al.*, 1982; Lindemann *et al.*, 1981), and soybean (Leben, 1965; Leben *et al.*, 1968).

Based on the pathogenic characteristics, strains of P. syringae are subdivided into pathovars (Young *et al.*, 1978). This species now contains approximately 40 distinct pathovars (Fahy and Lloyd, 1983), which are a very significant group of pathogens and attack a large number of plant species (Sands *et al.*, 1980). Specificity is also shown within pathovars which may consist of distinct races that are cultivar-specific. Existence of nine races of P. syringae pv. glycinea on soybean (Crosse *et al.*, 1966; Thomas and Leary, 1980; Fett and Sequeira, 1981); six races of P. syringae pv. pisi on pea (Taylor *et al.*, 1989); and at least three races of P. syringae pv. phaseolicola on bean (Walker and Patel, 1964; Taylor *et al.*, 1987) have been reported.

Bacterial blight of peas is a seed-borne disease which has been reported in most of the pea-growing areas of the world. P. syringae pv. pisi is the organism generally accepted as its causal agent. It has been said that the disease causes significant losses when environmental conditions favour its development (Lawyer, 1984), and particularly troublesome under cool wet conditions (Smith and Close, 1966; Young *et al.*, 1969). It is known that weather conditions are critical to the expression of the

disease and even to the type of symptom induced (Sackett, 1916; Harrison, 1964; Boelema, 1967; Watson and Dye, 1971).

It has been shown, however, that P. syringae pv. syringae can cause a disease of peas similar to, or indistinguishable from, that caused by P. syringae pv. pisi (Hoitink and Hagedorn, 1966; Butler and Fenwick, 1970). Nevertheless, P. syringae pv. syringae is a less virulent pathogen; it occurs under more restricted environmental conditions and is sometimes associated with frost damage. However, it is able to cause severe disease following hail damage or under excessively wet conditions (Wimalajeewa and Nancarrow, 1984).

The two pathogens are very closely related and associated with peas. Some of the tests reported in the literature to distinguish them are utilization of homoserine (Hildebrand, 1973), inoculation of young bean pods and immature lemons (Wimalajeewa and Nancarrow, 1984), and stem inoculation of specific pea cultivars (Malik *et al.*, 1987). Other reported methods of distinguishing the two organisms are by a set of typing phages and by serology (Taylor, 1972b; Taylor and Dye, 1972).

The pea (Pisum sativum) is an inbreeding annual with numerous cultivars. The genetics of the relationship between races of P. syringae pv. pisi and pea cultivars in terms of a gene-for-gene relationship was established by Taylor *et al.* (1984). The presence of additional races in the USA was also suggested by Lawyer (1984). Currently six races of P. syringae pv. pisi can be distinguished by their differential reactions on pea cultivars (Taylor *et al.*, 1989).

Genetics studies on the pathogen, P. syringae pv. pisi, (Malik *et al.*, 1987) and on the resistance of the host (Vivian *et al.*, 1989) have also been reported. Isolation and characterization of cloned DNA conferring specific avirulence in P. syringae pv. pisi to pea cultivars which possess the resistance allele (R2), has been recently described (Vivian *et al.*, 1989).

In southern Australia frost appears to influence the incidence of bacterial blight of peas (Fahy and Lloyd, 1983) and also the identity of the pathogens isolated,

whether P. syringae pv. syringae or P. syringae pv. pisi (S.C. Wimalajeewa, personal communication).

Frost damage

Frost injury occurs as a result of physical stress (low temperature) to frost-sensitive agricultural plants. This abiotic disease has been described as one of the main limiting factors to crop production in many locations in the temperate zone (Cary and Mayland, 1970; Chandler, 1958). This is because unlike frost-hardy plants, frost-sensitive plants have no ability (significant mechanism) to tolerate ice formation within their tissues (Burke *et al.*, 1976; Levitt, 1972; Mazur, 1969; Olien, 1967).

Some liquids such as water can be supercooled to several degrees C below the melting point of the solid phase and freeze only by the addition of a suitable catalyst for transition of the liquid to the solid phase (Bigg, 1953). These catalysts which change the water to the ice phase are known as ice nuclei. At low temperatures the catalysts are called homologous ice nuclei whereas those at warmer temperatures are known as heterologous ice nuclei (Lindow, 1983b).

Damage to frost-sensitive plants under natural conditions usually occurs between -2 and -5^{o}C (Modlibowska, 1962). Many plant species can be supercooled to temperatures below -5^{o}C and often below -10^{o}C without inducing ice formation in their tissues and thus becoming frost-injured (Kaku, 1966, 1973; Lucas, 1954; Marcellos & Single, 1976).

The presence of an interaction between certain leaf surface bacteria and occurrence of frost damage to some plants has been reported. This damage which is not because of low temperature stress, usually occurs at temperatures of -2 to -5^{o}C by initiating damaging ice crystal formation within the plant tissues and its spread inter-and intracellularly throughout the plants causes the frost damage (Anderson *et al.*, 1982, 1984; Arny *et al.*, 1976; Lindow *et al.*, 1978 a & c, 1982 a & b; Weaver, 1978; and Yankofsky *et al.*, 1981b).

Certain epiphytic bacteria broadly distributed on plants, are active in ice nucleation. They prevent supercoolling of plants. It has been shown by Arny *et al.* (1976) that seedling corn plants lacking certain bacteria were not damaged above -8°C, whereas plants with leaf surface populations of these bacteria were damaged at -3.5 to -4°C. Moreover, the amount of injury at -4°C was directly proportional to the logarithm of the population of an ice nucleation active strain of bacterium present on corn leaves (Lindow *et al.*, 1978b, 1982b).

The most common nonbiological sources of heterologous ice nuclei are mineral particles, particularly certain mineral clays. Silver iodide is an efficient ice nucleator but its abundance in nature is very low. These are active in ice nucleation only at temperatures lower than -8°C to -15°C (Mason and Hallet, 1957; Mason and Maybank, 1958; Schnell and Vali, 1972; Vonnegut, 1949; Zettlemoyer *et al.*, 1961).

When grown under greenhouse condition, ice nucleation activity in plants appears to be very rare at temperatures above -5°C (Arny *et al.*, 1976; Lindow et al., 1978a, 1982a). Significant ice nucleation activity is observed on plants grown under greenhouse condition only at temperatures lower than -8°C to -10°C (Kaku, 1964, 1975; Marcellos and Single, 1976, 1979).

Bacterial ice nuclei

Atmospheric ice nuclei were assumed to be the sources of ice nuclei on plants. It was observed that the concentration of ice nuclei in the atmosphere at a given location increases with increasing organic matter content of the soil at that location (Vali, 1968, 1971). One source of abundant ice nuclei is decaying vegetation (Schnell and Vali, 1976; Vali and Stansbury, 1966) and it was shown that the bacterium Pseudomonas syringae, associated with decaying leaf material is active in ice nucleation (Maki *et al.*, 1974).

Ice nucleation active (INA) strains of Pseudomonas syringae, Pseudomonas fluorescens, Pseudomonas viridiflava, Erwinia herbicola, and Xanthomonas campestris pv. translucens have been shown to promote frost damage to sensitive

crops (Hirano *et al.*, 1978; Kim *et al.*, 1987; Lindow *et al.*, 1978b; Maki *et al.*, 1974; Maki and Willoughby, 1978; Paulin and Luisetti, 1978; Yankofsky *et al.*, 1981a). Among these species, P. syringae, P. fluorescens and Erwinia herbicola are generally the most common INA bacteria on plants (Hirano *et al.*, 1982; Kim *et al.*, 1987; Lindow *et al.*, 1978b, 1982a). These bacteria are active in ice nucleation at temperatures of -2 to -5°C, but ice nucleation activity is detectable in other bacterial strains only at temperatures approaching -10°C (Hirano *et al.*, 1978; Makino, 1982; Paulin and Luisetti, 1978).

Approximately 50% of the many pathovars of P. syringae examined including P. syringae pv. syringae, P. syringae pv. pisi, P. syringae pv. lachrymans and P. syringae pv. coronafaciens are active in ice nucleation. This indicates that INA bacteria have a worldwide distribution (Hirano *et al.*, 1978; Paulin and Luisetti, 1978).

Not every cell of these INA bacteria is active as an ice nucleus at a given time. With decreasing temperatures below -1°C, the fraction of cells that have ice nucleation activity increases rapidly (Hirano, 1985; Lindow, 1982 a & b; Lindow *et al.*, 1976, 1978 b & c; Maki and Willoughby, 1978; Yankofsky *et al.*, 1981b). In addition to the temperature effect, the medium composition and temperature of growth also affect the ice nucleation efficiency of cells of INA bacteria (Lindow, 1983b; Yankofsky *et al.*, 1981 a & b). It has been shown that an INA strain of Xanthomonas campestris grown in nutrient broth medium causes more severe frost damage on spinach seedlings, compared with one grown in potato-semisynthetic broth medium; also frost damage was more intensive with 2 to 4-day old nutrient cultures compare with those from 8-day old ones (Goto *et al.*, 1988).

Ice nucleation active bacteria on plants

On many plant species, large epiphytic populations of INA bacteria are present (Hirano *et al.*, 1978; Lindemann *et al.*, 1981, 1982; Lindow, 1981; Lindow, 1982b; Lindow *et al.*, 1976, 1982b; Paulin and Luisetti, 1978; Yankofsky et al., 1981a).

The species involved appear to differ from one location to another. For example on field beans and brassica crops in Central Alberta, Canada, INA strains were identified as P. fluorescens, and neither E. herbicola nor P. syringae was detected (Kaneda, 1986). But in Queensland, Australia, on wheat, barley and other grasses, P. syringae and E. herbicola were identified as the most abundant INA organisms (Newton and Hayward, 1986). In New Zealand, P. viridiflava was an effective ice nucleation agent on the kiwifruit vine (Young, 1987). In the United States, P. syringae is reported to be the predominant INA organism (Lindow, 1982a, 1985; Lindow *et al.*, 1975).

Populations of P. syringae and E. herbicola as phytopathogenic bacteria, have been reported on many plants (Billing and Baker, 1963; Freigoun and Crosse, 1975; Gibbins, 1978; Leben, 1965). However, numbers of INA bacteria on plant surfaces vary between different plant species. Large seasonal variation of INA bacterial numbers has been observed on both annual and perennial plants. Generally, low numbers of INA bacteria are found on over-wintering plant tissues of deciduous plants or on emerging cotyledons or leaves of annual plants (Lindow, 1983b). Also, INA bacterial numbers on plant surfaces varied on a given species; large variation was observed on flowers, leaves, and fruit of healthy pear (Haefele and Lindow, 1982; Lindow, 1983b).

In vitro, the fraction of cells that are active in ice nucleation, is not the same as in vivo. For example, in a strain of P. syringae, ice nucleation activity on leaves was lower than in culture, but it increased on leaves with decreasing temperature from -1.5 to -6°C (Lindow, 1982a, 1983b).

Bacterial ice nucleation and frost injury

In the field, the presence of INA bacteria on plant surfaces, causes ice formation on and in the plants, and subsequently, frost damage at temperatures above -5°C occurs (Hendershott, 1962; Modlibowska, 1962; Proebsting *et al.*, 1982; Yelenosky, 1975; Young, 1966). In the absence of INA bacteria, many frost-

sensitive plants can supercool and avoid damaging ice formation at temperatures above -5°C (Anderson *et al.*, 1982; Arny *et al.*, 1976; Lindow *et al.*, 1978c, 1982a). Studies have shown that at least 95% ice nuclei on leaf surfaces active at -5°C or above, are of bacterial origin (Lindow, 1983b).

There is a direct relationship between the extent of frost injury and the number of INA bacteria. It has been shown that with increasing numbers of INA bacteria on the plant, frost damage increases (Lindow *et al.*, 1976, 1978 a & b, 1982b). This damage is more directly related to the numbers of cells that are active in ice nucleation than to the population of INA bacteria (Lindow, 1982 a & b, 1983b).

A relationship between INA bacteria, freeze damage, and genotype in oats has been shown by Marshall (1988). He has suggested that INA bacteria as well as nonmicrobial factors are responsible for freeze damage in oats; additionally, oat cultivars were found to differ in their resistance to INA bacteria.

Bacterial ice nucleation in plant disease

Frost injury can facilitate the infection of some plants by P. syringae (Olien and Smith, 1981; Panagopoulos and Crosse, 1964; Sabet, 1953; Süle and Seemüller, 1987). Infection by P. syringae pv. syringae in pear blossoms which supercooled to -2°C, was severe compared with inoculated unfrozen flowers (Panagopoulos and Crosse, 1964). Frost injury has also been found to aid in the development of bacterial blight of pea caused by P. syringae pv. pisi (Boelema, 1972); bacterial canker of apricot (Klement, 1974), and bacterial canker of poplar (Sabet, 1953) both caused by P. syringae pv. syringae. It has been shown that freezing and the presence of P. syringae pv. syringae were both required for development of bacterial canker of peach (Weaver, 1978; Weaver *et al.*, 1981). However, it has been reported that the majority of ice nuclei associated with peach were of nonbacterial origin, and INA bacteria were unlikely to be the primary ice nuclei associated with them (Anderson *et al.*, 1987; Ashworth *et al.*, 1985).

Infection by P. syringae often occurs after injury to a host plant. As P. syringae has the capacity to predispose plant tissues to ice damage, it also allows subsequent bacterial penetration and disease development (Lindow, 1983b).

Molecular basis of ice nucleation

It has been shown that ice nucleation activity is not detected in extracellular byproduct of INA bacteria; it is associated with the outer membranes of these INA bacteria (Lindow *et al.*, 1989; Maki and Willoughby, 1978; Maki *et al.*, 1974; Sprang and Lindow, 1981).

Work has shown that a protein is responsible for ice nucleation activity (Kozloff et al., 1983; Lindow, 1983b, Sprang and Lindow, 1981). However, some evidence from the study of antifreezing glycoproteins suggests that more than one substance, such as protein and carbohydrate or protein and lipid, may be required for ice nucleation (Feeney and Yeh, 1978). Morever, some studies have shown that ice nuclei appear to be complex structures comprising phospholipids (Govindarajan and Lindow, 1988a; Kozloff *et al*, 1983; Wolber *et al.*, 1986).

Several ice nucleating bacterial strains, including E. herbicola, P. syringae, and P. fluorescens isolates have been examined for their ability to shed ice nuclei into the growth medium (Phelps *et al.*, 1986). Only E. herbicola isolates shed cell-free ice nuclei active at -2 to -10^{o}C. It will shed ice nuclei into the growth medium when grown at 15^{o}C. These cell-free nuclei exhibited a freezing spectrum similar to that of ice nuclei found on whole cells, both above and below -5^{o}C. Partially purified cell-free nuclei were examined by density gradient centrifugation, chemical and enzymatic probes, and electron microscopy. Ice nucleating activity in these cell-free preparations was associated with outer membrane vesicles shed by cells and was sensitive to protein-modifying reagents (Phelps *et al.*, 1986). Recently, the preparation of relatively good yields of active cell-free ice nuclei from P. syringae has been reported (Pooley and Brown, 1990).

Genes encoding the INA phenotype from two different P. syringae strains (INA"Z" and INA"C") (Green and Warren, 1985; Orser *et al.*, 1985), P. fluorescens (INA"W") (Warren et al., 1986), and E. herbicola (Orser *et al.*, 1985) were cloned as a single restriction fragment in the bacterium Escherichia coli. Cloned genes responsible for ice nucleation activity expressed similar ice nucleation activity in E coli, which is normally INA$^-$, both quantitatively and qualitatively to that in the original source strains (Corotto *et al.*, 1986; Orser *et al.*,1983, 1985).

A single DNA fragment of approximately 4500 base pairs from P. syringae; of 5700 base pairs from E. herbicola and P. fluorescens, imparted ice nucleation activity to E. coli (Warren, 1987; Warren and Wolber, 1987; Corotto *et al.*, 1986). Furthermore, it has been shown that cloned DNA sequences with the ability of conferring ice nucleation can complement ice nucleation deficient mutants (Lindow and Staskawicz, 1981; Lindow, 1983b).

Two DNA fragments conferring ice nucleation activity from Pseudomonas have been sequenced. Each fragment contained a single gene (Green and Warren, 1985). The INA"Z" gene from P. syringae was similar to the INA"W" from P. fluorescens (Warren and Wolber, 1987). Orser *et al.* (1985) have shown that another INA gene from P. syringae has homology to the INA-conferring region from E. herbicola.

Attempts at cell fractionation have resulted in loss of at least 99.9% of the active nuclei (Maki *et al.*, 1974; Yankofsky *et al.*, 1981a; Lindow *et al.*, 1989). The ice nuclei active at -5^{o}C and above have molecular masses of up to 19000kD (Govindarajan and Lindow, 1988b), equivalent to more than fifty individual INA"Z" protein molecules.

Warren and Wolber (1987) have demonstrated a relationship between the amount of INA protein synthesized by a bacterial strain, and the frequencies of ice nucleation activity. Both in Pseudomonas and E. coli, the activity increased with increasing the number of cloned INA genes per cell.

Derivatives of the transposon Tn3 that allow an ice nucleation gene (INA"Z") to be used as reporter of the transcriptional activity have been constructed (Lindgren *et*

al., 1989). The INA"Z" fusion system provides a convenient tool for the study of bacterial gene expression. Also, detection of bacteria by transduction of ice nucleation genes has been demonstrated (Wolber and Green, 1990).

Plasmids in P. syringae

Strains of Pseudomonas spp. may harbour extrachromosomal DNA in the form of plasmids (Chakrabarty, 1976). It has been shown that many pathovars of P. syringae contain single or multiple plasmids (Bender and Cooksey, 1986; Comai and Kosuge, 1980; Curiale and Mills, 1977, 1983; Currier and Morgan, 1983; Gantotti et al., 1979; Gonzalez and Vidar, 1979 a & b, 1980; King, 1989; Panopoulous and Peet, 1985; Panopoulous *et al.*, 1978; Piwowarski and Shaw, 1982; Quant and Mills, 1984; Sato, *et al.*, 1982). Most of these plasmids are phenotypically cryptic. However, an association between the presence of plasmid and pathogenicity or resistance to toxin metal ions, in some pathovars of P. syringae has been reported.

a) Plasmid and pathogenicity

It has been demonstrated that the production of the phytohormone indoleacetic acid (IAA), a compound essential for gall development in the oleander host following inoculation with P. syringae subsp. savastanoi, is encoded by a plasmid; but in olive isolates the genes encoding the IAA biosynthesis enzyme are located on the chromosome (Comai and Kosuge, 1980, 1982; Comai *et al.*, 1982; Palm *et al.*, 1989).

Moreover, it is known that production of the phytotoxin coronatine in P. syringae pv. atropurpurea (Sato *et al.*, 1983), P. syringae pv. glycinea (Willis and Leary, 1984), and P. syringae pv. tomato (Bender *et al.*, 1989) is determined by plasmids.

Also, the involvement of plasmid DNA in the production of phytotoxins syringomycin in P. syringae (Gonzalez and Vidaver, 1979 a & b, 1980) and

phaseolotoxin in P. syringae pv. phaseolicola (Gantotti *et al.*, 1979) has been reported. However, further work casts doubt on the involvement of the plasmid in the pathogenicity or syringomycin production of P. syringae. Gonzalez *et al.* (1980, 1984) have determined that plasmid-free derivative strains still produced the phytotoxin syringomycin. Also, Currier and Morgan (1983) have shown that although all strains examined which contained plasmids, were able to produce the toxin syringomycin, plasmidless strains exist which are also able to produce syringomycin or an unidentified toxin. Moreover, they showed that when a syringomycin-producing strain was cured of its plasmid, toxin production or pathogenicity were unaffected. There are conflicting reports, also, for phaseolotoxin production by P. syringae pv. phaseolicola. Jamiesen *et al.* (1981) have shown that there was no correlation between phaseolotoxin production and the presence of plasmid in twelve P. syringae pv. phaseolicola strains examined.

Moreover, by transposon mutagenesis, it has been found that chromosomal rather than plasmid genes encode syringotoxin and phaseolotoxin synthesis (Morgan and Chatterjee, 1985; Peet *et al.*, 1986; Quigley *et al.*, 1985).

b) Conjugative plasmid and toxic metal ions resistance

Conjugative plasmids have been identified in some of the pathovars of P. syringae. pBPW1 is a conjugative plasmid isolated from P. syringae pv. tabaci BR2 (Staskawicz *et al.*, 1984), and similarly pCG131 which was isolated from P. syringae pv. syringae (Gonzalez *et al.*, 1984). Both of these plasmids are phenotypically cryptic but an association between the presence of a conjugative plasmid and streptomycin resistance in P. syringae pv. papulans (Burr *et al.*, 1988) and copper resistance in P. syringae pv. tomato (Bender and Cooksey, 1986) has been reported. Also, Sundin *et al.* (1989) have shown that copper resistance in P. syringae pv. syringae recovered from blossoms from cherry orchards is plasmid-borne, and the plasmid was conjugative to three copper-sensitive recipient strains of P. syringae pv. syringae isolated from the orchards. However, repeated

attempts to transfer copper resistance into copper sensitive P. syringae pv. morsprunorum isolated from blossom samples were unsuccessful.

Also, plasmid-determined resistance to toxic metal ions for many bacterial species has been demonstrated (Foster, 1983; Summers and Silver, 1978).

Plasmid labelling for transfer

Most P. syringae plasmids lack a selectable marker. They must be labelled with a drug resistance transposon to enable plasmid transfer to be detected. The construction of Tn5-Mob, a transposon that carries the RP4-specific Mob site has been described by Simon (1984). This transposon allows in Gram-negative strains, the labelling of any cryptic plasmid with a selectable antibiotic resistance marker as well as its high frequency conjugative transfer. Thus, Tn5-Mob is a useful system to facilitate the study of plasmid properties. Moreover, with the same procedure, chromosomal DNA may also be mobilized (Simon, 1984). Use of transposons for labelling of plasmids in P. syringae has been reported (Bender and Cooksey, 1986; Gonzalez *et al.*, 1984).

Scope of this project

The distribution and abundance of INA bacteria in South Australia have not been previously reported. For these reasons one objective of this project was to determine the distribution and seasonal population changes of INA bacteria. This is described in section A. 2 of this thesis.

A second objective of this project was to investigate the interference of various cryoprotectants (chemical agents which protect plants from frost injury) with INA strains of bacteria that were isolated. This is described in section A. 3.

Strains of Pseudomonas syringae isolated from a pea field in South Australia (this study, section A. 2), were active in ice nucleation. The identity of the pathovar(s) involved in ice nucleation is important but the solution of an even more important pathological problem depends on the infra sub-specific identity of strains

of P. syringae in South Australia. As it was found difficult to distinguish P. syringae pv. syringae from P. syringae pv. pisi, the methods currently used to distinguish them were tested and evaluated (section B. 1). Immunological techniques were employed to develop a much more rapid and convenient test to differentiate these two pathovars (section B. 2).

As serology could not distinguish pathogenic and non-pathogenic strains, the plasmid complement of both pathogenic and non-pathogenic strains of the two pathovars was examined; this was followed by a study on plasmid transfer. The possibility that genes encoding pathogenicity are plasmid located, was also investigated (section C).

Section A: Ice nucleation active bacteria

A. 1 Materials and methods

a) Leaf sample collection

Leaf samples were collected every two weeks during the growing season (July to October 1986) from a pea field at Waite Agricultural Research Institute. At each sampling date ten leaf samples were taken at random, each sample consisting of one whole leaf from five adjacent plants.

b) Detection of ice nucleation activity

Leaf samples were weighed, cut into pieces about 5 cm^2 and placed in Erlenmeyer flasks containing washing buffer [sterile potassium phosphate buffer; 0.1M, pH 7.0, supplemented with 0.1% (w/v) peptone; (Lindemann *et al.*, 1984)]. Approximately 10 ml buffer per each gram of leaf tissue was used. Leaf pieces were washed for 2 h on a rotary shaker (250 rpm) at 22°C. Leaf washings were centrifuged at 2000 g for 10 min and the pellet suspended in 1 ml sterile buffer (potassium phosphate buffer, 0.01M,pH 7.0)(Gross *et al.*, 1983); 0.5 ml was placed in a constant temperature bath at -5°C (Fig 1). After 30 min, results were recorded as positive for a frozen suspension or negative for an unfrozen suspension (Fig 2).

c) Determination of numbers of INA bacteria

Centrifuged leaf washings were serially diluted in the sterile buffer and 0.1 ml and 0.01 ml aliquots plated on nutrient agar containing 2.5% (v/v) glycerol (NGA) (Lindow *et al.*, 1978b). Cycloheximide (100mg per liter) was added to prevent fungal growth.

To detect E. herbicola , dilutions were also plated on nutrient agar plus 5% NaCl, a medium selective for E. herbicola (S.E. Lindow, personal communication). After 3 days growth at 24°C, individual colonies were tested for ice nucleating activity by a test tube technique (Gross *et al.*, 1983). Individual colonies were

Figure 1. A HETOFRIG cooling bath type CB7.
Temperature range: approximately -20°C to 100°C. The bath was equipped with a circulation pump and thermostat to regulate the temperature.

Figure 2. Detection of ice nucleating activity.
Half ml suspension of pelleted pea leaf washings was placed in a test tube and kept for 30 min in a constant temperature bath at -5°C. Frozen and unfrozen suspensions are shown as (+) and (-) respectively.

Figure 3. Determination of numbers of ice nucleation active bacteria by test tube technique.
Serially diluted pea leaf washings were plated and individual colonies suspended in 0.5 ml sterile buffer in a test tube and placed in a constant temperature bath at -5°C. Results were recorded as (+) for a frozen suspension and (-) for an unfrozen suspension.

Fig 1

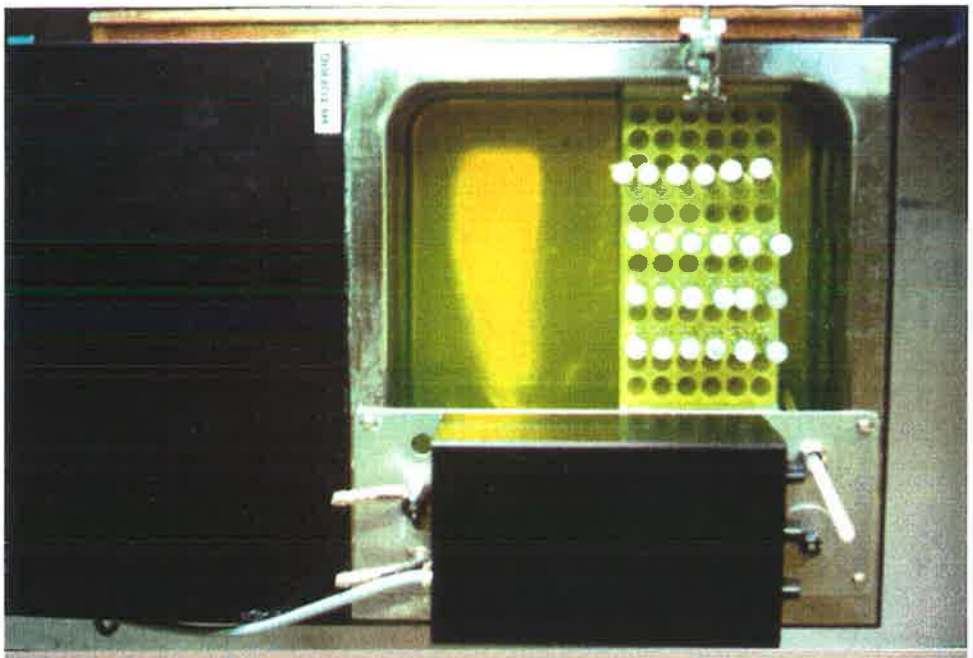


Fig 2

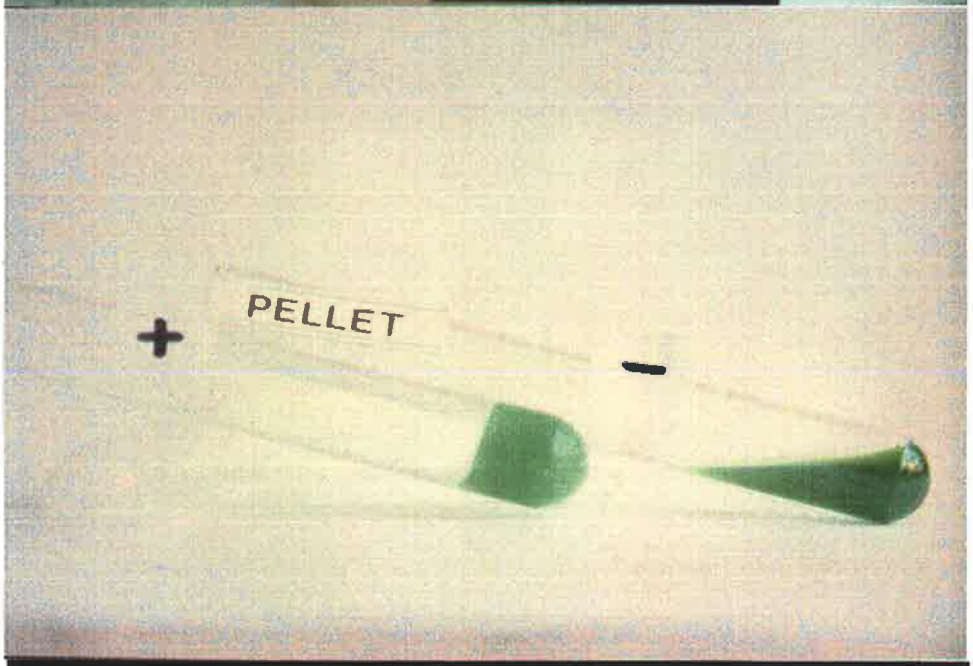
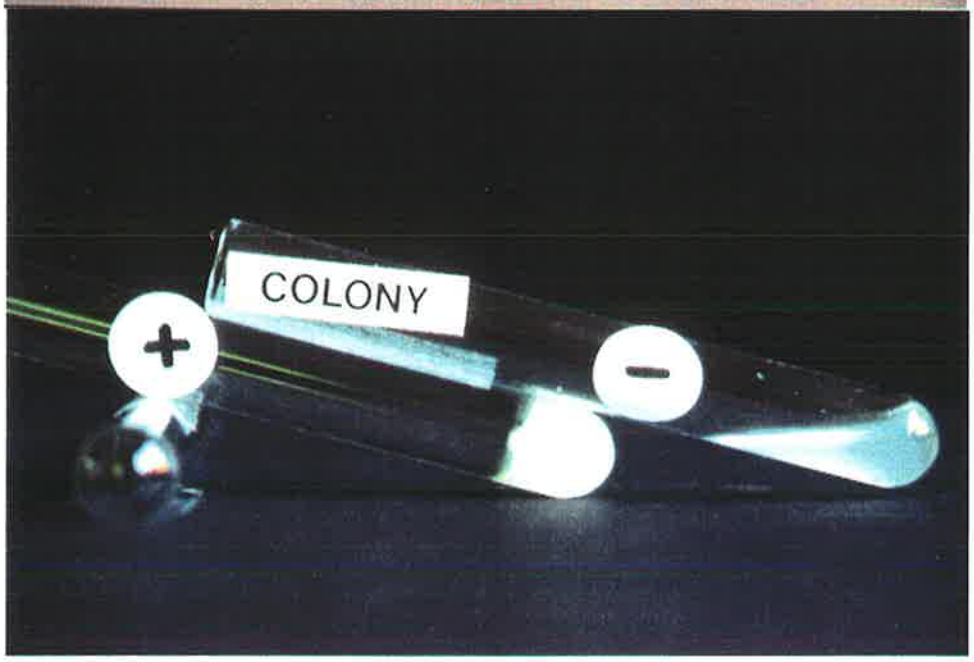


Fig 3



removed from agar in petri dishes and suspended in 0.5 ml sterile buffer and put in a constant temperature bath at -5°C for 30 min. Results were recorded as positive for a frozen suspension or negative for an unfrozen suspension in test tube (Fig 3). Also numbers were tested for ice nucleating activity by an aluminium foil technique (Lindow *et al.*, 1978a). The surface was prepared by spraying aluminium foil with a 1% solution of paraffin in xylene; the xylene was removed at 55°C in an oven, and the foil folded into a flat-bottomed "boat", which was floated on a constant temperature bath at -5°C. Discrete 3 days old colonies from agar plates were removed with a tooth pick and suspended in 0.1 ml of sterile distilled water to yield a turbid suspension (higher than 10^8 cells/ml). Five 10 µl droplets of suspension from each colony were placed on the -5°C aluminium foil test surface. A colony was considered to contain nuclei active at -5°C if one or more of the 5 droplets froze within 30 sec (Fig 4).

d) Identification of INA bacteria

Frozen individual colonies were streaked on King's B medium (King *et al.*, 1954), and individual colonies obtained from this medium subjected to the following tests according to Fahy and Lloyd (1983) and Lelliott *et al.* (1966).

1 - Production of fluorescence on King's B medium.

2 - Levan production.

3 - Oxidase test.

4 - Arginine dihydrolase.

5 - Tobacco hypersensitivity.

6 - Nitrate reductase.

7 - Gelatin hydrolysis.

8 - Utilization of sucrose, trehalose and homoserine as a sole source of carbon.

e) Greenhouse experiments

For all greenhouse experiments, randomized complete block designs with 4 replicates were used (except where otherwise indicated). Each plot consisted of one pea seedling in one pot. On the first day, all pea plants (except for the controls) were sprayed with a fresh suspension of INA bacteria. The fresh suspension was obtained from 2 days growth on nutrient glycerol agar (NGA) medium at 24°C. The bacterial suspension was adjusted to 0.7 optical density at 600 nm, which is approximately equal to 10^8 cells/ml.

On the second day, plants were sprayed with chemicals. On the third day, they were tested for ice nucleation activity by:

i) Tube nucleation test (Hirano *et al.*, 1985; Ashworth and Davis, 1984)

ii) Conductivity meter (Ketchie *et al*, 1972).

i) Tube nucleation test

Five ml of sterile distilled water were placed in a test tube and tested for the absence of heterogeneous ice nuclei active at -10°C for 60 minutes. At the end of this period the tubes were shaken vigorously. All the tubes in which the water had frozen were discarded and the others were allowed to equilibrate to ambient temperature (about 24°C) before use in the tube nucleation test. Three leaves (one from the top, one from the middle and one from near the base of the plant) were taken from each plant and were placed in a test tube prepared as described above. For each set of test leaves, sterile forceps were used to completely submerge the leaves in the sterile distilled water in each test tube. The test tubes were put in the bath at -5°C for 30 min and the number of tubes in which the water had frozen was recorded as positive, or as negative for unfrozen water (Fig 5).

ii) Conductivity meter

From each pea plant , two young leaves from the top of the plant were taken, one for a frozen sample and one for an unfrozen sample. The leaves were weighed,

Figure 4. Determination of numbers of ice nucleation active bacteria by aluminium foil technique.

Left) 10 µl droplets from suspension of individual colonies were placed on a paraffin coated aluminium foil "boat" floating on a constant temperature bath at -5°C. A colony was considered to contain active nuclei if one or more than one droplet froze within 30 sec.

Figure 5. Detection of ice nucleating activity by tube nucleation test.

Three pea leaves were placed in a test tube containing 5 ml sterile distilled water and kept for 30 min in a constant temperature bath at -5°C. Results were recorded as (+) and (-) for frozen and unfrozen water respectively.

Fig 4

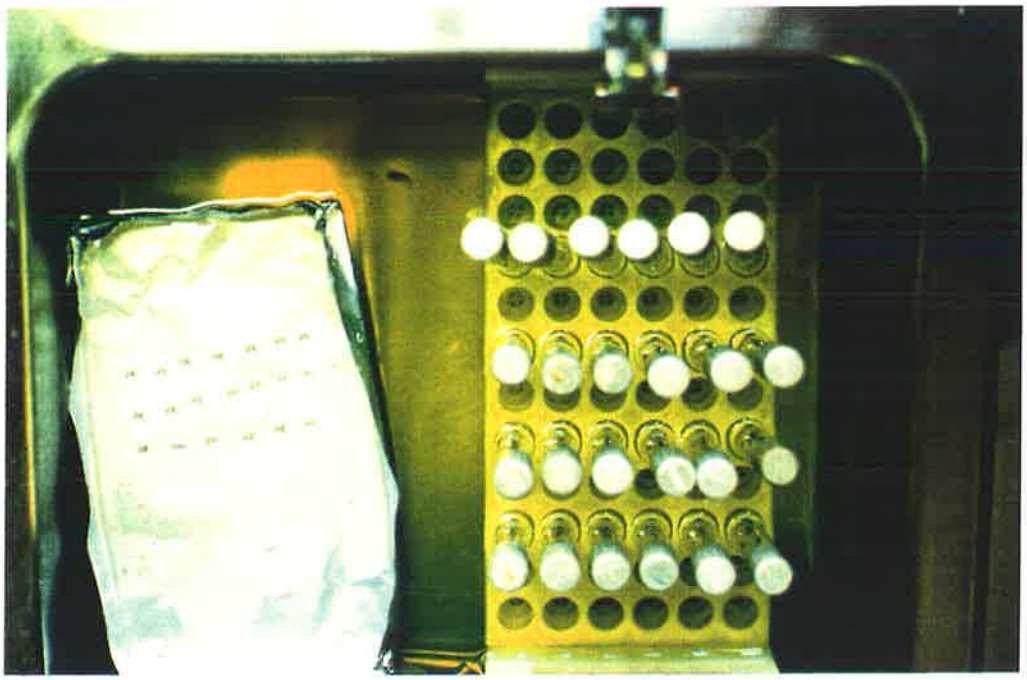
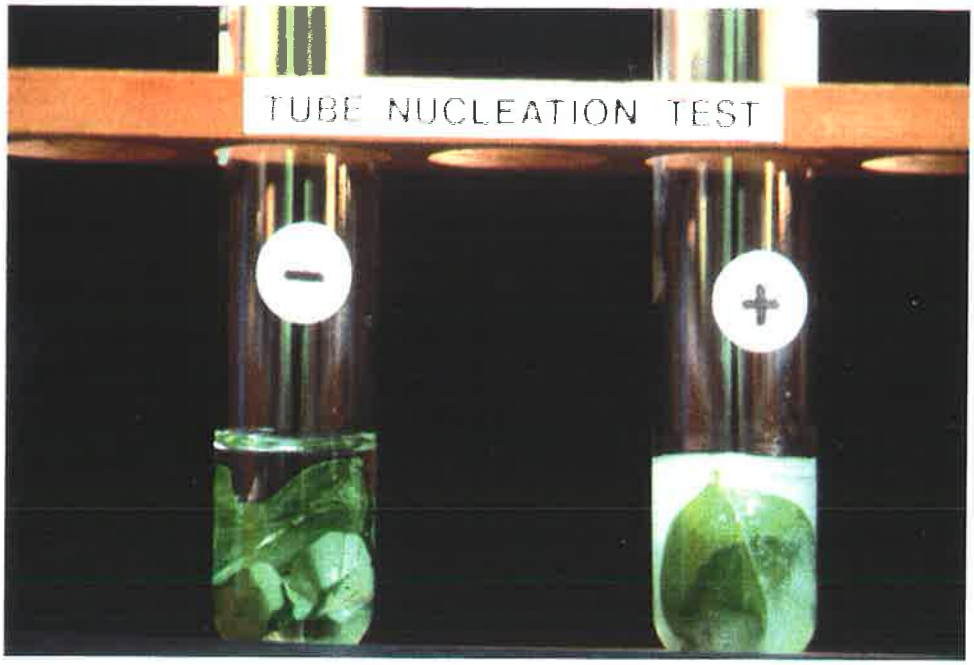


Fig 5



then, for the frozen samples, put in a test tube without liquid and the test tubes placed in a bath (-5°C) for 30 min. After 1 h thawing at room temperature, 1 ml deionized water was added per 0.01 g of tissue for all frozen and unfrozen samples. The tubes were allowed to stand at room temperature for 24 h. Conductance of solutions from both frozen and unfrozen samples was read by conductivity meter. All samples were then boiled for 3 min, immediately made to original volume with deionized water, and allowed to stand at room temperature for 24 h. Conductance of the boiled samples was read by conductivity meter.

From these measurements an index of injury for each treatment was calculated by the following formula according to Flint *et al.* (1967).

$$It = 100 [Lc - Lo] / [Lk - Lo]$$

It = Index of injury resulting from exposure to temperature -5°C.

Lc = Conductance of solution from frozen sample (at -5°C).

Lo = Conductance of solution from unfrozen sample.

Lk = Conductance of solution from frozen sample at -5°C and then heat killed.

f) Laboratory experiments

For testing the effect of chemicals on INA bacteria in laboratory, a fresh suspension of INA bacteria was obtained from a 2 days old culture on NGA medium at 24°C.

For the first group of chemicals a fresh suspension of INA bacteria was adjusted to optical densities at 600nm ($O.D_{600}$) of 0.4, 0.6 and 0.7. They were then mixed with known concentrations of the chemicals in a test tube. After 1 h at room temperature, the test tubes were put in the constant temperature bath at -5°C for 30 min. Results were recorded as positive for frozen liquid or negative for unfrozen liquid.

For other groups of chemicals a fresh suspension of INA bacteria was adjusted to an $O.D_{600}$ of 0.7 and a ten-fold dilution series prepared. Then a known

concentration of chemicals was added to each dilution. The serial dilutions were incubated at room temperature for 1 h and then placed in the bath (-5°C). After 30 min, results were recorded as positive for frozen liquid or negative for unfrozen liquid.

A. 2 Identification and seasonal population changes of INA bacteria.

a) Detection of ice nucleation activity

Leaf samples were collected every two weeks during the growing season from a pea field at the Waite Agricultural Research Institute and detection of INA bacteria attempted by exposing pelleted leaf washings to -5°C. Results are shown in Table 1.

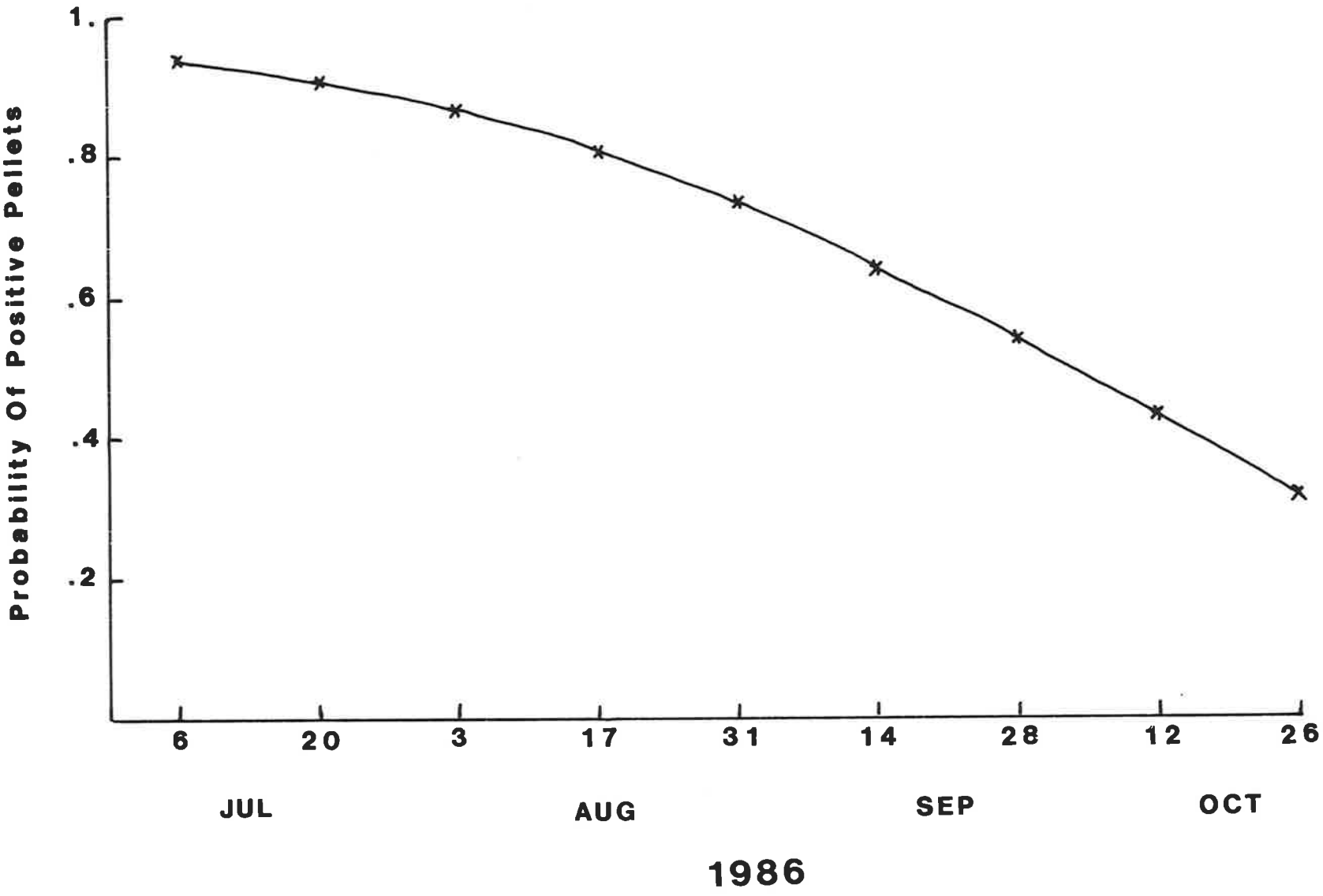
Table 1. Detection of ice nucleating activity in washings from pea leaves.

Sampling date 1986	**Number of positive pellets (out of ten)**
6th July	9
20th July	10
3rd August	8
17th August	8
31st August	8
14th September	7
28th September	5
12th October	3
26th October	4

The number of positive pellets from 10 samples at each sampling date from July 6 to October 26 decreased with time (Table 1). Assuming a binomial distribution, these data were used to calculate the probability of detecting INA bacteria over the sampling period (Fig 6). Probability decreased with time and also with increasing crop maturity.

Figure 6. Probability of detecting ice nucleating activity in pelleted pea leaf washings.

Pelleted pea leaf washings were exposed to -5°C. Probability of positive (frozen) pellets from 10 samples at each sampling date during the growing season was calculated.


Probability Of Positive Pellets
1.
.8
.6
.4
.2
6
20
3
17
31
14
28
12
26
JUL
AUG
SEP
OCT
1986

b) Numbers of INA bacteria

Numbers of INA bacteria were counted (Table 2). As might be expected numbers also decreased with time (Fig 7), although there was a secondary peak on September 14 which coincided with the onset of flowering. This may or may not be relevant. There was no influence of rainfall. But there was some effect of temperature. With decreasing temperature, numbers increased and with increasing temperature numbers decreased to an undetectable level on 12th and 28th October (Fig 7).

c) Identification of INA bacteria

All INA bacteria were identified as either P. syringae or P. fluorescens biotype 1 (Table 3).

Although a selective medium for isolation of E. herbicola was used, no INA strain of E. herbicola was detected. It was either absent or its presence was at a very low level (undetectable level).

Although no quantitative data are available, a clear impression was obtained that P. fluorescens was the predominant INA organism in that pea field. At all sampling dates, except on 28th September, P. fluorescens was isolated, whereas P. syringae was not detected on several dates (Table 4). Also at the time when both of them were detected the majority of isolates belonged to P. fluorescens.

As heavy frosts were not experienced and as no bacterial blight was detected, the influence of INA bacteria on crop damage by these two agencies could not be determined.

Table 2. Numbers of ice nucleation active (INA) bacteria per gram fresh weight of pea leaves (mean of 10 samples).

Sampling date (1986)	**No. of INA bacteria/g (mean of ten samples)**
6th July	3.9×10^2
29th July	7.5×10^2
3th August	2.2×10^2
17th August	1.6×10^2
31th August	1.3×10^2
14th September	5.4×10^2
28th September	1.6×10^2
12th October	Not detectable
26th October	Not detectable

Figure 7. Number of ice nucleation active bacteria detected in washings from pea leaves, July to October, along with data on rainfall and temperature.

A) Each point represents a log mean cells/g fresh weight of ten replicates. Bacterial data were transformed by ln(x+1). Vertical bars indicate the standard error. B) Data on rainfall (mm) per day. C) Average air temperature per week.

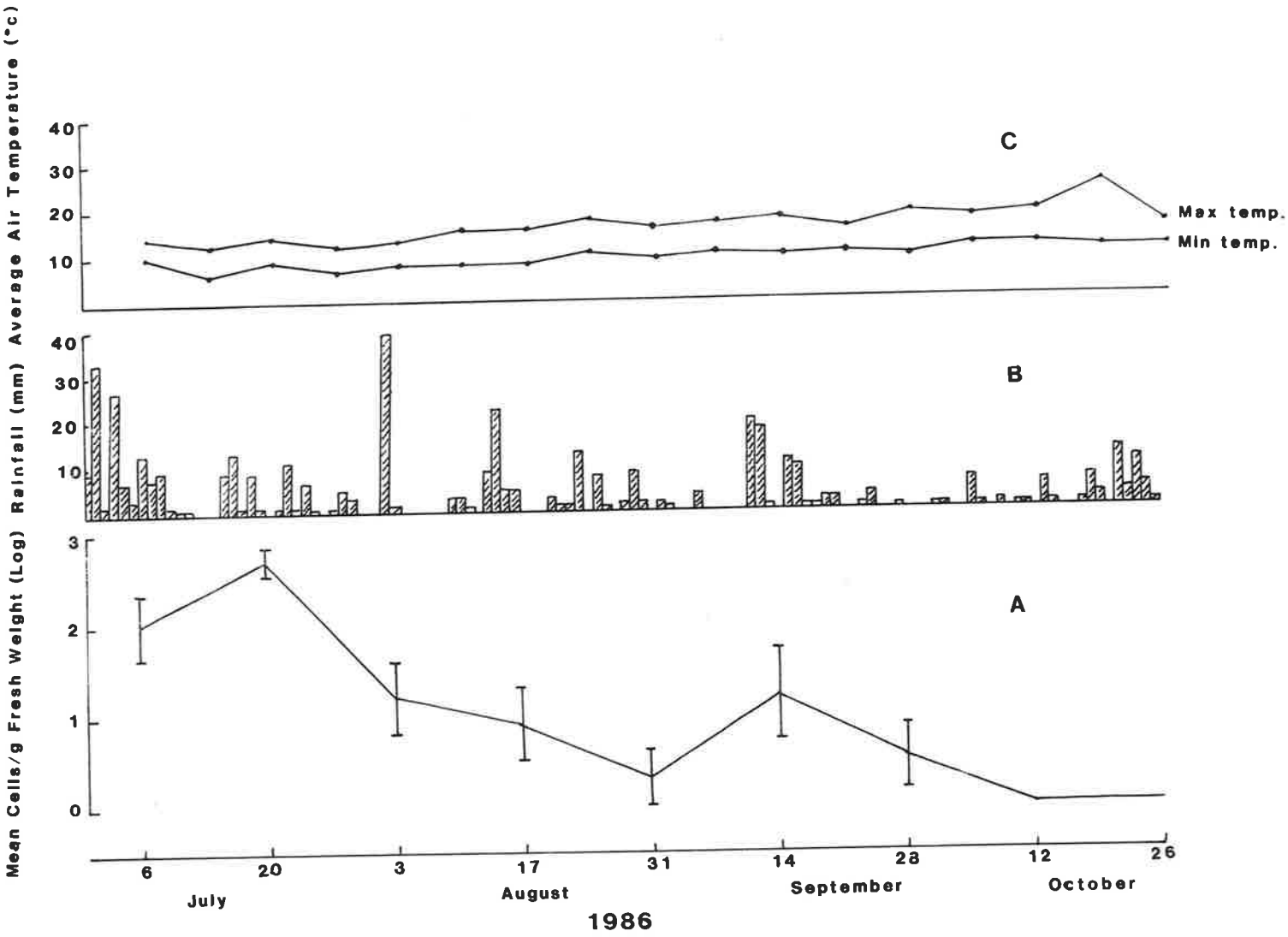

Mean Cells/g Fresh Weight (Log) Rainfall (mm) Average Air Temperature (°c)
C
Max temp.
Min temp.
B
A
40
30
20
10
3
2
1
0
6
20
3
17
31
14
28
12
26
July
August
September
October
1986

Table 3. Characteristics of ice nucleation active bacteria obtained from pea leaves.

Test	*P. syringae*	*P. fluorescens* biotype 1
Fluorescent on King's B medium	+	+
Levan production	+	+
Oxidase	-	+
Arginine dihydrolase	-	+
Tobacco hypersensitivity	+	-
Nitrate reductase	-	-
Gelatin hydrolysis	-	+
Utilization of:		
Sucrose	+	+
Trehalose	-	+

Table 4. Detection of ice nucleating activity in washings from pea leaves and presence of ice nucleation active strains of P. syringae and P. fluorescens biotype 1.

Sampling date (1986)	No. of positive pellets (out of 10)	P. syringae	P. fluorescens biotype 1
6th July	9	-	+
20th July	10	+	+
3rd August	8	+	+
17th August	8	+	+
31st August	8	+	+
14th September	7	-	+
28th September	5	+	-
12th October	3	-	+
26th October	4	-	+

A. 3 Cryoprotectants

a) Introduction

Cryoprotectants are chemical agents which protect plants from frost injury. The effectiveness of some chemicals as cryoprotectants (e.g. some teric and tween chemicals) has been shown on some plants (blackcurrants) in the field (Wilson and Jones, 1983a). It has been observed that the use of cryoprotectants reduces leaf water potential and reduction of leaf water potential increases resistance of plants to frost injury (Wilson and Jones, 1983b). Also the effect of some other chemicals such as glycerol, polyvinyl pyrrolidone (PVP 40), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) have been checked as cryoprotectants on apple and pear trees (Ketchie and Murren, 1976). They observed that use of the chemicals (cryoprotectants) increased cold resistance of plants.

However, cryoprotectants may also work by preventing ice crystal formation in plant tissues by an effect on INA bacteria. As pointed out by Lindow (1983a), several chemicals may reduce ice crystal formation by inactivating bacterial ice nuclei. The chemicals included heavy metal cations and certain cationic detergents. The effect of these and other possible cryoprotectants on INA strains of Pseudomonas syringae and Pseudomonas fluorescens biotype 1 that were isolated from the pea field (section A. 2) were checked in the laboratory as well as in the greenhouse.

b) First group of cryoprotectants

The first group of cryoprotectants to be tested were Tween 60, 80, and 85 (Sigma); Teric PE62, PE64, and 12A23B (ICI); Glycerol (BDH); Ethylene Glycol, EG, (Sigma); Polyvinyl Pyrrolidone-360, PVP, (Sigma); Dimethyl sulfoxide, DMSO, (BDH); Ca $(NO_3)_2$ (BDH); Mannitol (BDH); and Sorbitol (Ajax).

i) Laboratory tests

For testing the effect of these chemicals in the laboratory, fresh suspensions of INA bacteria were adjusted to $O.D_{600}$ of 0.4, 0.6, and 0.7. Each was mixed in test

tubes with concentrations 0.5, 1, 2, 4, 8, 10% of six tween and teric chemicals, and concentrations 5, 8, 10, 15, 25% of glycerol, EG, PVP, DMSO, Ca $(NO_3)_2$, mannitol and sorbitol.

After 1 h, the test tubes were placed in a constant temperature bath at -5°C. After 30 min results were recorded as positive for frozen liquid or negative for unfrozen liquid. Results are shown in Table 5.

For six teric and tween chemicals all the liquids in the test tubes with three different densities of INA bacteria ($O.D_{600}$ of 0.4, 0.6, and 0.7) froze after 30 min in the bath at -5°C (Table 5). For other chemicals, concentrations of more than 10% glycerol , EG, DMSO and Ca $(NO_3)_2$ reduced ice nucleating activity (Table 5).

ii) Greenhouse tests

For testing the effects of six tween and teric chemicals in the greenhouse, one experiment was done for each chemical with concentrations 0.5, 1, 2, 4, 8, 10%. Each plot consisted of one pea plant. Pea plants were sprayed with a fresh suspension of INA bacteria on day one, and were then sprayed with the chemicals on day two; on day three they were checked for ice nucleating activity by tube nucleation test. As might be expected from the laboratory tests, the six teric and tween chemicals had no effect. All the liquids in the test tubes containing three pea leaves were frozen after 30 min at -5°C.

For testing the effect of glycerol, EG, DMSO, PVP, Ca $(NO_3)_2$, mannitol and sorbitol in the greenhouse, a concentration of 15% of all chemicals was chosen. The procedure was the same as in the previous experiment, except that 8 replicates were used in this experiment. Results of the tube nucleation test are shown in Table 6 and Fig 8.

As might be expected from the laboratory tests, PVP, sorbitol and mannitol had no effect in the greenhouse test. However, some tubes containing glycerol, EG, DMSO and $Ca(NO_3)_2$ remained unfrozen after 30 min at -5°C (Table 6 & Fig 8).

Table 5. Effect of chemical agents on ice nucleation active strains of P. syringe and P. fluorescens biotype 1* in vitro.

Chemical agents	Concentration	(INA bacteria) OD at 600 nm		
		0.4	0.6	0.7
Control		+	+	+
Tween 60	0.5-10%	+	+	+
Tween 80	"	+	+	+
Tween 85	"	+	+	+
Teric PE62	"	+	+	+
Teric PE64	"	+	+	+
Teric 12A23B	"	+	+	+
PVP	5-25%	-	+	+
Mannitol	"	-	+	+
Sorbitol	"	-	+	+
Glycerol	15-25	-	-	+
EG	"	-	-	-
DMSO	"	-	-	-
Ca $(NO_3)_2$	"	-	-	-

* = There was no difference between the effect of the chemicals on these two strains of ice nucleation active bacteria.

+ = Ice crystal formation; - = No ice crystal formation.

Table 6. Effect of chemical agents (at 15% concentration) on an ice nucleation active strain of P. syringae in planta, using the tube nucleation test.

Replicates	*	**	PVP	Sorbitol	Mannitol	Glycerol	EG	DMSO	Ca $(NO_3)_2$
1	-	+	+	+	+	+	+	+	-
2	-	+	+	+	+	+	-	+	+
3	-	+	+	+	+	+	-	-	+
4	-	+	+	+	+	+	-	-	-
5	-	+	+	+	+	+	-	-	-
6	-	+	+	+	+	-	-	+	-
7	-	+	+	+	+	+	+	-	-
8	-	+	+	+	+	-	+	+	-

* = Unsprayed pea plants.

** = Pea plants were sprayed with ice nucleation active bacteria alone.

\+ = Ice crystal formation; - = No ice crystal formation.

PVP = Polyvinyl Pyrrolidone

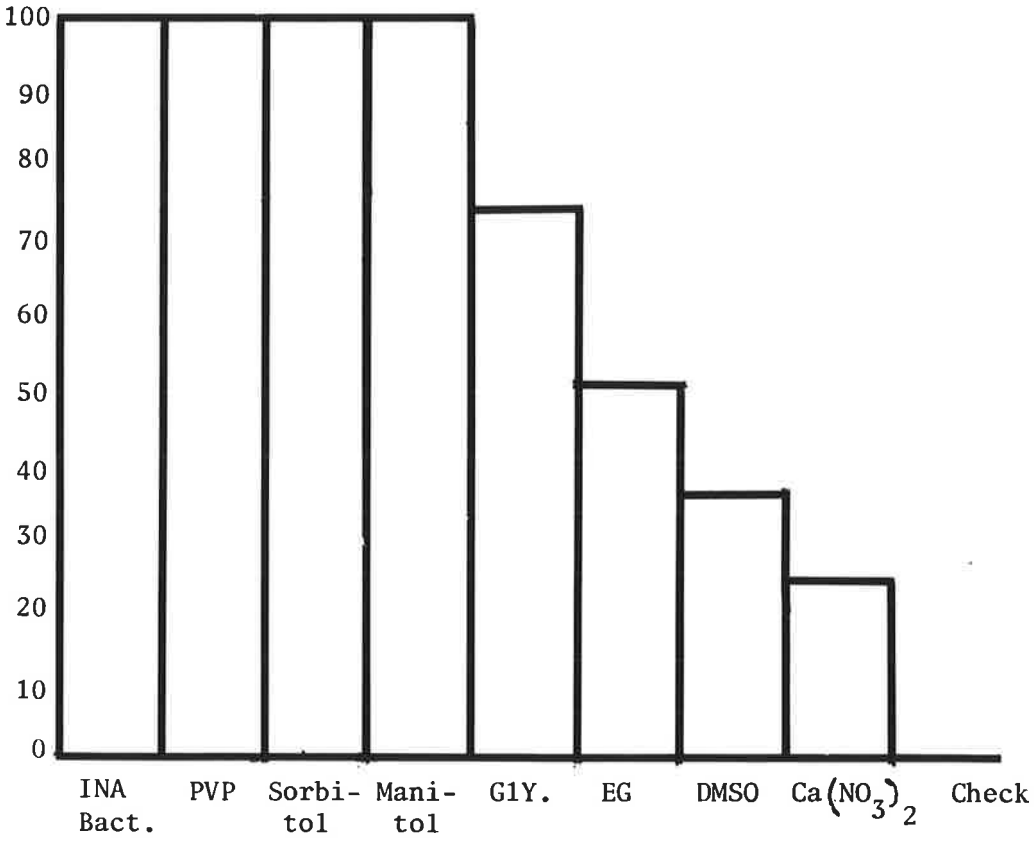
EG = Ethylene Glycol

DMSO = Dimethyl sulfoxide

Figure 8. Effect of chemicals (at 15% concentration) on an ice nucleation active strain of Pseudomonas syringae in planta, using tube nucleation test.

INA bacteria (first bar of the graph) = Pea plants were sprayed with ice nucleation active bacteria alone. Check (last bar of the graph) = Unsprayed pea plants. PVP = Polyvinyl Pyrrolidone, Gly = Glycerol, EG = Ethylene Glycol, and DMSO = Dimethyl sulfoxide.

Vertical line = Percentage of tubes frozen at -5^{o}C


100
90
80
70
60
50
40
30
20
10
0
INA Bact.
PVP
Sorbi-tol
Mani-tol
G1Y.
EG
DMSO
$Ca(NO_3)_2$
Check
Treatments

Since EG, DMSO, and glycerol produced some leaf damage on the pea plants, they were eliminated and only Ca $(NO_3)_2$ was chosen for further experiments.

c) Second group of cryoprotectants

A second group of cryoprotectants tested in this study were NH_4NO_3; $(NH_4)_2SO_4$; $MgSO_4$; $Mg(NO_3)_2$; KNO_3; $Ca(NO_3)_2$; $MnSO_4$; $ZnSO_4$; and $Al(NO_3)_3$ (BDH).

i) Laboratory tests

In this experiment a fresh suspension of INA bacteria was adjusted to OD_{600} of 0.7. A ten times dilution series was prepared. To each dilution 0.1M concentration of each chemical was added and left at room temperature for 1 h. Then the serial dilutions were placed at -5°C for 30 min. Results were recorded as positive for a frozen suspension or negative for an unfrozen suspension (Table 7).

Only $ZnSO_4$ and $Al(NO_3)_3$ prevented freezing at all dilutions (Table 7). KNO_3, $Ca(NO_3)_2$ and $MnSO_4$ prevented freezing at a bacterial dilution of 10^{-2} but not at 10^{0} and 10^{-1}. All other chemicals had no effect compared with the control. However, these data are only qualitative. More reliable information may be obtained from a quantitative assay. Also, it is possible that the chemicals influenced bacterial numbers, rather than interfering with ice nucleation. So, to obtain a quantitative assay, serial dilutions of bacteria were prepared following treatment and plated on King's B medium. After 3 days' growth at 24°C, total number of colonies was counted (Table 8).

ii) Greenhouse tests

To test the effect of these chemicals on ice nucleating activity in a greenhouse experiment, a conductivity meter rather than the tube nucleation test was used. With a conductivity meter it is possible to compare the effect of frost injury by a quantitative amount.

Table 7. Effect of various chemicals (0.1M) on cell suspensions of an ice nucleation active strain of P. syringae serially diluted, in vitro.

	Bacterial dilutions			
Treatment	**10^{0}**	**10^{-1}**	**10^{-2}**	**10^{-3} - 10^{-8}**
Control	+	+	+	-
$NH_4 NO_3$	+	+	+	-
$(NH_4)_2 SO_4$	+	+	+	-
$MgSO_4$	+	+	+	-
$Mg (NO_3)_2$	+	+	+	-
KNO_3	+	+	-	-
$Ca (NO_3)_2$	+	+	-	-
$MnSO_4$	+	+	-	-
$ZnSO_4$	-	-	-	-
$Al (NO_3)_3$	-	-	-	-

+ = Ice crystal formation; - = No ice crystal formation.

Table 8. Effect of various chemicals (0.1M) on the viability of an ice nucleation active strain of P. syringae.

Treatment	Total number of colonies
Control (no treatment)	70×10^7
$NH_4 NO_3$	70×10^7
$(NH_4)_2 SO_4$	60×10^7
$MgSO_4$	20×10^7
$Mg (NO_3)_2$	50×10^7
KNO_3	60×10^7
$Ca (NO_3)_2$	70×10^7
$MnSO_4$	40×10^7
$ZnSO_4$	13×10^3
$Al (NO_3)_3$	10×10^3

There is a close relationship between electrolytic conductance and frost injury of plants (Ketchie *et al.*, 1972). When plant cells are damaged, they release leachate. The more leachate, the higher the conductivity of the solution. This can be measured and by using suitable controls, a quantitative estimate of tissue damage can be obtained, This is known as the index of injury (Flint *et al.*, 1967).

The experiment involved the chemicals at a concentration of 0.1M, with 4 replicates per treatment. On day one, all pea plants were sprayed with a fresh suspension of INA bacteria which were adjusted to $O.D_{600}$ of 0.7. On day two, they were sprayed with the chemicals. After 24 h, each pea plant was checked for ice nucleation activity by conductivity meter. Results are shown in Table 9 and Fig 9.

To determine if different salts of the same metal behaved in the same way, the effect of $ZnSO_4$ and $Zn(NO_3)_2$ at two concentrations 0.05 and 0.1M were tested on INA bacteria both in the laboratory and the greenhouse. In the laboratory, the chemicals were tested on serial dilutions 10^0 to 10^{-8} (10^0 = $O.D_{600}$ of 0.7) of a bacterial suspension. Results were measured after 30 min at -5°C (Table 10).

There was no difference between $Zn(NO_3)_2$ and $ZnSO_4$ at either concentration of 0.1M and 0.05M (Table 10). Both prevented freezing at all bacterial dilutions. From each 10^0 dilution (after treatment), 20 10-µl droplets were placed on paraffin coated aluminium foil "boats" floating on the constant temperature bath at -5°C. After two min the number of drops that froze were recorded. In the control (INA bacteria without treatment) all 20 droplets were frozen; in the treated samples, no drops were frozen (Table 11).

All the serial dilutions (after 1h treatment) were plated on King's B medium and after 3 days' growth at 24°C total number of colonies was determined (Table 12).

The effect of the chemicals was tested in a greenhouse by the conductivity meter method. A concentration of 0.05M for $Zn(NO_3)_2$ and $ZnSO_4$ was used and there were four replicates of each treatment. Results are shown in Table 13.

Table 9. Index of Injury to plants kept at -5°C for 30 min following treatment with an ice nucleation active strain of P. syringae and various cryoprotectants (0.1M).

Replicates	*	**	$MnSO_4$	$Al(NO_3)_3$	$ZnSO_4$	$Ca(NO_3)_2$	KNO_3	$MgSO_4$	$Mg(NO_3)_2$	$(NH_4)_2SO_4$	NH_4NO_3
1	2.31	71.74	19.29	15.29	19.40	20.00	31.08	39.29	59.65	50.82	55.78
2	4.69	50.48	11.94	21.38	24.73	25.97	38.89	30.43	56.36	47.29	59.39
3	5.88	48.28	21.84	23.64	30.65	39.02	44.44	30.30	30.77	51.57	61.89
4	3.51	52.35	22.41	24.66	31.03	33.33	39.50	26.32	35.98	50.72	61.40
Mean	4.10	55.71	18.8	21.24	26.45	29.58	38.48	31.59	45.69	50.10	59.62

* = Unsprayed pea plants. (Not included in statistical analysis).

** = Pea plants were sprayed with ice nucleation active bacteria alone.

Mean values were significally different ($P \leq 0.01$).

Figure 9. Effect of various chemical (0.1M) on an ice nucleation active (INA) strain of Pseudomonas syringae in planta, using conductivity meter.

An index of injury was calculated following conductivity measurements. The histograms represent mean index of injury for four replicates. Controls consist of pea plants sprayed with INA bacteria alone (second bar of the graph) and also uninoculated plants (the last bar of the graph).

Vertical line = Index of injury.

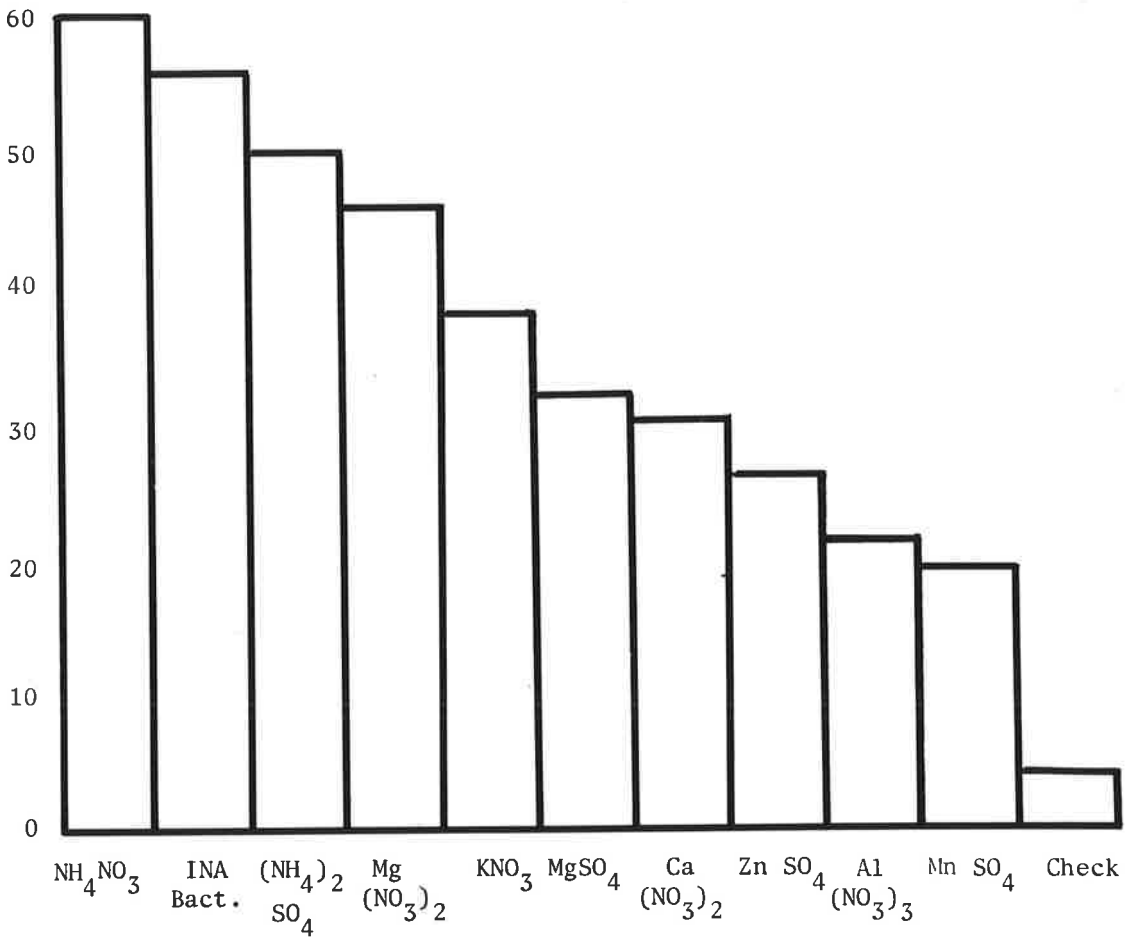

60
50
40
30
20
10
0
NH_4NO_3
INA Bact.
$(NH_4)_2SO_4$
$Mg(NO_3)_2$
KNO_3
$MgSO_4$
$Ca(NO_3)_2$
Zn SO_4
Al $(NO_3)_3$
Mn SO_4
Check
Treatments

Table 10. Effect of chemical agents on an ice nucleation active strain of P. syringae serially diluted, in vitro.

Treatment	10^{o}	10^{-1}	10^{-2}	10^{-3} - 10^{-8}
Control (no treatment)	+	+	+	-
$ZnSO_4$ - 0.1M	-	-	-	-
$Zn(NO_3)_2$ 0.1M	-	-	-	-
Control (no treatment)	+	+	+	-
$ZnSO_4$ 0.05M	-	-	-	-
$Zn(NO_3)_2$ 0.05M	-	-	-	-

+ = Ice crystal formation; - = No ice crystal formation.

Table 11. Number of frozen droplets placed on a paraffin-coated aluminium foil floating on a constant temperature bath (-5^{o}C). The droplets (10µl) were taken from a 10^{o} dilution of bacterial cells treated with 0.1M $ZnSO_4$ and Zn $(NO_3)_2$.

Treatment	**Number of positive drops (out of 20)**
Control (no treatment)	20
$ZnSO_4$	0
Zn $(NO_3)_2$	0

Table 12. Effect of chemical agents, $ZnSO_4$ and $Zn(NO_3)_2$ (0.1M), on viability of an ice nucleation active strain of P. syringae.

Treatment	Total number of colonies
Control (no treatment)	80×10^7
$ZnSO_4$	13×10^3
$Zn(NO_3)_2$	12×10^3

Table 13. Index of injury to plants kept at -5°C for 30 min following treatment with an ice nucleation active strain of P. syringae and various cryoprotectants (0.05M).

Replicates	Control*	Control**	$ZnSO_4$	$Zn(NO_3)_2$
1	2.31	59.55	38.31	28.21
2	3.69	51.94	35.36	32.86
3	8.62	53.74	32.94	39.04
4	3.03	64.09	40.33	35.00
Mean	4.41	57.33	36.74	33.78

* = Unsprayed pea plant. (Not included in statistical analysis).

** = Pea plants were sprayed with ice nucleation active bacteria alone.

Mean values were significally different ($P \leq 0.01$).

To determine the lowest effective concentration of the chemicals, Ca $(NO_3)_2$, $MnSO_4$, $ZnSO_4$, Zn $(NO_3)_2$, and Al $(NO_3)_3$ were tested in the laboratory at concentrations of 0.1M, 0.01M, 0.005M, and 0.001M for each chemical.

First a fresh suspension of INA bacteria was diluted 10^0 to 10^{-5} (10^0 suspension was adjusted to $O.D_{600}$ of 0.7) then a known concentration of each chemical was added to each bacterial dilution. After 1 h treatment at room temperature, all the bacterial dilutions were placed in a bath (-5°C) for 30 min. Results were recorded as positive for frozen suspensions or negative for unfrozen suspensions (Table 14).

At concentrations lower than 0.1M, Ca $(NO_3)_2$ and $MnSO_4$ lost their effect. However, other chemicals were still effective even at the highest dilution.

d) Third group of cryoprotectants

The last group of chemicals tested in this study were $FeCl_2$; $FeCl_3$; $CoCl_3$; and $NiCl_2$ (BDH).

i) Laboratory tests

Different concentrations (0.1, 0.05, 0.01, and 0.005 M) of the chemicals were tested on bacterial dilutions of 10^0 to 10^{-5} (10^0 suspension was adjusted to $O.D_{600}$ of 0.7). After 30 min in the bath at -5°C, results were recorded as positive for frozen suspension or negative for unfrozen suspension in test tube (Table 15).

After 1h treatment, all bacterial dilutions treated with chemicals at 0.1M were plated on King's B medium and after 3 days' growth at 24°C, total number of bacteria were counted (Table 16).

It seems that these chemicals have some bactericidal properties. Cobalt and nickel substantially reduced numbers but the effect was less than with iron, both ferrous and ferric.

Table 14. Effect of different concentrations of chemical agents on an ice nucleation active strain of P. syringae serially diluted, in vitro.

Treatment		Bacterial dilution			
		10^{o}	10^{-1}	10^{-2}	10^{-3} - 10^{-5}
Control (no treatment)		+	+	+	-
Ca $(NO_3)_2$	0.1M	+	+	-	-
	0.01M	+	+	+	-
	0.005M	+	+	+	-
	0.001M	+	+	+	-
Control		+	+	+	-
$MnSO_4$	0.1M	+	+	-	-
	0.01M	+	+	+	-
	0.005M	+	+	+	-
	0.001M	+	+	+	-
Control		+	+	+	-
Al $(NO_3)_3$	0.1M	-	-	-	-
	0.01M	+	-	-	-
	0.005M	+	-	-	-
	0.001M	+	-	-	-
Control		+	+	+	-
Zn $(NO_3)_2$	0.1M	-	-	-	-
	0.01M	-	-	-	-
	0.005M	+	-	-	-
	0.001M	+	-	-	-
Control		+	+	+	-
$ZnSO_4$	0.1M	-	-	-	-
	0.01M	-	-	-	-
	0.005M	+	-	-	-
	0.001M	+	-	-	-

+ = Ice crystal formation; - = No ice crystal formation.

Table 15. Effect of chemical agents with various concentrations on an ice nucleating active strain of P. syringae serially diluted, in vitro.

Treatment		Bacterial dilutions			
		10^{0}	10^{-1}	10^{-2}	10^{-3} - 10^{-5}
Control (no treatment)		+	+	+	-
$FeCl_2$	0.1M	-	-	-	-
	0.05M	-	-	-	-
	0.01M	-	-	-	-
	0.005M	+	-	-	-
Control		+	+	+	-
$FeCl_3$	0.1M	-	-	-	-
	0.05M	-	-	-	-
	0.01M	-	-	-	-
	0.005M	+	-	-	-
Control		+	+	+	-
$CoCl_2$	0.1M	-	-	-	-
	0.05M	-	-	-	-
	0.01M	+	-	-	-
	0.005M	+	-	-	-
Control		+	+	+	-
$NiCl_2$	0.1M	-	-	-	-
	0.05M	-	-	-	-
	0.01M	+	-	-	-
	0.005M	+	-	-	-

+ = Ice crystal formation; - = No ice crystal formation.

Table 16. Effect of chemical agents (0.1M) on viability of an ice nucleation active strain of P. syringae.

Treatment	Total number of colonies
Control (without treatment)	6.0×10^8
$FeCl_2$	8.0×10^3
$FeCl_3$	2.3×10^4
$CoCl_2$	4.0×10^5
$NiCl_2$	5.0×10^5

ii) Greenhouse tests

The effect of the chemicals was tested in the greenhouse using both the tube nucleation test and the conductivity meter. The experiment had four replicates per treatment and all chemicals were tested at a concentration of 0.1M.

1. The tube nucleation test

In this procedure, pea leaves were added to 5ml sterile distilled water; after 30 min in the bath at -5°C, ice formation was determined at two different times:

A - One day after spraying the pea plants with the chemicals (Table 17, A).

B - Three days after spraying the pea plants with the chemicals (Table 17, B).

2. The conductivity meter

The conductivity meter was used and an index of injury for each treatment was calculated (Table 18).

Although all of these chemicals had an ameliorating effect on ice nucleation at 0.1M concentration both in qualitative (Table 17) and quantitative (Table 18) assays, some of the chemicals such as $CoCl_2$, $NiCl_2$ and especially Fe in both forms (ferric and ferrous) caused severe leaf damage to pea plants after spraying in greenhouse tests; this probably explains the very variable results obtained with these chemicals (see Table 18).

To obtain the most effective dose of the chemicals without leaf damage, the lowest effective doses of the chemicals in the laboratory (Table 14 &15) were chosen for testing the effect in the greenhouse.

Lowest effective doses tested for various chemicals were 0.01M, 0.001M for Al $(NO_3)_3$, Zn $(NO_3)_2$, $NiCl_2$; and 0.01M for $CoCl_2$ and $FeCl_2$.

Both assays, the tube nucleation test and the conductivity meter, were used. Results are shown in Tables 19 and 20, respectively. Summary of results from conductivity meter are shown in Table 21.

Table 17. Effect of chemical agents (0.1M) on an ice nucleation active strain of P. syringae in planta, using the tube nucleation test.

Replicates		Control*	Control**	$Al(NO_3)_3$	$Zn(NO_3)_2$	$CoCl_2$	$NiCl_2$	$FeCl_2$	$FeCl_3$
A	1	-	+	-	-	-	-	+	-
	2	-	+	-	-	-	-	-	+
	3	-	+	-	-	-	-	+	+
	4	-	+	-	-	-	-	-	-
B	1	-	+	+	+	-	-	+	+
	2	-	+	+	-	-	+	+	+
	3	-	+	-	-	-	-	+	+
	4	-	+	-	-	+	-	-	-

* = Unsprayed pea plants .

** = Pea plants were sprayed with ice nucleation active bacteria alone .

A = Using the tube nucleation test , one day after spraying with chemicals .

B =" " " " , Three days after spraying with chemicals .

\+ = Ice crystal formation; - = No ice crystal formation.

Table 18. Effect of chemical agents (0.1M) on an ice nucleation active strain of P. syringae in planta. Index of Injury to plants was measured using a conductivity meter.

Replicates	Control*	Control**	$Al(NO_3)_3$	$Zn(NO_3)_2$	$CoCl_2$	$NiCl_2$	$FeCl_2$	$FeCl_3$
1	4.55	60.85	27.91	26.67	45.83	60.00	80.00	71.70
2	3.45	47.83	26.40	37.50	35.48	55.56	24.00	22.33
3	5.88	45.28	25.00	41.38	60.00	40.00	27.42	38.24
4	2.94	77.27	20.00	33.33	31.82	23.53	50.53	42.31
Mean	4.21	57.81	24.83	34.72	43.28	44.77	45.36	43.65

* = Unsprayed pea plants. (Not included in statistic analysis).

** = Pea plants were sprayed with ice nucleation active bacteria alone.

Mean values were significally different ($P \leq 0.01$).

Table 19. Effect of chemical agents (0.01 and 0.001M) on an ice nucleation active strain of P. syringae in planta, using the tube nucleation test.

			Concentration (M)							
Replicates	*	**	0.01 $Al(NO_3)_3$	0.001 $Al(NO_3)_3$	0.01 $Zn(NO_3)_2$	0.001 $Zn(NO_3)_2$	0.01 $NiCl_2$	0.001 $NiCl_2$	0.01 $CoCl_2$	0.01 $FeCl_2$
1	-	+	+	+	-	+	-	+	-	+
2	-	+	+	+	+	+	-	-	+	+
3	-	+	-	+	-	-	+	-	+	+
4	-	+	+	+	+	+	+	+	+	+

* = Unsprayed pea plants.

** = Pea plants were sprayed with ice nucleation active bacteria alone.

+ = Ice crystal formation; - = No ice crystal formation.

Table 20. Effect of chemical agents (0.01 and 0.001M) on an ice nucleation active strain of P. syringae in planta. Index of injury to plants was measured using a conductivity meter.

			Concentration (M)							
			0.01	**0.001**	**0.01**	**0.001**	**0.01**	**0.001**	**0.01**	**0.01**
Replicates	*	**	$Al(NO_3)_3$	$Al(NO_3)_3$	$Zn(NO_3)_2$	$Zn(NO_3)_2$	$NiCl_2$	$NiCl_2$	$CoCl_2$	$FeCl_2$
1	4.55	62.50	42.11	60.87	35.00	42.22	35.71	42.50	38.10	52.56
2	5.45	55.10	40.00	48.72	42.50	49.52	32.73	39.53	44.84	52.86
3	2.33	52.38	40.00	55.00	38.89	40.30	39.47	40.89	55 00	52.63
4	3.45	55.31	55.00	54.76	41.00	43.18	38.57	43.15	40.40	42.22
Mean	3.95	56.32	44.28	54.84	39.35	43.81	36.62	41.51	44.59	50.07

* = Unsprayed pea plants. (Not included in statistic analysis).

** = Pea plants were sprayed with ice nucleation active bacteria.

Mean values were significally different ($P \leq 0.01$).

Table 21. Summary of means of the index of injury of the chemical agents which may be promising as cryoprotectants for frost control.

	Mean Index of Injury		
	Concentration (M)		
Treatment	**0.1**	**0.01**	**0.001**
Al $(NO_3)_3$	24.83	44.28	54.84
Zn $(NO_3)_2$	34.42	39.35	43.81
$NiCl_2$	44.77	36.62	41.52
$CoCl_2$	43.28	44.59	N.T
$FeCl_2$	55.36	50.07	N.T
Control *	4.21	3.95	3.95
Control **	57.81	56.32	56.32

* = Unsprayed Pea plants.

** = Pea plants were sprayed with ice nucleation active bacteria.

N.T = Not tested.

Although by reducing the concentration of chemical, the cryoprotectant effect is also reduced (Table 21), some of the chemicals such as $NiCl_2$ and $Zn(NO_3)_2$ were effective at low concentrations compared with control pea plants which were sprayed with INA bacteria alone.

For comparison of the effect of the $Ca(NO_3)_2$, $MnSO_4$, $Al(NO_3)_3$ and $Zn(NO_3)_2$ on two strains of INA bacteria in the laboratory, one ice nucleation active strain of P. syringae isolated from the pea field in South Australia was compared with an ice nucleation active strain of P. syringae isolated in New Zealand.

First fresh suspensions of the two strains were adjusted to $O.D_{600}$ of 0.7. A ten fold dilution series was prepared from these suspensions and there were 4 replicates. A final concentration of 0.1M of the chemicals was adjusted for each bacterial dilution. After 1 h, the tubes were placed in a bath at -5°C for 30 min. Results were recorded as positive for a frozen suspension or negative for an unfrozen suspension (Table 22).

The results indicate that the New Zealand strain is more active in ice nucleation than the South Australian strain. In the untreated controls, the NZ strain was active at a dilution of 10^{-3} compared with activity at 10^{-2} dilution for the South Australian strain (Table 22). For two of the chemicals, $Ca(NO_3)_2$ and $MnSO_4$, the cryoprotectant effect was lower on the NZ strain. However in both strains, $Al(NO_3)_3$ and $Zn(NO_3)_2$ could stop freezing at all dilutions (Table 22).

Following treatment, the serial dilutions were plated on King's B medium for 3 days' growth at 24°C. Total colonies for each treatment were counted (Table 23).

The results confirm previous findings that the cryoprotectant effect of $Ca(NO_3)_2$ and $MnSO_4$ is not because of bactericidal activity. In contrast, $Al(NO_3)_3$ and $Zn(NO_3)_2$ were markedly bactericidal.

Table 22. Effect of chemical agents (0.1M) on two* ice nucleation active strains of P. syringae serially diluted, in vitro.

	Bacterial dilutions								
	SA strain				NZ strain				
Treatment	10^{o}	10^{-1}	10^{-2}	10^{-3} - 10^{-8}	10^{o}	10^{-1}	10^{-2}	10^{-3}	10^{-4} - 10^{-8}
Control	+	+	+	-	+	+	+	+	-
Ca $(NO_3)_2$	+	+	-	-	+	+	+	-	-
	+	+	+	-	+	+	+	+	-
	+	+	+	-	+	+	+	+	-
	+	+	-	-	+	+	+	+	-
Control	+	+	+	-	+	+	+	+	-
$MnSO_4$	+	+	-	-	+	+	+	-	-
	+	+	-	-	+	+	+	+	-
	+	+	+	-	+	+	+	+	-
	+	+	+	-	+	+	+	+	-
Control	+	+	+	-	+	+	+	+	-
Al $(NO_3)_3$	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
Control	+	+	+	-	+	+	+	+	-
Zn $(NO_3)_2$	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-

* = One strain from South Australia (strain L) and one from New Zealand (strain 2796).

\+ = Ice crystal formation; - = No ice crystal formation.

Table 23. Effect of chemical agents on the viability of two* ice nucleation active strains of P. syringae in vitro.

Treatment	SA strain Total number of colonies	NZ strain Total number of colonies
Control**	3.2×10^7***	3.2×10^7
$Ca(NO_3)_2$	2.6×10^7	2.7×10^7
$MnSO_4$	2.9×10^7	2.8×10^7
$Al(NO_3)_3$	9.5×10^3	9.5×10^3
$Zn(NO_3)_2$	3.5×10^2	6.0×10^2

* = One strain from South Australia (strain L) and one from New Zealand (strain 2796).

** = Without treatment.

*** = Each number represents mean for 4 replicates.

Discussion

During the growing season of a pea crop, numbers of INA bacteria decreased with time. This could be due to environmental changes or to changes in crop maturity. Rainfall had no detectable affect on numbers of INA bacteria but numbers were negatively correlated with temperature. It seems in the hot weather the numbers and activity of INA bacteria are at a low level or even at an undetectable level in contrast to cold weather when numbers are high.

The detection of P. syringae and P. fluorescens (and not other ice nucleation active strains of bacteria) in this study, indicates that the appearance of the species depends on environmental conditions or on the plant host. It is not possible to distinguish these two factors because we have not examined hosts other than pea; no studies on peas have been reported from other areas.

The control of frost injury could be achieved by the prevention of ice formation by removing or inhibiting most, or all, ice nucleation activity. Control of frost damage using bactericidal chemicals to destroy INA bacteria and using bacterial ice nucleation inhibitors, chemicals that quickly inactivate the ice nucleus associated with INA bacteria without necessarily killing bacterial cells, has been reported for several commercial crop plants (Anderson *et al.*, 1984; Lindow, 1983a, 1984). Also, results reported by Young (1987), have shown that ice nucleation activity in the washings from Kiwifruit leaves was reduced after treatment with lethal doses of $CuSO_4$ and carbonyl cyanide m-chlorophenyl hydrazone. Moreover, it has been reported that broccoli leaves treated with the bactericide Kasugamycin showed the lower level of frost injury (Goto, *et al.*, 1988).

Among all the chemical agents that were tested as cryoprotectants (both in vitro and in planta), it would appear that some specific metal cations such as Mn, Al, Zn, Ca, Ni, Co, and Fe have some effect on INA bacteria, either because of bactericidal properties (Al, Zn, Ni, Co, and Fe), or by an effect on ice nucleating activity (Ca and Mn). The anions of $(SO_4)^{--}$ or $(NO_3)^{-}$ compounds appeared to have no influence, because treatments with NH_4NO_3 and $(NH_4)_2SO_4$, had no effect. Also, there was

no difference between the cryoprotectant effects of $MgSO_4$ and $Mg(NO_3)_2$ or between $ZnSO_4$ and $Zn(NO_3)_2$. The results with bactericidal chemicals suggests that death of cells must alter the ice nucleation protein on the bacterial surface. This is in contrast to the results of Lindow (1983a), who reported that cells killed by various chemicals lost ability to nucleate ice very slowly. It is possible that in the present study, cell killing and alteration of the ice nucleation protein are two independent events. The chemicals used in the two studies were different. Although, by reducing the concentration of chemicals (to lower than 0.1M), Ca and Mn lose their cryoprotectant effectivity, others are still active, some such as Al and Zn at 0.001M. As there appeared to be no evidence of phytotoxicity caused by Al $(NO_3)_3$ and Zn $(NO_3)_2$ at this concentration, these chemicals would probably be worth testing in the field for control of frost damage. They certainly hold more promise than recognized cryoprotectants such as teric and tween chemicals which were ineffective.

Section B: Pathovars of Pseudomonas syringae on peas

Introduction

During studies on ice nucleating bacteria (section A), it was established that Pseudomonas syringae had ice nucleation activity and was common on the pea crop being monitored. However, the pathovar of the isolates was not determined. Two very closely related plant pathogenic bacteria are associated with peas, one Pseudomonas syringae pv. pisi (Fig 10) is seed-borne and causes significant economic damage especially during spring and summer; the other Pseudomonas syringae pv. syringae (Fig 10) is a less virulent pathogen but can cause severe disease following hail damage or under excessively wet condition (Wimalajeewa and Nancarrow, 1984). The identity of the pathovar(s) involved in ice nucleation is important but the solution of an even more important pathological problem depends on the infra sub-specific identity of strains of P. syringae in South Australia. Several countries have import restrictions against peas grown in areas where P. syringae pv. pisi occurs; P. syringae pv. syringae is probably ubiquitous and no restrictions based on its occurrence have been imposed.

Until recently, only P. syringae pv. syringae had been recorded in South Australia and consequently export of pea seed was not restricted. However, a recent report (Newton and Hayward, 1986) listed P. syringae pv. pisi as present in South Australia. Also P. syringae pv. pisi has been recorded in Victoria and it is claimed that samples collected in South Australia and processed in Victoria contained this organism (D. Cartwright, personal communication).

The two organisms are difficult to distinguish. Some of the tests reported in the literature as discriminatory are shown in Table 24. However, there is considerable doubt about the reliability of these tests. Another reported method of distinguishing the two organisms is by a set of typing phages, viruses which kill bacteria and by serology (Taylor, 1972b; Taylor and Dye, 1972).

Figure 10. Bacterial cells of Pseudomonas syringae pv. pisi and Pseudomonas syringae pv. syringae viewed by transmission electron microscopy (A, x 10000; B, x 18000; C, x 16000; D, x 20000).

A and B) Pseudomonas syringae pv. pisi

C and D) Pseudomonas syringae pv. syringae

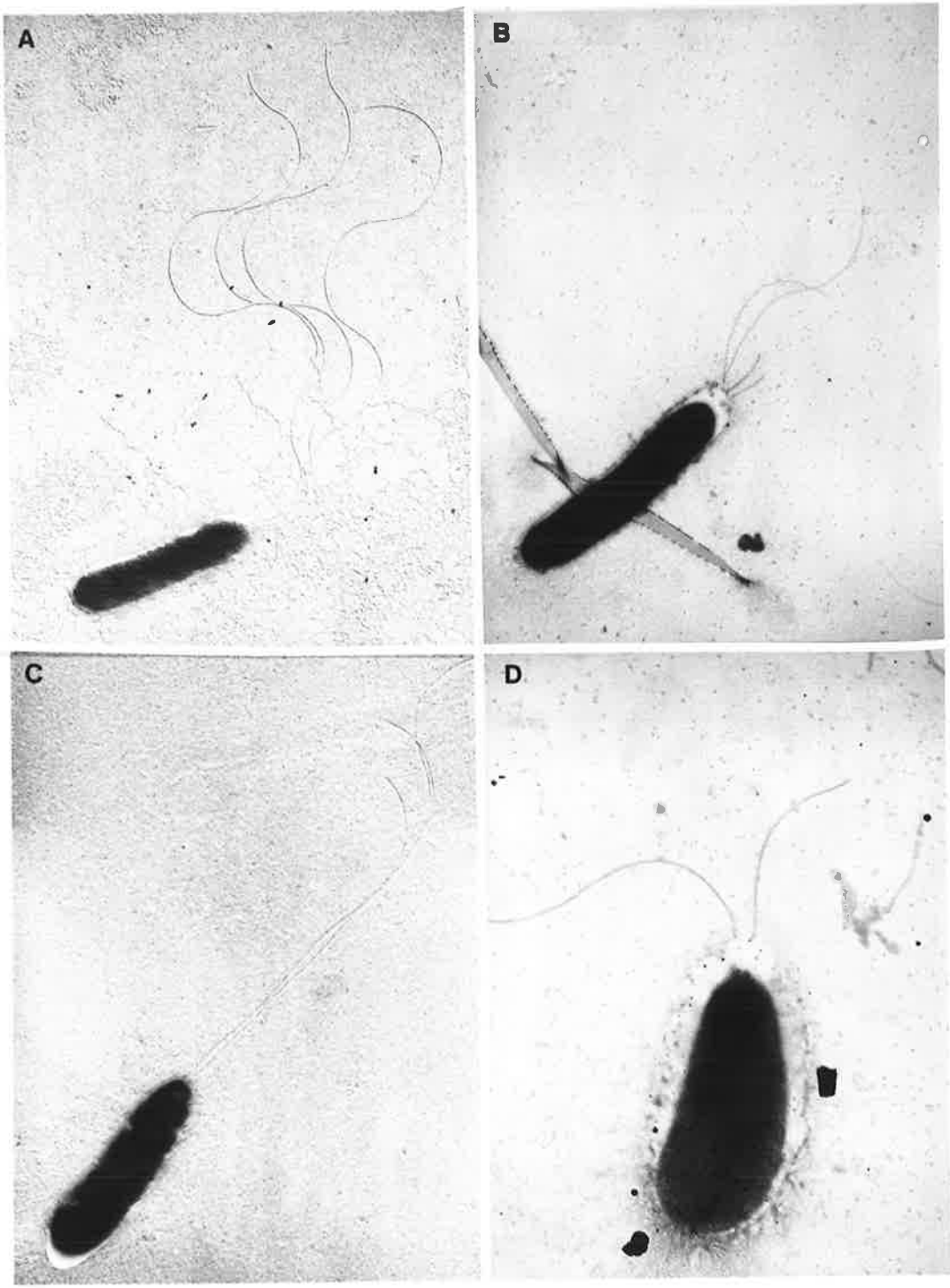

A
B
C
D

Table 24. Tests reported as distinguishing Pseudomonas syringae pv. syringae from Pseudomonas syringae pv. pisi.

Test	P. syringae pv. syringae	P. syringae pv. pisi
1-Utilization of homoserine (Hildebrand, 1973)	-	+
2-Lesion produced on young bean pods, and immature lemons (Harrison and Freeman, 1985)	+	-
3-Stem inoculation of sensitive host cultivars (Malik *et al.*, 1987)	-	water soaking

In this section, the methods reported to distinguish P. syringae pv. syringae from P. syringae pv. pisi with the exception of bacteriophage have been evaluated and a much simpler screening process based on polyclonal antibodies has been developed.

Materials and methods

a) Cultures

Reference cultures of P. syringae pv. syringae and P. syringae pv. pisi were obtained from New Zealand, Victoria, Queensland, Sydney and England and compared with South Australian strains of P. syringae collected from a pea field at Waite Institute (section A). All were subjected to the tests reported as distinguishing the two pathovars. Strains are listed in Table 25.

b) Growth conditions and maintenance of isolates

All the samples were recultured on King's B medium for 48 h at 24°C, then streaked on King's B medium and individual colonies obtained from this medium were maintained on nutrient agar slants at 4°C.

B. 1 Nutritional and pathogenicity tests

a) Utilization of homoserine

Filter-sterilized homoserine was incorporated (0.1% w/v) into a mineral-base medium as the sole carbon source. Mineral-base medium (Palleroni and Doudoroff, 1972) consisted of: Na-K phosphate buffer, pH 6.8, M/30; NH_4Cl, 0.1%; $MgSO_4.7H_2O$, 0.05%; ferric ammonium citrate, 0.005%; $CaCl_2$, 0.0005%; Ionagar, 1%. A bacterial suspension (0.03ml) was placed on the medium, and the capacity to grow was observed after 2 weeks

b) Pathogenicity

i) Bean pod and lemon fruit inoculation

Table 25. Cultures of Pseudomonas syringae used in this investigation.

Strains	Pathovar designation	Source
2796; 3319; 3543; 3939; 5055	syringae	New Zealand (Dr J.M. Young)
815; 2452; 3575; 5310; 5316	pisi	"
3576; 3940; 3941; 5319	?	"
DAR26811;DAR35712; DAR41320	syringae	Sydney (Dr P. Fahy)
DAR30566;DAR33368; DAR33369;DAR33379; DAR58730	pisi	"
UQM213; UQM227	syringae	Queensland (Dr A.C. Hayward)
UQM551[a]; RYE9	pisi	"
DN20; DN28	?	"
P47; P54; P55	syringae	Victoria (Dr D.L.S. Wimalajeewa)
P52; P53; P60	pisi	"
1212	syringae	England (Dr J.D. Taylor)
1217	pisi	"
L; N; O	?	South Australia (M. Mazarei)

a = Originally isolated in South Australia by M.V. Carter.

Young bean pods and light yellowish-green lemon fruits were inoculated by placing drops of an aqueous bacterial suspension (about 10^8 cells/ml) on the surface and pricking lightly through the drops with a sterile needle. Incubation was in moist containers at 25°C for 7 days.

ii) Stem inoculation

Two susceptible pea cultivars, Rovar and Blue-Prussian, (Taylor, 1972a) were grown (10-14 days) in pots. Bacteria cultured (16 h) on King's B media were scraped from the surface with a sterile needle and stabbed into the main stem at its junction with the stipules at the youngest two nodes (two inoculations per plant). The plants were maintained for 10 days before recording the results. There were two replicates for each strain on both cultivars.

Results

a) Utilization of homoserine

The majority of the reference cultures of P. syringae pv. syringae did not utilize homoserine as a sole carbon source. In contrast most of the reference cultures of P. syringae pv. pisi utilized homoserine (Fig 11). However there was a significant number of exceptions (Table 26).

b) Pathogenicity

i) Bean pods and lemon fruits

Inoculation of bean pods was not a reliable test. Most of the reference cultures of the two pathovars produced small reddish-brown depressed lesions (Fig 12). On lemon fruits all pathogenic strains of P. syringae pv. syringae produced brown depressed lesions whereas all pathogenic strains of P. syringae pv. pisi produced no symptoms (Fig 13).

Results are shown in Table 26.

Table 26. Responses of strains of P. syringae pv. syringae and pv. pisi to various diagnostic tests.

Strain	Inoculation of: Utilization of homoserine	Lemon fruits	Pea stems of cultivar: Rovar	Blue-Prussian	Pathovar identification
815, 1217 2452, 3575 5055, 5310 5316, DAR30566 DAR 33369, DAR 58730, UQM 551	+	-	WS	WS	pisi
2796, 3319, DAR 26811, DAR 41320, L, N, O, DN20, DN28*	-	+	N	N	syringae
1212, 3576, 5319, DAR33379 P54*	+	+	N	N	syringae
DAR 35712, UQM 227*	-	-	N	N	syringae?
3543, P47, P52	+	-	-	-	non pathogenic
3939*, P53*, P60, UQM 213	-	-	-	-	non pathogenic
DAR33368, RYE9	+	-	G	-	non pathogenic?
3940, 3941, P55	-	+	G	-	non pathogenic?

WS = Water soaked.

N = Extensive necrosis and collapse.

\- = Null response (only inoculation wounds were visible).

G = Skin cracking with production of galls.

* = Production of galls on cultivar Rovar.

Figure 11. Utilization of homoserine as a sole carbon source by Pseudomonas syringae.

Left : Pseudomonas syringae pv. syringae (strain L).

Right : Pseudomonas syringae pv. pisi (strain UQM 551).

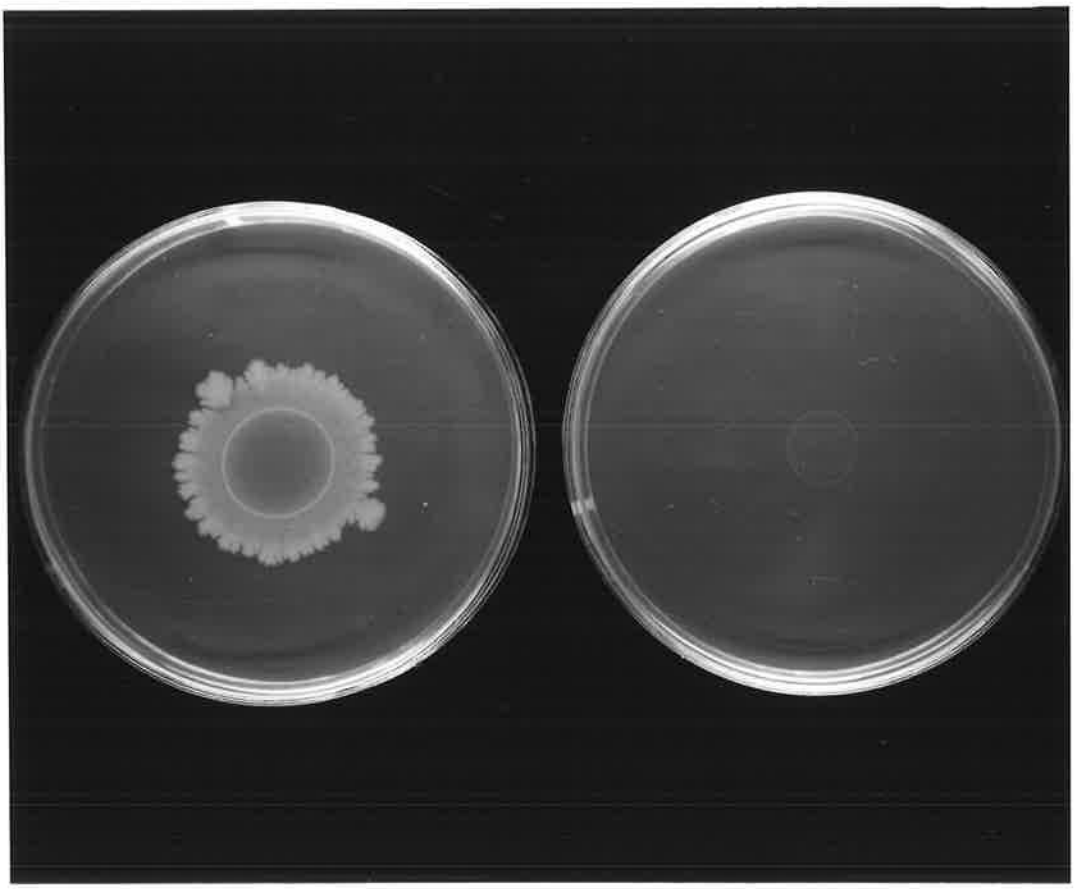


Figure 12. Pathogenicity of Pseudomonas syringae on bean pods.

Left : Pseudomonas syringae (pv. pisi or syringae).

Right : Control (water alone).

Figure 13. Pathogenicity of Pseudomonas syringae on lemon fruits.

Left : Pseudomonas syringae pv. syringae (strain L).

Right : Pseudomonas syringae pv. pisi (strain UQM 551).

ii) Stem-inoculation

Inoculation with P. syringae pv. pisi resulted in water-soaked tissue (Fig 14) spreading from the site of inoculation for most pathogenic strains. In contrast, inoculation with P. syringae pv. syringae resulted in extensive necrosis and collapse with stunting (Fig 15) on the two cultivars Rovar and Blue-Prussian. The pathogenic responses were clearer and more extensive on cultivar Blue-Prussian than on cultivar Rovar. Occasionally small galls were produced on cultivar Rovar (Fig 16).

Non pathogenic strains of P. syringae produced a null response in which no symptoms other than the inoculation wounds were visible (Fig 17).

Results are shown in Table 26.

Figure 14. A pathogenic response of water-soaking spreading from the site of stem inoculation on pea cultivar Blue-Prussian, produced by Pseudomonas syringae pv. pisi.

Figure 15. A pathogenic response of extensive necrosis and collapse produced by Pseudomonas syringae pv. syringae, after stem inoculation on pea cultivar Blue-Prussian.

Fig 14

Fig 15

Figure 16. A response of skin cracking with production of yellow galls produced by Pseudomonas syringae, after stem inoculation on pea cultivar Rovar.

Figure 17. A null response by Pseudomonas syringae, after stem inoculation on pea cultivar Blue-Prussian, in which no symptoms other than the inoculation wounds are visible.

Fig 16

Fig 17

Discussion

Nutritional and pathogenicity tests

A comparison of published tests (utilization of homoserine, pathogenicity on lemon fruits and stem-inoculation of appropriate host cultivars) to distinguish two pathovars of P. syringae (pv. syringae and pv. pisi), indicated that stem-inoculation, especially of cultivar Blue-Prussian, was most useful for distinguishing the two pathovars. Results can be confirmed by inoculation of lemon fruit. Those strains producing a water-soaked lesion after stem-inoculation with no response on lemon fruits can be identified as P. syringae pv. pisi. In contrast, those strains producing extensive necrosis and collapse after stem-inoculation of peas and a depressed brown lesion on lemon fruits can be identified as P. syringae pv. syringae.

Although most of the reference strains of P. syringae pv. pisi utilized homoserine as a sole carbon source, some P. syringae pv. syringae strains (e.g. 1212 and P 54) also utilized homoserine. This test alone is not, therefore, sufficient to distinguish the two pathovars.

Some reference strains had been misidentified. For example, strain DAR 33379 was received as P. syringae pv. pisi. Although it utilized homoserine as a sole carbon source, its other characteristics, especially necrosis following stem inoculation on both pea cultivars (Rovar and Blue-Prussian) and the brown depressed lesion following lemon inoculation, are typical of P. syringae pv. syringae. Identification of the strain was confirmed serologically which is described in the following section.

B. 2 Serology

Introduction

Although nutritional and pathogenicity tests (section B. 1) for differentiation of P. syringae pv. syringae from P. syringae pv. pisi are effective, they are also time consuming and the pathogenicity test requires a lot of greenhouse space. A serological differentiation of the two pathovars could be much more cost-effective (Taylor, 1972b; Taylor and Dye, 1972). The possibility of developing a much more rapid and cost-effective screening for P. syringae pv. syringae and P. syringae pv. pisi based on polycolonal antibodies was examined.

Materials and methods

a) Selection of isolates

From all the reference strains (Table 25), two strains were chosen which showed the typical responses of the two pathovars (Table 26).

Both strains were isolated from South Australia:

1- Strain UQM 551(P. syringae pv. pisi), was isolated from a pea field in 1966.

2- Strain L (P. syringae pv. syringae), was isolated from a pea field in 1986.

b) Growth conditions of strains for serological tests

The two strains were streaked twice on King's B medium, single colonies selected and grown for 48 h on a shaker at 25°C in a liquid medium (Lucas and Grogan, 1969) which consisted of: 10 g glucose, 6 g Difco casamino acids, 2 g KH_2PO_4, 1 g K_2HPO_4, and one liter distilled water.

The bacterial cells were sedimented by centrifugation at 4°C for 10 min at 10000g. The pellets were suspended in 5ml sterile distilled water and centrifuged again. The pellets were washed twice more and, after final centrifugation, resuspended in 5ml sterile distilled water. These cells were the source of the antigenic preparations of bacterial cells.

c) Preparation of bacterial antigens

i) Glutaraldehyde fixed antigen

Bacterial cells were fixed with 0.25% glutaraldehyde (Allan and Kelman, 1977).

ii) Sonicated antigen

Bacterial cells were sonicated at 100W for 5 min with 1 min treatments separated by 1 min cooling periods.

iii) Heat-killed antigen

Bacterial cells were heat-killed at 121°C for 15 min.

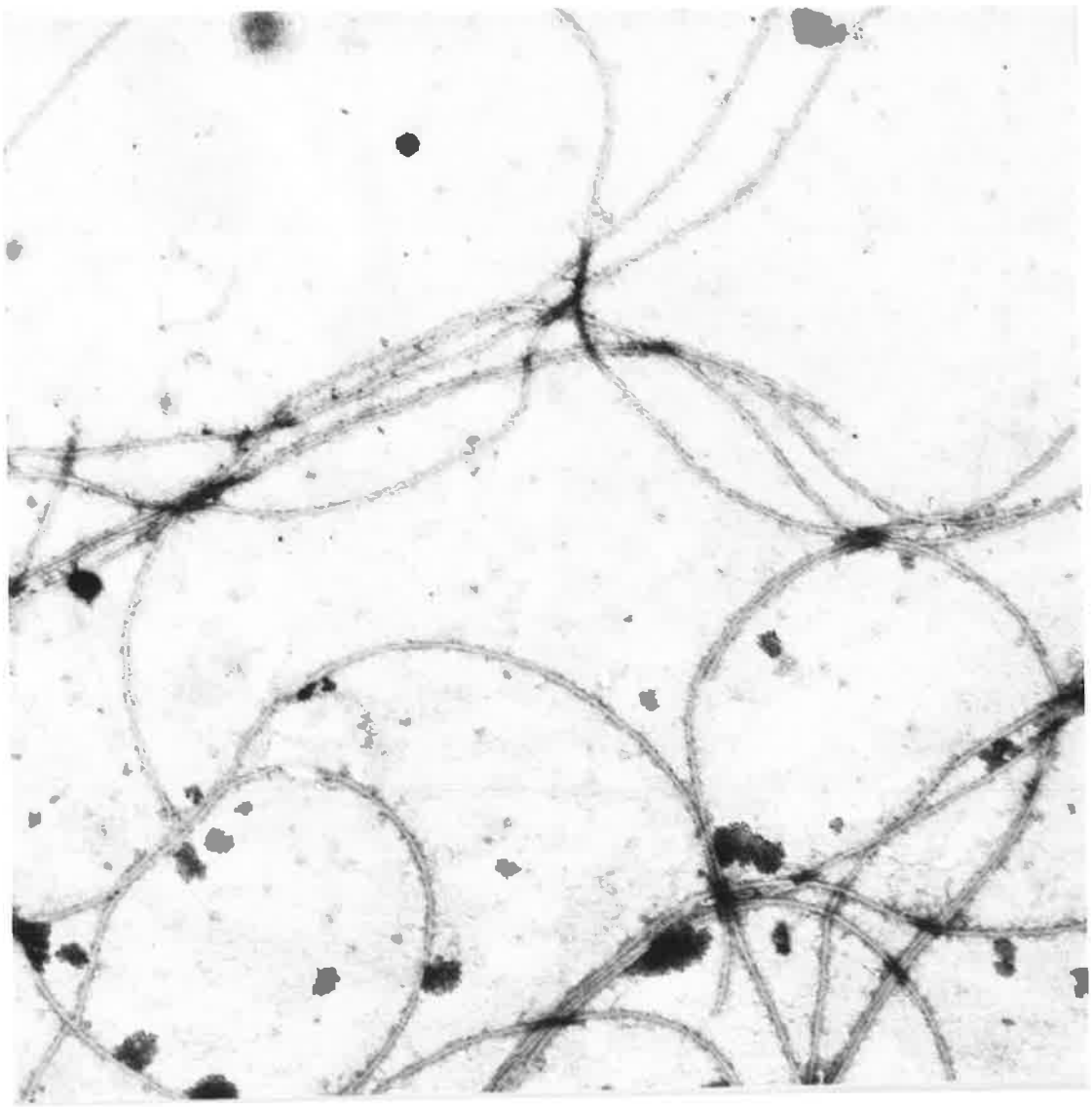
iv) Flagellar antigen

For preparation of flagella, the procedure of Guffanti and Eisenstein (1983) was used; it is based on the fact that bacterial cells sediment more rapidly than flagella. Cells were grown in liquid medium on a rotary shaker and highly motile cultures in the late-exponential phase of growth were harvested by centrifugation at 12000g for 10 min. The pellets were suspended in SDW (30 g/liter) and shaken for 10 min with a twist action shaker to shear the flagella. Intact cells were spun down at 6000g for 30 min and the resulting supernatant was centrifuged at 16000g for 20 min. The supernatants were pooled and the flagella and cellular fragments were separated by two series of differential centrifugations, first at 12500g for 30 min and then at 80000g for 1 h. The clear, gelatinous flagella in the upper part of the pellet were carefully removed and suspended in SDW and allowed to stand at 4°C overnight. The flagellar suspension was viewed by transmission electron microscopy (Fig 18).

v) Cell wall antigen

This was prepared by both hot phenol and cold trichloracetic acid (TCA) methods.

Figure 18. Flagellar suspension of Pseudomonas syringae viewed by transmission electron microscopy (x 30000).



The hot phenol procedure of Lucas and Grogan (1969) was used; twice washed bacterial cells were pelleted, weighed, and suspended in 100ml SDW (65°C) per 5 g of bacterial wet cells. To this solution, an equal volume of 90% warm (65°C) phenol was added. The emulsion was stirred for 30 min at 65°C, cooled in an ice bath, and centrifuged at 1000g for 30 min. During the centrifugation, the emulsion separated into three phases: a) an aqueous phase on top, b) a precipitate at the interface and c) a phenol phase at the bottom of the tube. The aqueous phase was collected; the precipitate and phenol phases were extracted again with 100 ml of SDW (65°C), centrifuged as above and the aqueous phase collected and combined with the first extract. It was then dialysed against SDW for two days at room temperature and the antigen precipitated at -20°C overnight by the addition of six volumes of cold ethanol. The precipitate was collected by centrifugation, and dissolved in SDW.

Cell wall antigen was also prepared by a cold TCA method using a similar procedure to that described by Digat and Cambra (1976). The twice washed bacterial cells in distilled water were centrifuged and the packed cells weighed and suspended in 2 times their weight of ice cold SDW. Then an equal volume of cold 0.5N TCA was added with stirring continued for 3 h at 4°C. After centrifugation at 12000g for 30 min, the supernatant was neutralized to pH 7.0 with concentrated NaOH. Following addition of 2 volumes of cold ethanol and incubation at -20°C overnight, the antigen was precipitated by centrifugation and dissolved in 0.1 of the original volume of SDW and dialysed against SDW for 4 days at 4°C. Finally it was centrifuged at 27000g for 15 min, and the supernatant which contained cell wall antigen was retained.

vi) Membrane protein complex antigen

This was prepared as described by Yakrus and Schaad (1979). The harvested washed bacterial cells were centrifuged, the pellet weighed and suspended in 200ml of 0.2M LiCl per 10 g wet weight of cells. To this suspension 3 ml of glass beads (3mm diameter) were added, and agitated on a rotary shaker for 2-3 h at 45°C. The

suspension was filtered through cheesecloth, and centrifuged at 12000g for 20 min. The supernatant fluid retained and centrifuged at 30000g for 40 min and the supernatant fluid containing the membrane protein complex centrifuged at 100000g for 2 h. The pellet was washed once with SDW and centrifuged at 100000g for 2 h, and the final pellet suspended in SDW and stored at 4°C.

vii) Glycoprotein antigen

This was prepared according to the method of Digat and Cambres (1976). The harvested bacterial cells in SDW were shaken vigorously for 30 min, centrifuged at 3000g for 15 min, and the supernatant centrifuged again at 6000g for 15 min. The supernatant was filtered through a millipore filter (0.45μm), neutralized to pH 7.0, and precipitated by addition of an equal volume of saturated ammonium sulfate at 4°C overnight. The precipitate was collected by centrifugation at 20000g for 15 min, dissolved in SDW, and dialysed against SDW for 4 days.

viii) Exopolysaccharide antigen

This antigen was prepared as described by Coleno *et al.*, (1976). Bacterial cells were grown on levan medium containing sucrose to produce exopolysaccharide (slime). The cells were harvested, suspended in SDW, and pelleted by centrifugation. The pellet was washed 3 times and the supernatants of the second and third washings combined. The slime was precipitated with ethanol (final concentration 60%) at -20°C overnight. The gelatinous pellet was taken up and dialysed against SDW overnight.

d) Measuring protein concentration

Protein concentrations were measured by Pierce (ILLINOIS, U.S.A) BCA Protein Assay Reagent according to the manufacturer's recommendations. To prepare BCA Protein Assay Working Reagent, 50 parts of Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1N

NaOH) was mixed with 1 part of Reagent B (copper sulfate). A set of protein standards of known concentration was prepared by diluting a stock solution of BSA (bovin serum albumin), in the same diluent as the unknown samples; 0.1 ml of each standard or unknown protein sample was pipetted into the test tube. For blanks, 0.1 ml of diluent was used. Two ml BCA Working Reagent was added to each tube, mixed well and all the tubes incubated at 37°C for 30 min. After incubation, all tubes were cooled to room temperature. The absorbance of the blank was subtracted from the values of the standards or unknowns. A standard curve was prepared by plotting the net (blank corrected) absorbance at 562nm vs. protein concentration. By using this standard curve, the protein concentration for each unknown protein sample was determined.

e) Electron microscopy

A drop of suspension was placed for 1 min on a 400-mesh copper grid, which was covered by a carbon-coated formvar film pretreated by glow discharge. Negative staining was done with 1% phosphotungstic acid for 30 seconds and rinsed with SDW for 30-60 seconds. Grids were dried on filter paper in petri-dishes and viewed under a transmission electron microscope (TEM) (EM400; Philips, Australia).

f) Polyacrylamide gel electrophoresis (PAGE)

The discontinuous buffer system of Laemmli (1970) with 12% of lower gel and 5% of stacking gel was used in vertical slab gels on a Bio-Rad electrophoresis apparatus.To prepare the lower gel, 13.9 ml distilled water, 10 ml Tris (18.17g Tris-base + 6 N HCl + 4 ml of 10% SDS pH 6.8 adjusted to 100 ml with distilled water) and 16 ml Acryl/Bis from stock solution (30% acrylamide, 0.8% Bisacrylamide W/V in distilled water) were mixed. To this solution 0.12 ml of ammonium persulfate (0.1g/ml) and 0.02 ml of TEMED were added. The mixture was carefully added to apparatus with a Pasteur pipette in order to avoid trapping bubbles, while leaving 1 cm from the top empty. A flat surface was ensured by overlaying with 1 ml water-

saturated-Butanol. The gel was allowed to polymerize for 2-3 h. The stacking gel was prepared by mixing 3.85 ml distilled water, 1.7 ml upper Tris (6.06g Tris-base + 6 N HCl + 4 ml 10% SDS, pH 6.8, adjusted to 100 ml with water) and 1.15 ml Acryl-Bis from stock solution. After addition of 0.04 ml ammonium persulphate and 0.01 ml TEMED to this solution, it was poured between sandwich glass plates. (Previously the overlying liquid above the lower gel had been poured off and the gel washed with distilled water). Finally the comb was inserted carefully to avoid trapping bubbles at the bottom of the teeth. Then the gel was allowed to polymerize. Bacterial cells of the two pathovars (strains UQM 551, P. syringae pv. pisi and L, P. syringae pv. syringae) were prepared in sample buffer of Laemmli (1970) containing 62.5 mM Tris-HCl pH 6.8, 3% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue and boiled for 5 min at 90-100°C. After the samples were cooled to room temperature, 10µl were applied to each sample well with a Hamilton syringe. The Tris-Glycine buffer (3.0g Tris-base, 14.3g glycine in 1 liter of distilled water) was used as electrophoresis buffer and the electrophoresis was performed at 4mA / gel for 16 h. After electrophoresis, the gel was stained.

i) Silver staining of protein gel

The technique has been described by Wray *et al.* (1981). Briefly, following electrophoresis, the gel was soaked in 50% methanol in double distilled water and left on a rocking platform overnight. This solution was changed three times. The silver staining solution was prepared as follows: 1.4 ml of fresh 14.8M ammonium hydroxide was added to 21 ml of 0.36% NaOH. Then 0.8 g silver nitrate (BDH chemical, Australia) dissolved in 4 ml of double distilled water was added dropwise to this solution and the total volume increased to 100 ml with double distilled water. This solution was always used within 5 min of preparation. (The gel was quickly washed for a maximum of 1-2 min in deionized water on a rocking platform, before adding the silver nitrate solution). The gel remained in the silver solution for 15 min with constant agitation at room temperature. Later the gel was washed with gentle

agitation for 2-5 min in double distilled water. The developing solution was prepared by mixing 2.5 ml of 1% citric acid and 0.25 ml of 38% formaldehyde in 500 ml of deionized water. This solution was always prepared fresh. The gel was soaked in this solution and with gentle agitation it was developed (5-15 min). Then it was washed with distilled water several times and incubated in Ilford rapid fixer (prepared 1:5 in double distilled water) in order to remove background which was deposited on both sides of the gel or to reduce the darkness of stained bands. This step was monitored very closely as it may remove all the stained bands. The gel was rinsed several times with distilled water and then incubated in hypocleaning agent for 30 min with agitation (agitation was necessary to prevent it from sticking to the container and breaking). During this treatment the gel swells and the residue of rapid fixer is washed away.

The gel was rinsed several times in distilled water and then agitated in 10% methanol and 5% glycerol in deionized water for at least 2 h. Finally the gel was photographed.

g) Production of polyclonal antibodies

Four kinds of antisera against both P. syringae pv. pisi (strain UQM 551) and P. syringae pv. syringae (strain L) were prepared; i) antisera to glutaraldehyde-fixed cells; ii) antisera to sonicated cells; iii) antisera to heat-killed cells and iv) antisera to glutaraldehyde-fixed flagella of bacterial cells.

For preparation of all antisera, New Zealand white rabbits were used. In all injections, except intravenous, the antigen was mixed with an equal volume of Freund's incomplete adjuvant. The rabbits were bled from the marginal ear vein at regular intervals. Before the first injection, preimmune antisera (control antisera) were obtained. Blood was clotted after incubation for 2-3 h at 37°C and left overnight at 4°C. The sera were recovered as supernatants after centrifugation at 2000g for 10 min. After each bleeding, antisera were titrated by an Ouchterlony gel double-diffusion test (Ouchterlony, 1962).

i) Antisera to glutaraldehyde-fixed bacterial cells

Strains UQM 551 (pv. pisi) and L (pv. syringae) were fixed with 0.25% glutaraldehyde, and used to inject two rabbits. Rabbits were injected 1 (optical density, OD = 1 at 600nm, subcutaneously), 5 (OD = 1 at 600nm, intramuscularly), and 12 (OD = 2 at 600nm, intramuscularly) weeks after the first injection (OD = 1 at 600nm, subcutaneously).

ii) Antisera to sonicated bacterial cells

Strains UQM 551 (pv. pisi) and L (pv. syringae) were sonicated, the protein concentrations measured, and used to inject two rabbits. Rabbits were injected 1 (500µg, subcutaneously), 5 (500µg, intramuscularly), and 12 (1000µg, intramuscularly) weeks after the first injection (250µg, subcutaneously).

iii) Antisera to heat-killed bacterial cells

Strains (UQM 551 and L) of the two pathovars (pisi and syringae) were heat-killed, protein concentrations measured, and used to inject two rabbits. Rabbits were injected 1 (250µg, intravenously), 2 (500µg, intramuscularly), 5 (500µg, intramuscularly), 10 (1000µg, intramuscularly), and 20 (500µg, intravenously) weeks after the first injection (250µg, intravenously).

iv) Antisera to glutaraldehyde-fixed flagella

Flagella from the two pathovars (strain UQM 551, pv. pisi and strain L, pv. syringae) were isolated as described before, fixed with 0.25% glutaraldehyde, protein concentrations measured, and used to inject two rabbits. Rabbits were injected 1 (250µg, intravenously), 2 (500µg, intramuscularly), 6 (500µg, intravenously), 10 (500µg, intramuscularly), and 14 (500µg, intravenously) weeks after the first injection (250µg, intravenously).

h) Absorption of antisera

Cross-absorption was by the method of Azad and Schaad (1988). One volume of each antiserum was mixed separately with an equal volume of either whole untreated, glutaraldehyde-fixed, sonicated, or heat-killed bacterial cells of the heterologous antigen. Mixtures were incubated for 1 h at 37°C in a water-bath shaker, centrifuged at 3000g for 10 min, and the pellets discarded. For use in indirect ELISA, antisera were absorbed as above three more times by further addition of antigens.

i) Serological techniques

i) Ouchterlony gel double-diffusion

Tests were done in 90mm plastic petri dishes containing 15 ml of 0.75% purified agar in 0.01M Na_2HPO_4 - NaH_2PO_4 buffer at pH 7.6 containing 0.9% NaCl and 0.02% NaN_3 added as a preservative. The plates were stored in a refrigerator until needed. Circular patterns with 6 wells surrounding a central antiserum well were used in gel diffusion tests. Holes were 3mm in diameter and 3.5mm apart. 15 µl of antigen was placed in each outer well and 15 µl of undiluted antiserum in the central well. Plates were maintained at 25°C and observed for precipitation lines after 2-3 days.

ii) Enzyme-linked immunosorbant assay (ELISA)

An indirect ELISA technique (Koenig, 1981) was used. Antigens were diluted in carbonate buffer, pH 9.6, and added to microtitre ELISA plates from Nunc (Denmark) (100µl per well). The protein concentrations for coating test antigens were measured as described before, and details of coating concentrations are presented in the results section. After incubation for 3 h at 25°C in a moist plastic container and three 4-min washings in PBS-Tween (0.8g NaCl, 0.2g KH_2PO_4, 1.15g Na_2HPO_4, 0.2g KCl, 0.5 ml Tween-20 and water to 1 liter, pH 7.4), a blocking solution (10g/liter bovin serum albumin, 0.1M NaCl) was added (375µl per

well) to block non-specific protein binding, and incubated for 1 h at 25°C. After washing the plates, antisera diluted in sample buffer (PBS-Tween, 2% PVP buffer, pH 6.0) were added (100µl per well) and then incubated overnight at 4°C. Details of antisera dilutions are presented in the results section. Plates were washed as before. Goat anti-rabbit immunoglobulin (Sigma) coupled with alkaline phosphatase diluted in conjugate buffer (PBS-Tween-2% PVP, 0.2% BSA buffer, pH 7.4) (1µl/ml) was added (100µl per well) and incubated for 3 h at 25°C. After washing, the substrate, p-nitrophenyl phosphate in substrate buffer (diethanolamine, pH 9.8) (1 mg/ml) was added. The absorbance was measured with an automatic ELISA reader (Bio-Rad, 2550) at 405nm after 1 h.

Results

a) Polyacrylamide gel electrophoresis

All the protein bands were common to both pathovars (pv. syringae and pv. pisi), although some showed quantitative differences (Fig 19). It seems these two pathovars cannot be differentiated by PAGE assay.

b) Serological tests

i) Ouchterlony gel double-diffusion

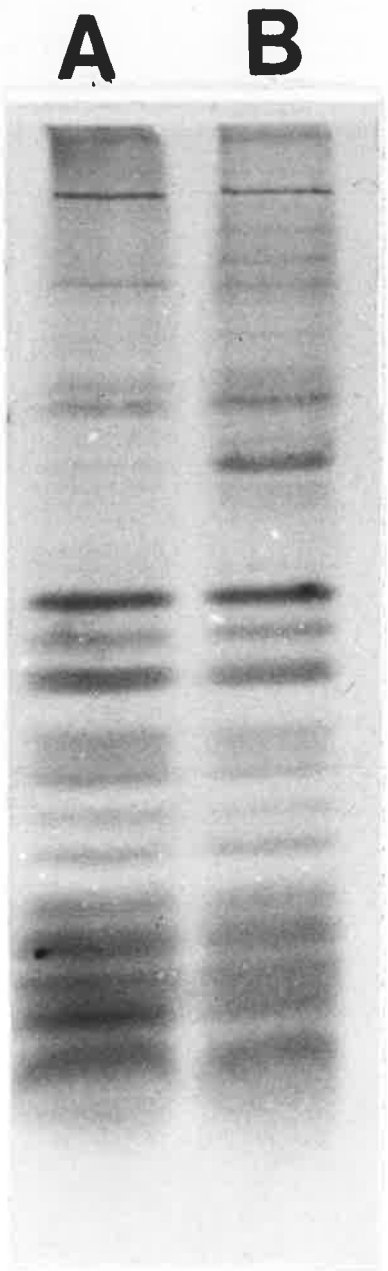
Each antiserum was tested in Ouchterlony gel double-diffusion test with its corresponding homologous and heterologous antigens, using whole untreated, fixed, sonicated, and heat-killed bacterial cells as antigens. With fixed cells, no reaction occurred either with homologous or heterologous antisera, presumably because fixed cells cannot move in the gel.

All antisera were tested against other pathovars, other species of Pseudomonas, and other genera; they were P. syringae pv. phaseolicola, P. syringae pv. coronafaciens isolated from oats, Pseudomonas fluorescens, Escherichia coli, Rhizobium, and Agrobacterium . Antigens were whole untreated, sonicated, and heat-killed bacterial cells. Pre-immune serum (control serum) of each immunized

Figure 19. Analysis of protein profiles of Pseudomonas syringae pv. syringae and Pseudomonas syringae pv. pisi.

A) P. syringae pv. syringae (strain L).

B) P. syringae pv. pisi (strain UQM 551).


A
B

rabbit was tested and no reaction was evident. All strains (Table 25) that had been previously checked by nutritional and pathogenicity tests (Table 26), were tested against these 8 antisera, using whole untreated, sonicated, and heat-killed cells as antigens.

1. Antisera to glutaraldehyde-fixed bacterial cells

The highest immunodiffusion titres of antiserum to isolate UQM 551 (pv. pisi) was 1/128, and to isolate L (pv. syringae) was 1/64. Antiserum to fixed strain UQM 551 and to fixed strain L had a high level of specificity. With heterologous antigens, using whole untreated cells as test antigen, a very weak precipitation line (halo) was observed. No reaction was evident, using sonicated and heat-killed cells as test antigen (Fig 20 a, b).

Reaction between the antisera and other pathovars was very weak, producing an indistinct halo only when whole untreated cells were used as antigen. No reaction with other species of Pseudomonas or other genera was evident, using all forms (whole untreated, sonicated, and heat-killed) of test antigen.

Results are presented in Table 27.

2. Antisera to sonicated bacterial cells

The highest immunodiffusion titres of antiserum to isolate UQM 551 (pv. pisi) was 1/64, and to isolate L (pv. syringae) was 1/32. Antisera gave a strong reaction against homologous antigens. With heterologous antigens no cross reactivity was observed, when heat-killed bacterial cells were used as test antigen. However, with other forms (whole untreated and sonicated cells) of test antigen, cross reactivity (weak precipitation line) was evident (Fig 20 c, d).

The antisera showed cross reactivity with other pathovars, and species of Pseudomonas, when whole untreated and sonicated cells were used as test antigen. With other genera no reaction was evident, using all forms (whole untreated, sonicated, and heat-killed cells) of test antigen.

Figure 20. Reaction of antisera to glutaraldehyde-fixed, sonicated and heat-killed cells and to flagella of strains L (pv. syringae) and UQM 551 (pv. pisi) of Pseudomonas syringae with homologous and heterologous antigens, in Ouchterlony gel double-diffusion test.

Central wells:

a) Antiserum to glutaraldehyde-fixed cells of strain L
b) Antiserum to glutaraldehyde-fixed cells of strain UQM 551

c) Antiserum to sonicated cells of strain L
d) Antiserum to sonicated cells of strain UQM 551

e) Antiserum to heat-killed cells of strain L
f) Antiserum to heat-killed cells of strain UQM 551

g) Antiserum to flagella of strain L
h) Antiserum to flagella of strain UQM 551

Outer wells:

1, whole untreated bacterial cells of antigen UQM 551; 2, heat-killed bacterial cells of antigen UQM 551; 3, sonicated bacterial cells of antigen UQM 551; 4, whole untreated bacterial cells of antigen L; 5, heat-killed cells of antigen L; 6, sonicated cells of antigen L.

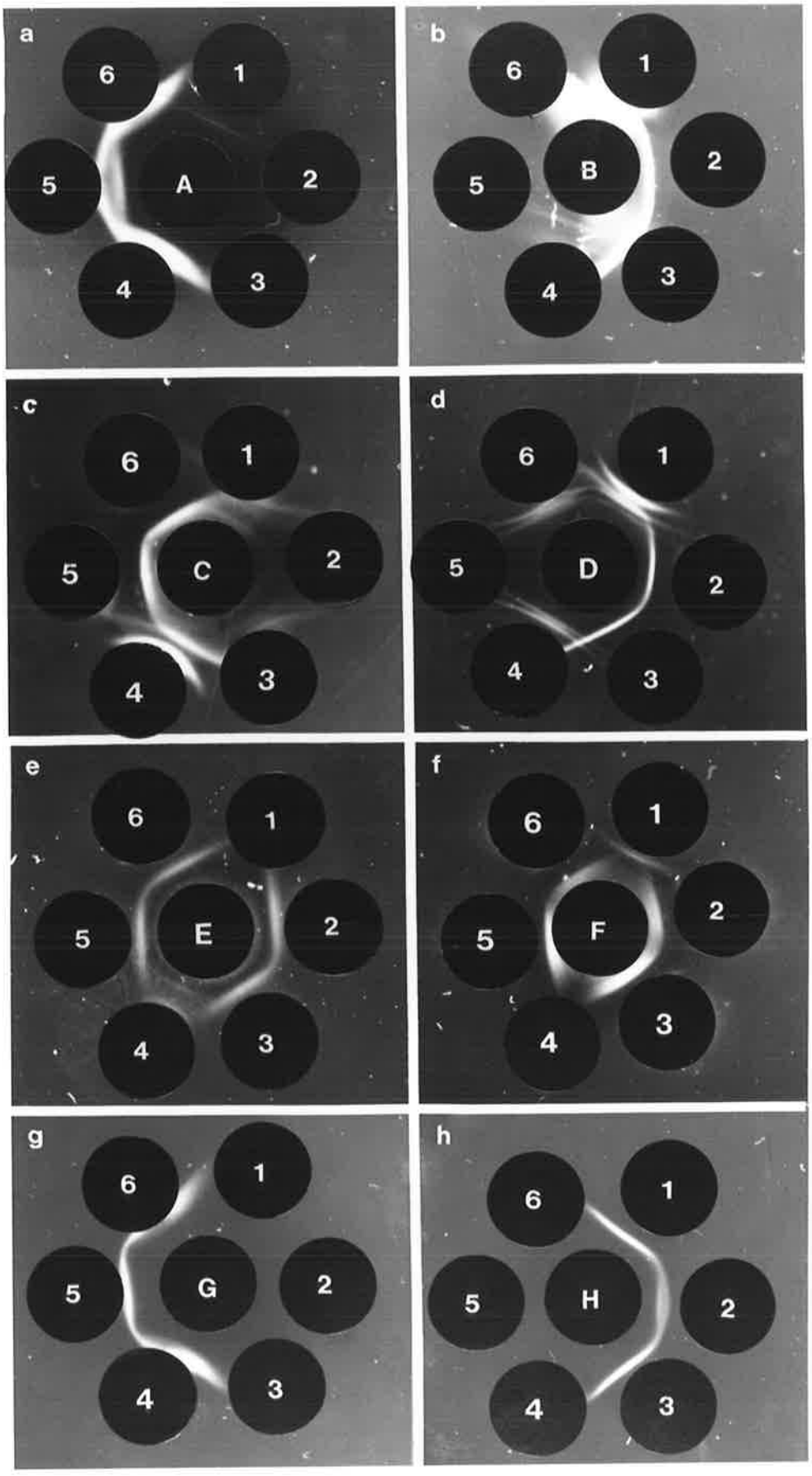

a
6
1
5
A
2
4
3
b
6
1
5
B
2
4
3
c
6
1
5
C
2
4
3
d
6
1
5
D
2
4
3
e
6
1
5
E
2
4
3
f
6
1
5
F
2
4
3
g
6
1
5
G
2
4
3
h
6
1
5
H
2
4
3

Table 27. Responses of several strains to antisera to glutaraldehyde-fixed cells of strains UQM 551 (P. syringae pv. pisi) and L (P. syringae pv. syringae), in Ouchterlony gel double-diffusion tests, using whole untreated, sonicated, and heat-killed cells as antigen.

	Antiserum to fixed cells of strain					
	UQM 551			**L**		
Antigen	untrea.	sonic.	heat-killed	untrea.	sonic.	heat-killed
UQM551	+	+	+	-*	-	-
L	-*	-	-	+	+	+
815,1217, 2452,3940, 3941,3575, 5055,5310, 5316, DAR30566, DAR33369 DAR58730	+	+	+	-*	-	-
1212,2796, 3543,3576, 5319, DAR33368, DAR33379, DAR35712, N,O, P47,P52,P60, RYE9, UQM213, UQM227	-*	-	-	+	+	+
3319,3939, DAR26811, DAR41320, DN20,DN28, P53,P55	-*	-	-	-*	-	-
P. syringae pv. phaseolicola pv. coronafaciens	-*	-	-	-*	-	-
P. fluorescens	-	-	-	-	-	-
Agrobacterium Escherichia coli Rhizobium	-	-	-	-	-	-

\+ = Presence of a strong precipitation line.
-* = Presence of a very weak (halo) precipitation line.
\- = No precipitation line was formed.

Reaction of antisera with reference cultures (Table 25 and 26) are shown in Table 28.

3. Antisera to heat-killed bacterial cells

The highest immunodiffusion titre of antiserum to UQM 551 (pv. pisi) was 1/64, and to L (pv. syringae) was 1/32. Antisera showed a weak reaction (precipitation line) with its homologous antigen, when whole untreated cells were used as test antigen. Although these antisera showed strong reactions with heat-killed and sonicated cells of homologous antigens, the same reaction was also observed when heat-killed and sonicated cells of heterologous antigen were used as test antigens (Fig 20 e, f).

Reaction of antisera with other pathovars of P. syringae was the production of a strong precipitation line, when heat-killed and sonicated cells were used as test antigens. No reaction with other species of Pseudomonas and other genera was evident, using all forms (whole untreated, sonicated, and heat-killed cells) of test antigen.

Results are summarized in Table 29.

4. Absorption of antisera

Antisera to glutaraldehyde-fixed cells were absorbed with bacterial cells of heterologous antigen to remove common antigenic determinant(s) which showed as a halo (very weak precipitation line), when whole untreated heterologous cells were used as test antigen (Fig 20 a, b). Antisera were cross absorbed with different forms (whole untreated, fixed, sonicated, and heat-killed cells) of heterologous antigen. Results of cross absorption showed that fixed bacterial cells did not absorb as efficiently as other forms, presumably because of their lack of solubility. There was no difference between antisera absorbed with other antigen forms, which could absorb the antisera and no precipitation line with heterologous antigen was evident following cross-absorption (Fig 21 a, b).

Table 28. Responses of several strains to antisera to sonicated cells of strains UQM 551 (P. syringae pv. pisi) and L (P. syringae pv. syringae), in Ouchterlony gel double-diffusion tests, using whole untreated, sonicated, and heat-killed cells as antigen.

	Antiserum to sonicated cells of strain					
	UQM 551			**L**		
Antigen	untrea.	sonic.	heat-killed	untrea.	sonic.	heat-killed
UQM551	+	+	+	+*	+*	-
L	+*	+*	-	+	+	+
815,1217, 2452,3940, 3941,3575, 5055,5310, 5316, DAR30566, DAR33369 DAR58730	+	+	+	+*	+*	-
1212,2796, 3543,3576, 5319, DAR33368, DAR33379, DAR35712, N,O, P47,P52,P60, RYE9, UQM213, UQM227	+*	+*	-	+	+	+
3319,3939, DAR26811, DAR41320, DN20,DN28, P53,P55	+*	+*	-	+*	+*	-
P. syringae pv. phaseolicola pv. coronafaciens	+*	+*	-	+*	+*	-
P. fluorescens	+*	+*	-	+*	+*	-
Agrobacterium Escherichia coli Rhizobium	-	-	-	-	-	-

\+ = Presence of a strong precipitation line.
+* = Presence of a weak precipitation line (cross reactivity).
\- = No precipitation line was formed.

Table 29. Responses of several strains to antisera to heat-killed cells of strains UQM 551 (P. syringae pv. pisi) and L (P. syringae pv. syringae), in Ouchterlony gel double-diffusion tests, using whole untreated, sonicated, and heat-killed cells as antigen.

	Antiserum to heat-killed cells of strain					
	UQM 551			L		
Antigen	untrea.	sonic.	heat-killed	untrea.	sonic.	heat-killed
UQM551	+*	+	+	-	+	+
L	-	+	+	+*	+	+
815,1217, 2452,3940, 3941,3575, 5055,5310, 5316, DAR30566, DAR33369 DAR58730	+*	+	+	-	+	+
1212,2796, 3543,3576, 5319, DAR33368, DAR33379, DAR35712, N,O, P47,P52,P60, RYE9, UQM213, UQM227	-	+	+	+*	+	+
3319,3939, DAR26811, DAR41320, DN20,DN28, P53,P55	-	+	+	-	+	+
P. syringae pv. phaseolicola pv. coronafaciens	-	+	+	-	+	+
P. fluorescens	-	-	-	-	-	-
Agrobacterium Escherichia coli Rhizobium	-	-	-	-	-	-

\+ = Presence of a strong precipitation line.
+* = Presence of a weak precipitation line.
\- = No precipitation line was formed.

Figure 21. Reaction of antisera to glutaraldehyde-fixed, sonicated, and heat-killed strain L (pv. syringae) and UQM 551 (pv. pisi) of Pseudomonas syringae, following cross absorption, to homologous and heterologous antigens, in Ouchterlony gel double-diffusion test.

Central wells:

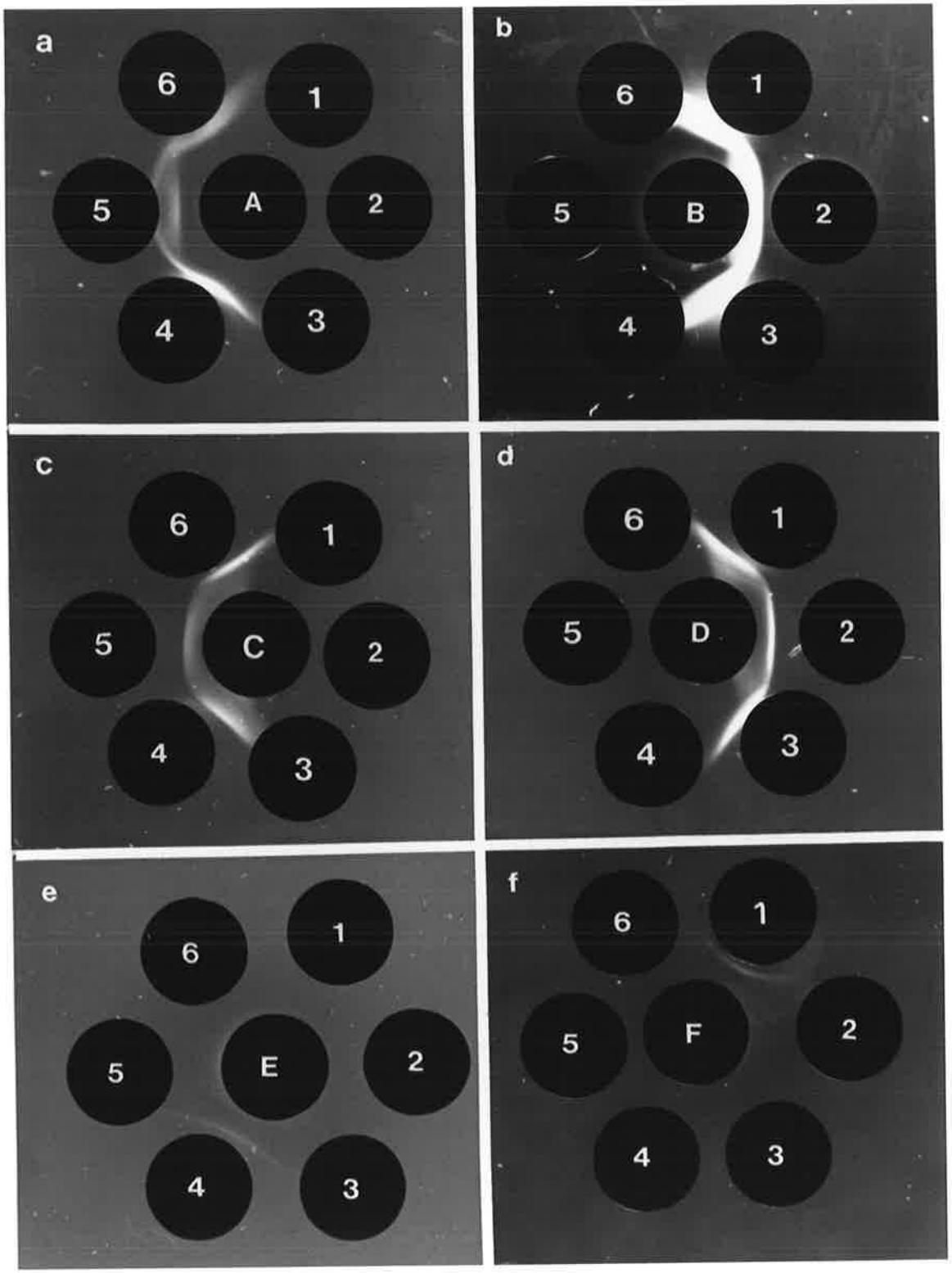
a) Antiserum to glutaraldehyde-fixed cells of strain L
b) Antiserum to glutaraldehyde-fixed cells of strain UQM 551

c) Antiserum to sonicated cells of strain L
d) Antiserum to sonicated cells of strain UQM 551

e) Antiserum to heat-killed cells of strain L
f) Antiserum to heat-killed cells of strain UQM 551

Outer wells:

1, whole untreated bacterial cells of antigen UQM 551; 2, heat-killed bacterial cells of antigen UQM 551; 3, sonicated bacterial cells of antigen UQM 551; 4, whole untreated bacterial cells of antigen L; 5, heat-killed cells of antigen L; 6, sonicated cells of antigen L.


a
6
1
5
A
2
4
3
b
6
1
5
B
2
4
3
c
6
1
5
C
2
4
3
d
6
1
5
D
2
4
3
e
6
1
5
E
2
4
3
f
6
1
5
F
2
4
3

Antisera to sonicated cells were absorbed with heterologous antigens. No cross reactivity was observed following cross absorption (Fig 21 c, d).

Absorbed antisera to heat-killed cells with whole untreated heterologous antigen, showed no change in their reactivity. However, cross-absorption with sonicated and heat-killed heterologous antigen, resulted in loss of precipitation line when either homologous or heterologous cells were used as test antigen (Fig 21 e, f).

Reactions of antisera with homologous and heterologous antigen following cross-absorption are shown in Table 30.

Table 30. Reaction of antisera to various antigenic preparations of P. syringae pv. syringae and pv. pisi following cross absorption with heterologous antigen, against their homologous and heterologous antigens in Ouchterlony gel double-diffusion tests.

	form of test antigen	Homologous	Heterologous
Antisera (cross-absorbed with whole untreated cells)			
to fixed cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to sonicated cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to heat-killed cells	whole untreated	+*	-
	heat-killed	+	+
	sonicated	+	+
Antisera (cross-absorbed with heat-killed cells)			
to fixed cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to sonicated cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to heat-killed cells	whole untreated	+*	-
	heat-killed	-	-
	sonicated	-	-
Antisera (cross-absorbed with sonicated cells)			
to fixed cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to sonicated cells	whole untreated	+	-
	heat-killed	+	-
	sonicated	+	-
to heat-killed	whole untreated	+*	-
	heat-killed	-	-
	sonicated	-	-

+ = Presence of a strong precipitation line.
+* = Presence of a weak precipitation line.
- = No precipitation line was formed.

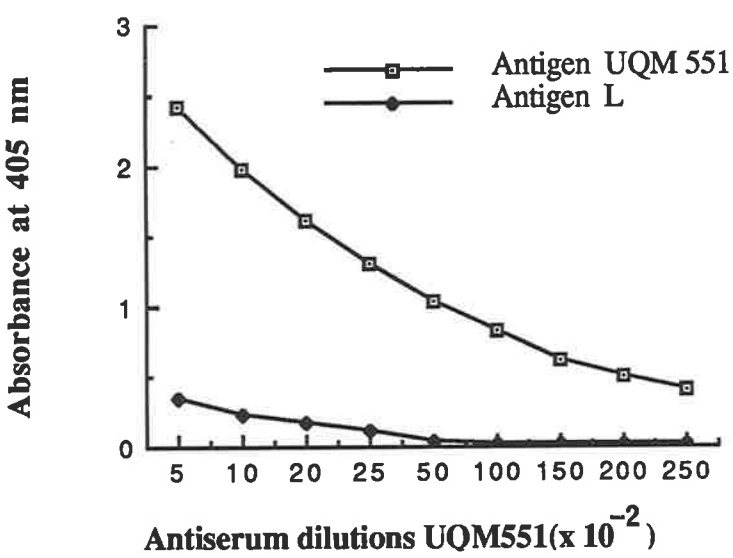
ii) Indirect ELISA

Different dilutions of cross absorbed antisera to glutaraldehyde-fixed strains UQM 551 and L were tested against homologous and heterologous antigens, using whole untreated, and heat-killed bacterial cells as antigen. A comparison of antisera absorbed with different heterologous antigenic forms (whole untreated, sonicated, fixed, and heat-killed cells), showed that, as in the Ouchterlony gel double-diffusion test, fixed bacterial cells did not absorb as efficiently as other forms. There was no difference between antisera absorbed with other forms. Both antisera had a high level of specificity against their homologous antigens.

The system was optimized with different antiserum and antigen concentrations. Antiserum dilutions from 1/500 to 1/25000 and antigen concentrations from 5 μg to 125 pg were tested. Weak positive reactions (light yellow colour) were obtained with 1/500-1/2500 dilutions of antisera with heterologous antigen. At 1/500 and 1/1000 dilutions, even other bacteria and controls (coating buffer without antigen) gave a weak positive response (light yellow colour). The best dilution was 1/5000, which could detect 15.6 ng of homologous antigen without cross reactivity with heterologous antigen, even with a 5-h incubation period after addition of substrate. Antiserum dilutions from 1/5000 to 1/25000 could still detect homologous antigen, but reaction at 1/25000 was weaker for antiserum to fixed L compared with antiserum to fixed UQM 551 (Fig 22).

All strains (Table 25) were tested by indirect ELISA with antiserum dilution 1/5000. Each sample was added to two wells and the experiment was repeated. Results are shown in Table 31 and Fig 23.

Figure 22. Interactions between cross-absorbed antisera to glutaraldehyde-fixed cells of strains UQM 551 (pv. pisi) and L (pv. syringae) of Pseudomonas syringae at several dilutions and homologous and heterologous antigens in indirect ELISA.


Absorbance at 405 nm
3
2
1
0
Antigen UQM 551
Antigen L
5 10 20 25 50 100 150 200 250
Antiserum dilutions UQM551(x 10^{-2})

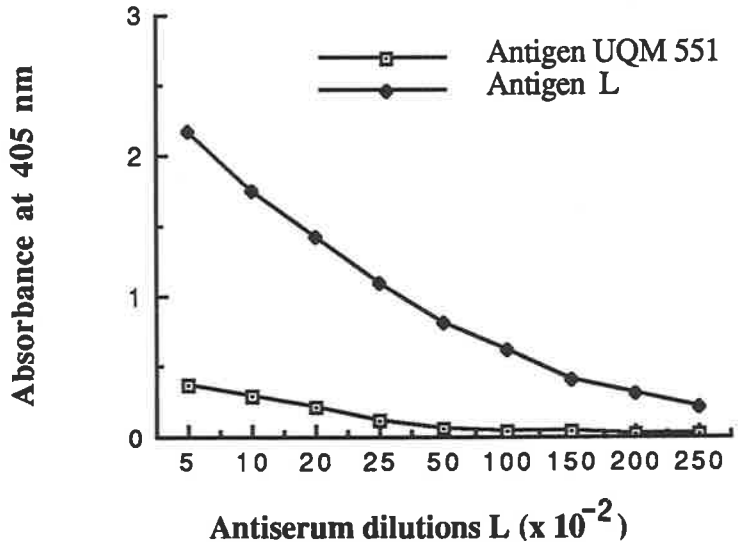

Absorbance at 405 nm
3
2
1
0
Antigen UQM 551
Antigen L
5 10 20 25 50 100 150 200 250
Antiserum dilutions L (x 10^{-2})

Table 31. Responses of strains of P. syringae pv. syringae and pv. pisi and other bacteria to antisera[a] raised against glutaraldehyde-fixed cells of strains UQM 551 (P. syringae pv. pisi) and L (P. syringae pv. syringae), in indirect ELISA.

	Absorbance at 405nm[c]	
Antigen[b]	Antiserum to fixed strain UQM 551	Antiserum to fixed strain L
UQM551	0.933	0.059
L	0.046	0.804
815,1217,2452,3940, 3941,3575,5055,5310, 5316,DAR30566, DAR33369,DAR58730	0.836-0.931	0.029-0.190
1212,2796,3543,3576, 5319,DAR33368,DAR33379, DAR35712,N,O,P47,P52,P54, P60,RYE9,UQM213,UQM227	0.033-0.085	0.715-0.820
3319,3939,DAR26811,DAR 41320,DN20,DN28,P53,P55	0.132-0.236	0.109-0.403
Other bacteria[d]	0.008-0.091	0.023-0.088
coating buffer (without antigen)	0.000-0.006	0.000-0.004

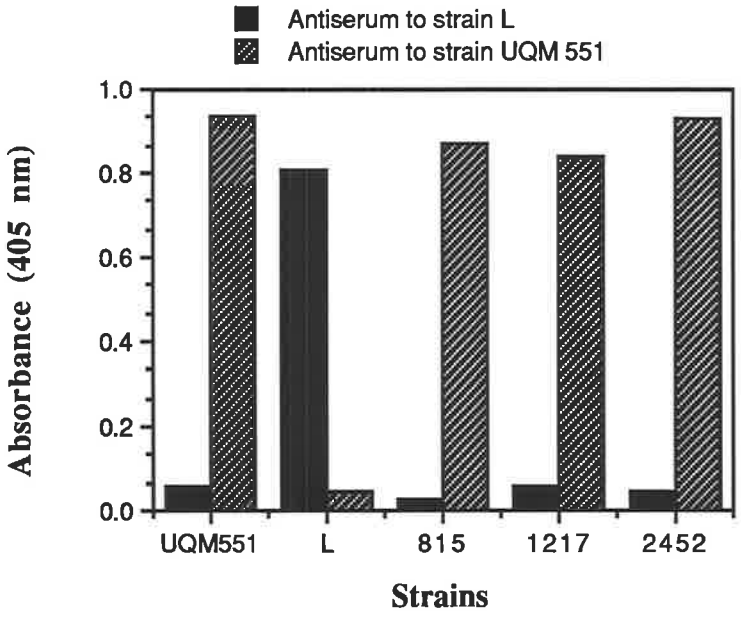
a = Antisera were used at 1/5000 dilution.

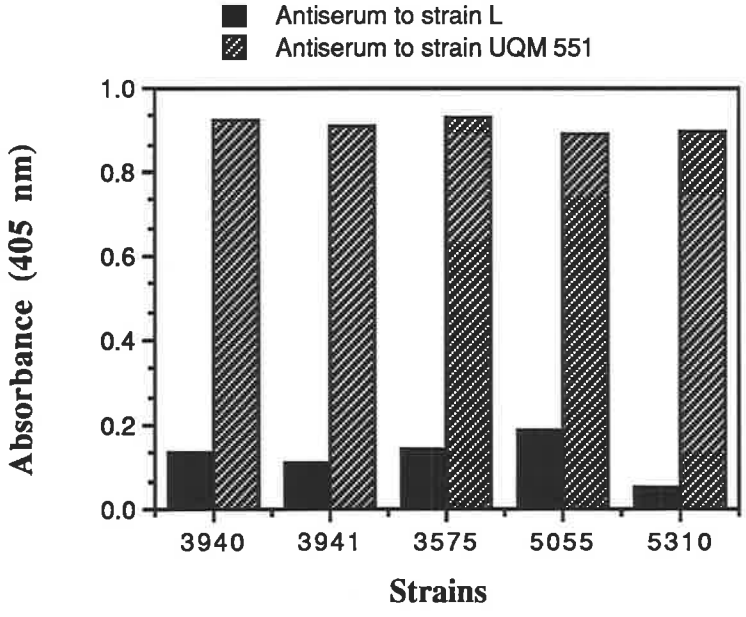
b = Antigen concentration was 5 µg/ml, and whole untreated cells were used as test antigen.

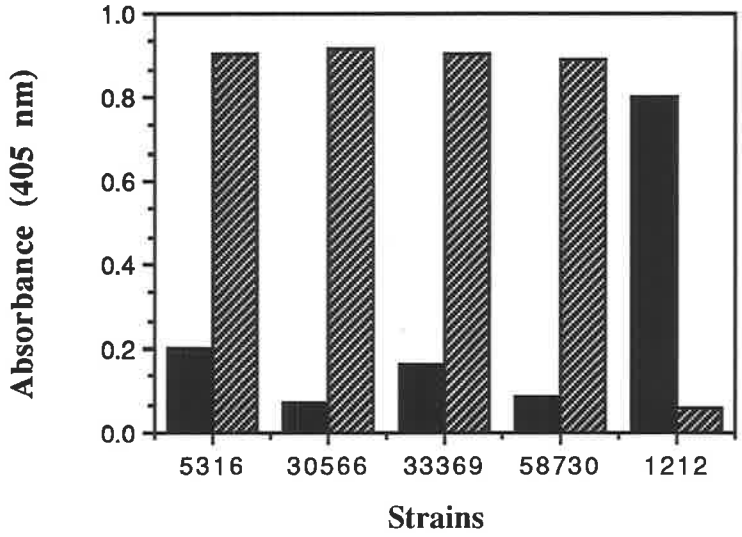
c = Absorbance values were recorded after 1 hour incubation of the substrate.

d = Pseudomonas syringae pv. phaseolicola, P. syringae pv. coronafaciens, P. fluorescens, Escherichia coli, Rhizobium, Agrobacterium.

Figure 23. Responses of strains of P. syringae pv. pisi and pv. syringae to cross-absorbed antisera raised against glutaraldehyde-fixed cells of strains UQM 551 (pv. pisi) and L (pv. syringae), in indirect ELISA. Antisera were used at 1/5000 dilution. Antigen concentration was 5 µg/ml and whole untreated cells were used as test antigen. Absorbance values were recorded after 1 hour incubation of the substrate.


Antiserum to strain L
Antiserum to strain UQM 551
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
UQM551
L
815
1217
2452
Strains


Antiserum to strain L
Antiserum to strain UQM 551
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
3940
3941
3575
5055
5310
Strains


Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
5316
30566
33369
58730
1212
Strains

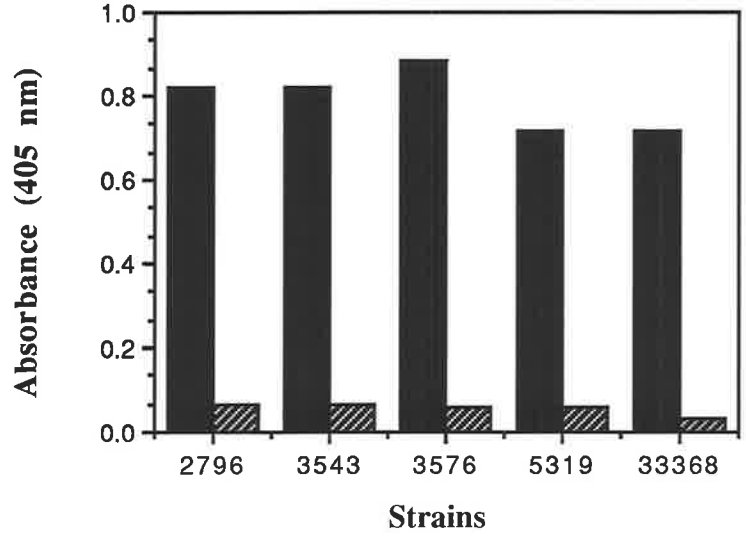
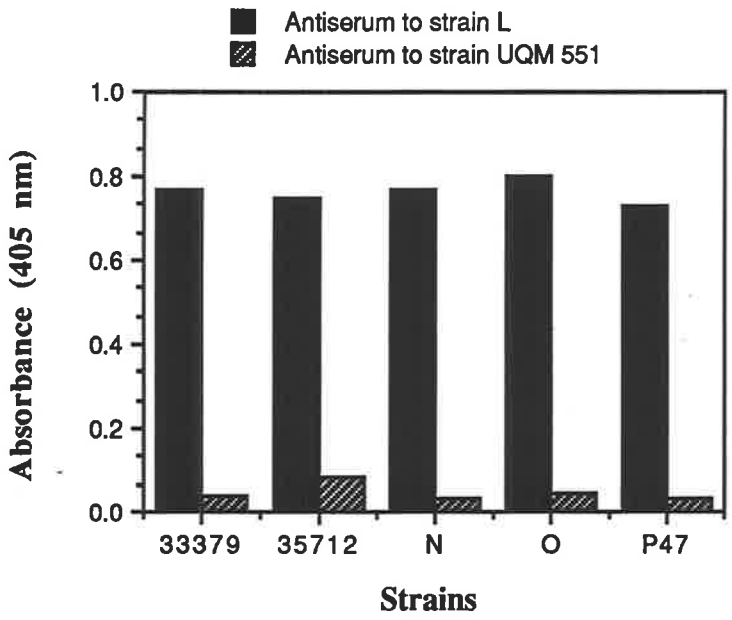
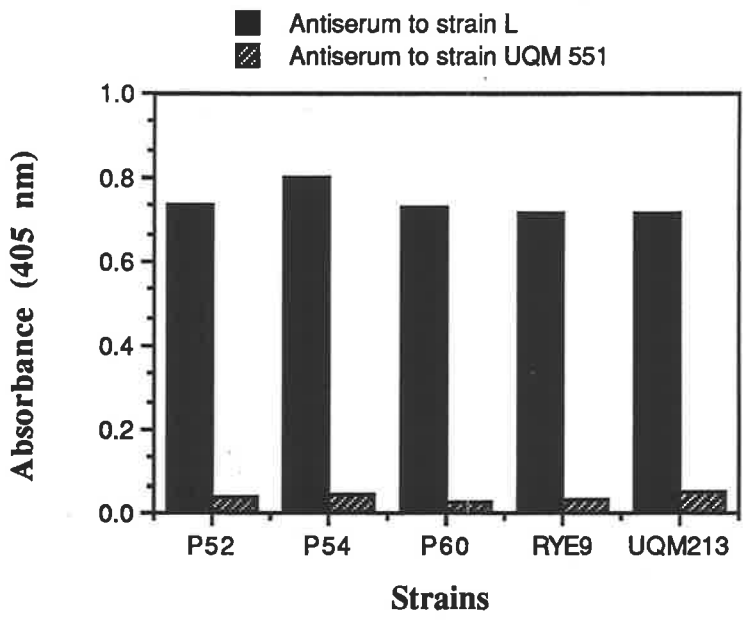
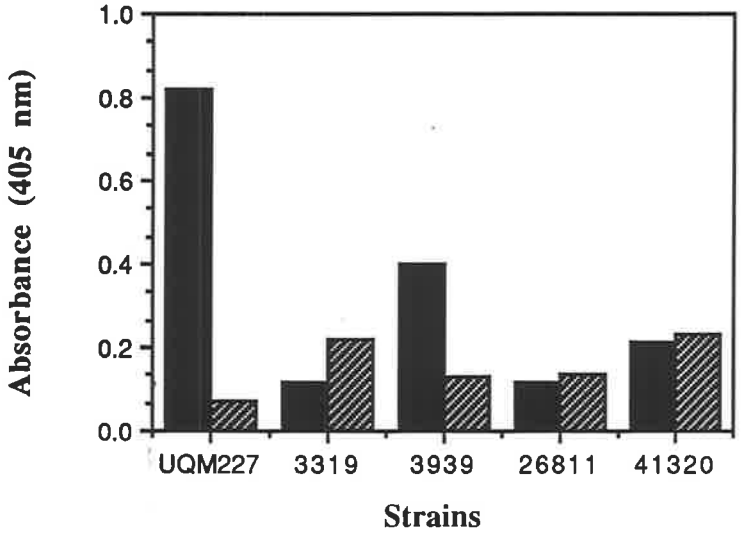
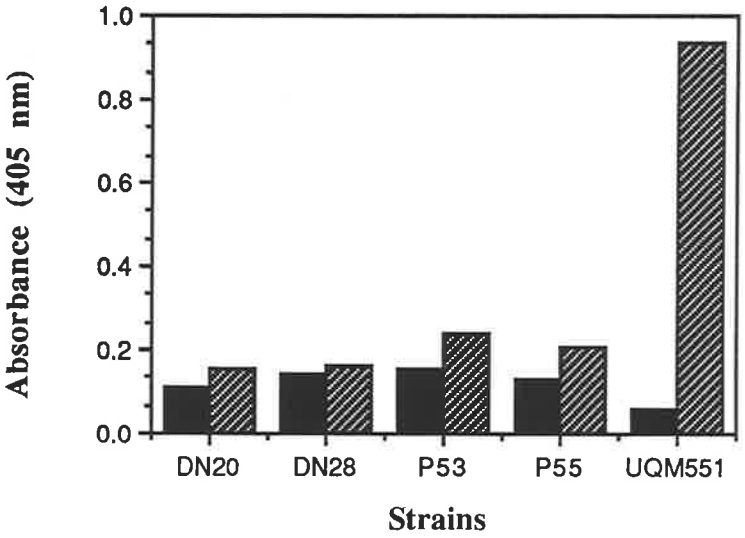

Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
2796
3543
3576
5319
33368
Strains

Figure 23, continued.


Antiserum to strain L
Antiserum to strain UQM 551
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
33379
35712
N
O
P47
Strains


Antiserum to strain L
Antiserum to strain UQM 551
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
P52
P54
P60
RYE9
UQM213
Strains


Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
UQM227
3319
3939
26811
41320
Strains


Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
DN20
DN28
P53
P55
UQM551
Strains

iii) High level of specificity of antisera to glutaraldehyde-fixed cells

The highly specific reaction of antiserum to glutaraldehyde-fixed cells was investigated further. Glutaraldehyde treatment stabilizes bacterial cells and prevents degradation in immunised rabbits. Such fixation preserves the natural antigenicity of the molecule (Reichlin, 1980). Upon immunization of rabbits, presumably most antibodies produced were directed against antigenic determinants located on the surface of bacteria.

According to Beveridge (1988) and Digat and Cambra (1976) surface antigens of a bacterial cell consist of: a) Flagella (protein). b) Exopolysacharide slime (polysaccharide). c) Glycoprotein. d) Cell wall: isolated by hot phenol (lipopolysaccharide); isolated by cold trichloracetic acid (lipopolysaccharide + protein). e) Membrane protein complex (phospholipid + protein) (see Fig 24).

Surface antigens of P. syringae pv. pisi (strain UQM 551) and P. syringae pv. syringae (strain L) were isolated according to the standard procedures described previously. Each antiserum was tested with its corresponding homologous and heterologous surface antigens, in Ouchterlony gel double-diffusion; untreated and heat-killed surface antigens were used. Unabsorbed antisera were used to determine if cross reaction occurred with heterologous antigens.

iv) Reaction of antisera with surface antigens

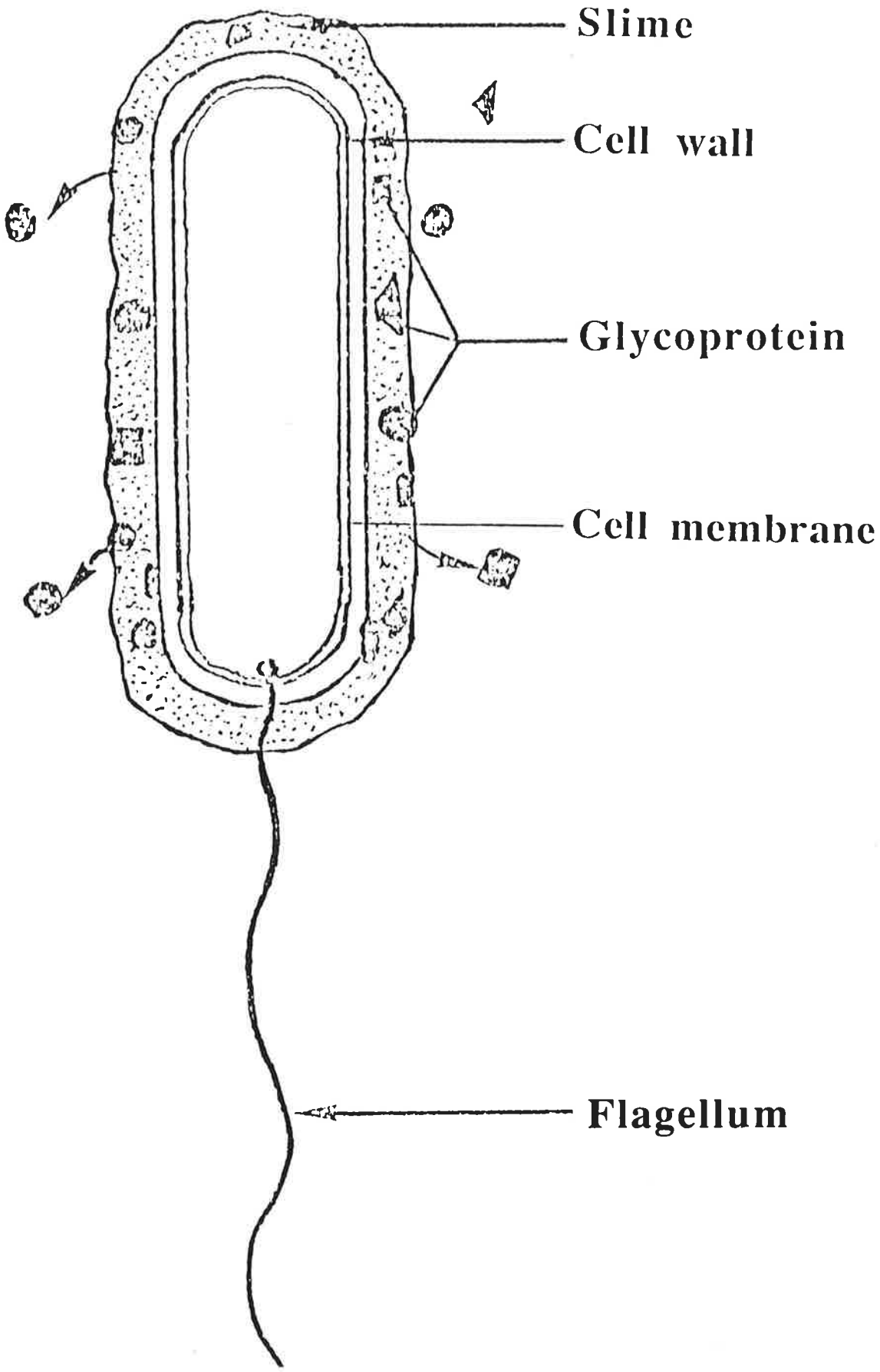
1. Homologous reaction

Each antiserum showed a strong precipitation line with its corresponding homologous surface antigen, except when slime and cell wall (isolated by hot phenol procedure) were used as antigens, weak reactions were observed.

2. Heterologous reaction

Antisera showed cross reactivity with the untreated heterologous surface antigens slime, glycoprotein and membrane protein complex. No cross reactivity was observed when heat-treated surface antigens were used. Flagella and cell wall were

Figure 24. A schematic diagram (Digat and Cambra, 1976) of the surface antigens in Pseudomonas.


Slime
Cell wall
Glycoprotein
Cell membrane
Flagellum

the only surface antigens which showed no cross reactivity, using both untreated and heat-killed forms as antigens.

v) Antisera to glutaraldehyde-fixed flagella

The highest immunodiffusion titre of antiserum to isolate UQM 551 (pv. pisi) was 1/128, and to isolate L (pv. syringae) was 1/64. Antiserum to fixed flagella of strain UQM 551 and to fixed flagella of strain L had a high level of specificity. With heterologous antigens (whole untreated, sonicated, and heat-killed cells), no cross reactivity was observed (Fig 20 g, h).

No cross-reaction was evident between these antisera and antigens from different pathovars, other species of Pseudomonas or other genera.

Reactions of antisera with reference cultures (Table 25, 26) are shown in Table 32.

Table 32. Responses of several strains to antisera to glutaraldehyde-fixed flagella of strains UQM551 (P. syringae pv. pisi) and L (P. syringae pv. syringae), in Ouchterlony gel double-diffusion tests, using whole untreated, sonicated, and heat-killed cells as antigen.

	Antiserum to flagella of strain					
	UQM 551			L		
Antigen	untrea.	sonic.	heat-killed	untrea.	sonic.	heat-killed
UQM551	+	+	+	-	-	-
L	-	-	-	+	+	+
815,1217, 2452,3940, 3941,3575, 5055,5310, 5316, DAR30566, DAR33369 DAR58730	+	+	+	-	-	-
1212,2796, 3543,3576, 5319, DAR33368, DAR33379, DAR35712, N,O, P47,P52,P60, RYE9, UQM213, UQM227	-	-	-	+	+	+
3319,3939, DAR26811, DAR41320, DN20,DN28, P53,P55	-	-	-	-	-	-
P. syringae pv. phaseolicola pv. coronafaciens	-	-	-	-	-	-
P. fluorescens	-	-	-	-	-	-
Agrobacterium Escherichia coli Rhizobium	-	-	-	-	-	-

\+ = Presence of a strong precipitation line.
\- = No precipitation line was formed.

Discussion

Polyclonal antisera against P. syringae pv. syringae and P. syringae pv. pisi have a high level of specificity. By using antisera to glutaraldehyde-fixed bacterial cells, both in Ouchterlony gel double-diffusion and in indirect ELISA following cross absorption, the two pathovars can be easily differentiated, using all forms (whole untreated, sonicated or heat-killed) of bacterial cells as test antigens.

Previously, polyclonal antisera were raised against P. syringae pv. pisi by Taylor (Taylor, 1972b; Taylor and Dye, 1972). He used whole untreated bacteria as immunogens and such antisera could differentiate pv. pisi from pv. syringae in agglutination tests only when heat-killed bacterial cells were used as test antigens. Consequently, their application under field conditions would be limited. In contrast, our antisera show a high level of specificity both in Ouchterlony gel double-diffusion and ELISA tests, even when whole untreated cells are used as test antigen.

Despite the difference in specificity of antiserum raised to whole untreated bacterial cells compared with those elicited by glutaraldehyde-fixed cells, both types of antisera demonstrated that the antigenic structure of each bacterium possesses unique as well as common antigenic determinant(s). Because glutaraldehyde fixation, stabilizes the surface of bacterial cells, presumably most antibodies produced were directed against antigenic determinant(s) located on the surface of the bacteria. On the other hand, antibodies in the antisera to whole untreated bacterial cells raised by Taylor (1972b), may have been targeted both to unique antigenic site(s) and internal common antigenic determinant(s), which presumably are destroyed by heat denaturation when bacterial cells used as test antigens are heat-treated. The same phenomenon can explain the reactions of antisera raised by sonicated cells. There again, specificity was obtained only when heat-treated cells were used as antigens.

This specificity of heat-treated test antigen, suggested that heat-killed bacterial cells of these two pathovars do not share common antigenic determinant(s). If so, antisera to heat-killed cells of the pathovars should react only with homologous antigens without cross reactivity with heterologous antigens, when whole untreated,

sonicated or heat-killed cells are used as test antigens. Unexpectedly, antisera to heat-killed cells showed a strong reaction (precipitation line) with both homologous and heterologous antigens when sonicated or heat-killed cells were used as test antigens (Table 29). Antisera to heat-killed bacterial cells could differentiate the two pathovars without cross reactivity, only when whole untreated cells were used as test antigen. However, it reacted weakly with its homologous antigen (Table 29). It is possible that because of this weak reaction with homologous antigen, reaction with heterologous antigen is too weak to be detected.

The most useful attribute of antiserum to heat-killed cells, is its ability to distinguish Pseudomonas syringae from other species of Pseudomonas and from other genera. All pathovars tested gave a positive reaction, but P. fluorescens, E. coli, Agrobacterium and Rhizobium did not react. Further investigation is warranted using other pathovars of P. syringae, other species of Pseudomonas and other genera to define more accurately the specificity.

Absorption of antisera with different forms (whole untreated, sonicated, and heat-killed) of heterologous antigen shows that, a relationship exists between antigen preparation as immunogen and antigen preparation as absorption antigen. Results of absorbed antisera to glutaraldehyde-fixed and to sonicated cells shows that all forms (whole untreated, sonicated, and heat-killed) of heterologous antigen could remove the cross reactivity (Table 30). This did not apply to antisera to heat-killed cells. Precipitation lines both in homologous and heterologous interactions disappeared following cross absorption of antisera to heat-killed cells with sonicated or heat-killed forms of heterologous antigens. This indicates that sonication and heat-killing of the cells of these two pathovars exposed common but not specific antigenic determinants recognized by antisera to heat-killed cells. On the contrary, the antigens gave a specific reaction with other antisera. The basic reason for these results is not known.

All these data show that cells of both pathovars pisi and syringae contain unique antigenic determinant(s), and common antigenic determinant(s); the

expression of unique and common antigenic determinant(s) is dependent on the kind of immunogen used and also on the form of the test antigen.

The conclusion that antisera to glutaraldehyde-fixed cells were directed against surface antigens prompted a more detailed examination of these antigens. Of the surface antigens purified, only cell wall and flagellar antigens did not produce cross reactivity with heterologous antiserum. Antisera to glutaraldehyde-fixed flagella of these two pathovars showed the same specificities as antisera to glutaraldehyde-fixed cells, except that the very weak precipitation line (halo) which was observed with antisera to fixed cells, when whole untreated cells of heterologous cells were used as test antigen, was never observed when antisera to flagella of these two pathovars were used.

These data indicate that specificity of antisera to glutaraldehyde-fixed cells could be the result of antibodies elicited to flagella.

All strains of the two pathovars that had been previously examined by nutritional and pathogenicity tests were checked against all the antisera and most gave the expected reaction. Eight strains, 3319, 3939, DAR26811, DAR41320, DN20, DN28, P53 and P55 showed the same reaction with all the antisera in Ouchterlony gel double-diffusion tests (Tables 27,28,29,32). In indirect ELISA, they showed a weak reaction with antisera to glutaraldehyde-fixed cells (Table 31, Fig 23). Strain P55 was pathogenic to lemon fruit, but not to pea stems. Strains 3319, DAR26811, DAR41320, DN20 and DN28 gave the typical P. syringae pv. syringae reaction following inoculation of peas and lemons. There would appear to be more than one serotype of pv. syringae. Because of the weak serological reaction of the pv. syringae strains to the antiserum to strain UQM 551 (pv. pisi), it is unlikely that they would interfere with a diagnostic test for pv. pisi using ELISA.

All pathogenic strains of P. syringae pv. pisi reacted strongly with homologous antiserum. A problem is posed by strains 3940 and 3941. They reacted strongly with antiserum to strain UQM 551 (pv. pisi), but did not produce typical water-soaked lesions on pea stems. The distribution and abundance of such strains would be an

important consideration in the development of a commercial diagnostic test. Characteristics of these two nonpathogenic strains of pv. pisi are described in section C.

Seed-borne infection of any pathogen is a very efficient method of its distribution. Hence, the need for seed health testing is of particular importance. Because of the high level of specificity with indirect ELISA, which is a very sensitive serological assay, a screening procedure for pea seed could probably be developed. At present antiserum to P. syringae pv. pisi can be used with confidence in the laboratory to detect P. syringae pv. pisi. However, the suitability of the antiserum under field conditions needs to be tested.

Section C: Plasmids and pathogenicity

Introduction

Pseudomonas syringae pv. syringae and Pseudomonas syringae pv. pisi, causal agents of bacterial blight of peas, are very closely related plant pathogenic bacteria. The two pathovars can be readily distinguished serologically (section B. 2), but some non-pathogenic strains also react with antisera to both pathovars. In an attempt to determine the difference between pathogenic and non-pathogenic strains with the same serological reaction, the plasmid complement of several strains of both P. syringae pv. syringae and P. syringae pv. pisi was determined. This was followed by studies on plasmid transfer, which established that plasmids in P. syringae pv. pisi carry genes for pathogenicity.

Materials and methods

a) Bacterial strains and plasmids

Strains of P. syringae pv. syringae and P. syringae pv. pisi obtained from several different sources, are listed in Table 25. Spontaneous mutants resistant to 50 μg/ml rifampicin were selected from strains 3939, 3940, 3941, P53, P55, P60 and UQM 213. Escherichia coli strain S17.1 (Simon, 1984; Simon *et al.*, 1983) containing the suicide plasmid vector pSUP5011 was used for Tn5 mutagenesis. In this plasmid, a mobilization site of 1.9 kilobases (kb) has been inserted into Tn5, making a total transposon of 7.4 kb with a 5.2 kb HindIII internal fragment. E. coli strain HB101 (R68.45) (Haas and Holloway, 1976), was used as a conjugative helper; it provided the transfer functions (Tra) on plasmid R68.45. A plasmid which contains the HindIII internal fragment of Tn5 subcloned in pUC18 (Messing, 1983) was used for probing.

b) Media and culture conditions

Pseudomonas strains were grown on mannitol glutamate (MG) minimal medium (Roberts and Kerr, 1974) at 25°C; E. coli strains were grown on Luria-Bertani (LB) agar medium (Maniatis et al., 1982) at 37°C. Selective antibiotic concentrations were as

follows; rifampicin (Rif), 50 μg/ml; kanamycin (Km), 50 μg/ml for P. syringae or 25 μg/ml for E. coli; tetracycline (Tc), 10 μg/ml; and chloramphenicol (Cm), 50 μg/ml.

c) Bacterial conjugations

Bacterial conjugations between P. syringae pv. pisi and E. coli were performed on tryptone yeast (TY) medium (Beringer, 1974). Conjugations between P. syringae strains were performed on MG medium. Overnight cultures of E. coli grown on LB agar plates and 36 to 48 h cultures of P. syringae grown on MG agar plates were used in surface matings.

For Tn5 mutagenesis, loopfuls of E. coli S17.1 and a P. syringae pv. pisi recipient were mixed in a 1:1 ratio on TY medium. The plates were incubated at 28°C for 16 h and cells suspended in sterile distilled water, serially diluted and plated onto MG agar plates containing Km. (Neither parent can grow on this medium). After incubation for 3 days at 25°C, several hundred Km-resistant (Km^r) transconjugants were pooled and were used as recipients in a second round of conjugation with E. coli, R68.45 (Tc^r), to provide them with the transfer genes. Donors and recipient were incubated for 6 h, then either plated onto MG agar plates containing kanamycin and tetracycline or the whole mixture used as a donor culture in another 16 h mating (third mating) with a Rif^r P. syringae (pv. pisi or pv. syringae) strain as recipient (Fig 25).

Transconjugants containing plasmids with Tn5 insertions were selected on MG medium containing rifampicin and kanamycin, and plasmid transfer confirmed by gel electrophoresis followed by probing with a HindIII internal fragment of Tn5.

d) Plasmid DNA isolation

Plasmid DNA was isolated by an alkaline miniprep procedure (Farrand *et al.*, 1985). The cell pellet from a 1.5ml culture was suspended in 1ml of TE buffer (50mM trisma base, 20mM Na_2 EDTA, pH 8.0), followed by the addition of 0.1ml 5M NaCl and 0.01ml 10% Na sarkosyl with gentle mixing. The suspension was pelleted by centrifugation for 2 min, and 0.1ml Birnboim 1 solution (50mM Glucose, 10mM Na_2

Figure 25. Illustration of transfer of the kanamycin resistance transposon Tn5 to P. syringae pv. pisi (strain DAR 58730), and mobilization of its plasmids (pDAR58730a and pDAR58730b) into P. syringae pv. syringae (strain UQM 213).

A) Escherichia coli, strain S17.1.

B) P. syringae pv. pisi, strain DAR 58730.

C) Tn5 insertion in chromosome.

D) Tn5 insertion in pDAR58730a.

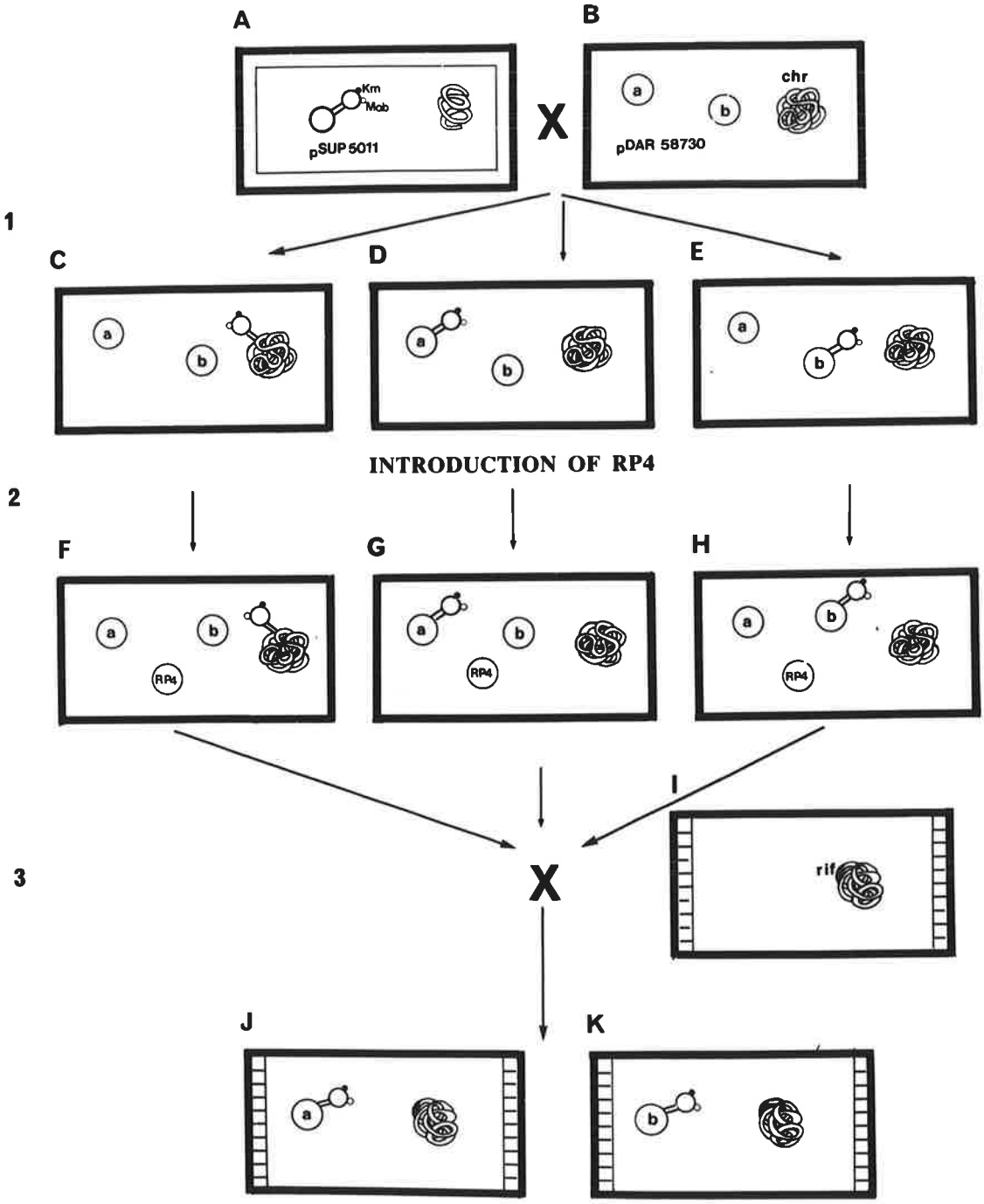
E) Tn5 insertion in pDAR58730b.

F, G, H) Introduction of RP4.

I) P. syringae pv. syringae, strain UQM 213 (rifampicin resistant).

J) Strain UQM 213 (pDAR 58730a : : Tn5).

K) Strain UQM 213 (pDAR 58730b : : Tn5).


A
B
Km
Mob
pSUP 5011
X
a
b
chr
pDAR 58730
1
C
D
E
INTRODUCTION OF RP4
2
F
G
H
RP4
I
3
X
rif
J
K

EDTA, 25mM trisma base, pH 8.0) containing lysozyme (2mg/ml) was added and left on ice for 5 min followed by the addition of 0.2ml Birnboim 2 solution (0.2N NaOH, 1% SDS) by gentle inversion for lysing. After standing for 15 min at room temperature, 2M Tris-HCl, pH 7.0 (0.05ml) was added and left for an additional 30 min at room temperature. The mixture was gently extracted by adding an equal volume of phenol saturated with 3% NaCl. After 5 min, the upper aqueous phase was obtained by a 10-min centrifugation, transferred to a fresh tube and two volumes of cold ethanol and 0.1 volume 3M sodium acetate (pH 5.2) added. The DNA precipitate was centrifuged for 15 min, dried under vacuum and resuspended in 0.02 - 0.03ml TE buffer. Plasmid DNA was separated by electrophoresis for 3 h at 70W through a horizontal 0.7% agarose gel submersed in Tris-borate buffer (89mM Trisma base, 89mM boric acid, 2.5 mM Na_2 EDTA; pH 8.3); the gels were stained for 30 min with ethidium bromide (0.5μg/ml) and photographed under 302-nm UV light.

e) Plasmid transformation

The method of Olsen *et al.* (1982) was used for plasmid transformation. Large-scale plasmid isolations for use in the transformation procedure were done by the method of Casse *et al.* (1979) and purified in ethidium bromide-cesium chloride density gradients (Maniatis *et al.*, 1982).

f) Digestion and separation of DNA fragments

Plasmid samples were digested with 90 units of restriction endonuclease HindIII in the presence of heat-treated RNase at 37°C for 3 h. Digests were separated by horizontal gel electrophoresis in 0.7% agarose gel at 25W for 16 h, using Tris-acetate buffer (Maniatis *et al.*, 1982).

g) DNA hybridization

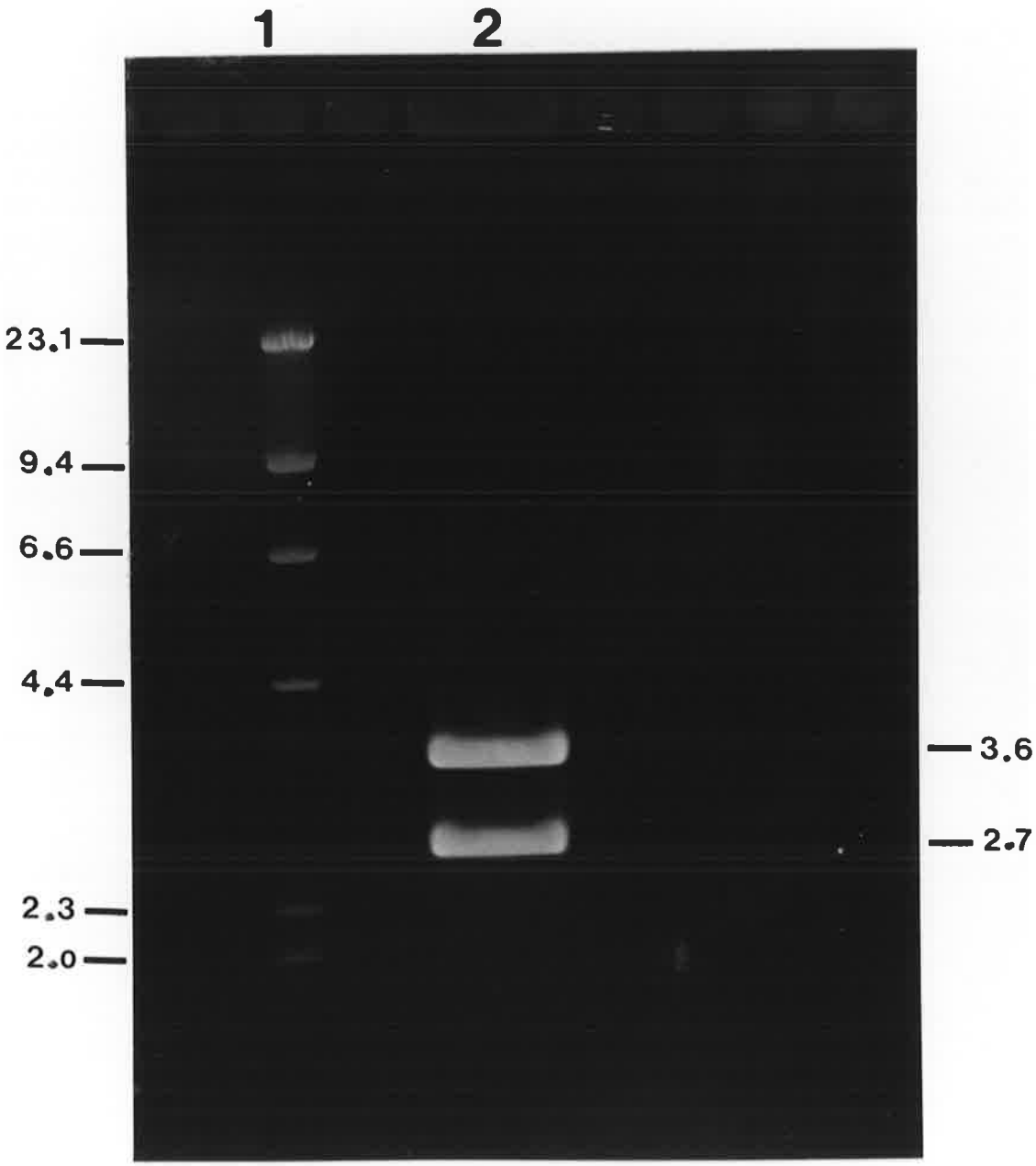
i) DNA used as a probe

A 3.6 kb HindIII internal fragment of Tn5 was used as a probe to verify plasmid labelling. This fragment was obtained by digesting a plasmid which contained the 3.6 kb HindIII internal fragment of Tn5 that had been subcloned into pUC18. DNA fragments were separated by gel electrophoresis at 25W for 16 h in a horizontal 0.7% agarose gel (Fig 26) submersed in Tris-acetate buffer and the 3.6 kb fragment recovered from the gel, using The Geneclean Kit, BIO 101 Inc., as follows. A DNA band was excised from ethidium-bromide stained agarose gel with a razor blade. The volume of the gel slice was determined by weight (1g equals approximately 1ml) and then transferred to a plastic tube. Three volumes of 6M sodium iodide was added to the piece of agarose gel that contained DNA. The tube was placed in a 55°C waterbath for 5 min to dissolve the agarose. To this solution 10 µl of glassmilk (silica matrix) was added, incubated for 5 min at room temperature and pelleted by centrifugation for 5 sec. The pellet was washed 3 times. After the last washing, the pellet was resuspended with 20 µl sterile distilled water, followed by incubation at 55°C for 3 min. The suspension was pelleted by centrifugation for 30 sec and supernatant containing the eluted DNA was carefully removed and placed in a new tube.

ii) DNA labelling

The eluted DNA was labelled with ^{32}P by nick translation (Maniatis *et al.*, 1982), using Nick-translation Kit (Bresatec). A mixture of the eluted DNA (6.5 µl), nucleotide/buffer cocktail C (4 µl), cold nucleotide solution (2 µl), DNA polymerize (5 µl), and ^{32}P (2.5 µl) was placed in a waterbath at 14°C for 90 min. Radiolabelled DNA was separated from the unincorporated nucleotides by loading the reaction mix directly onto a column of Sephadex G-50 suspended in TE buffer. Twenty drop (approximately 500 µl) fractions were collected and monitored for radioactivity. The fractions representing the first peak of radioactivity were pooled and used as a probe in Southern blots.

Figure 26. Fragment banding patterns produced by cleavage of a plasmid which contains HindIII internal fragment of Tn5 that had been subcloned in pUC18, with restriction endonuclease HindIII, followed by separation on a 0.7% agarose gel. Bands are identified from top to bottom. Lane 1, λ DNA HindIII fragments, as a size marker. Lane 2, pUC18 (internal fragment = 3.6 kb).


1
2
23.1
9.4
6.6
4.4
2.3
2.0
3.6
2.7

iii) Southern blot

Southern blots were done by the procedure of Maniatis *et al.* (1982). Plasmid DNA and DNA fragments obtained by restriction digests of plasmids were denatured by soaking the gels in several volumes of a solution of 1.5M NaCl in sterile distilled water containing 0.5N NaOH with constant gentle agitation for 45 min. The gels were rinsed briefly in deionized water, and then neutralized by soaking in several volumes of a solution of 1M Tris (pH 7.4) in sterile distilled water containing 1.5M NaCl at room temperature with constant gentle agitation. The neutralization solution was changed and soaking of the gels continued for a further 15 min as before. The DNA fragments were transferred to nitrocellulose filters by capillary transfer method (Southern, 1975) as outlined by Maniatis *et al.* (1982). The filters were then baked for 2 h at 80°C oven in a vacuum and were prehybridized by treating them in 20 ml of the prehybridization solution containing deionized formamide (50%); Denhardt's reagent (1x Denhardt's reagent contains 0.1g of Ficol, type 400, Pharmacia; 0.1g PVP; 0.1g bovin serum albumin; and water to 10 ml); SDS (0.1%); heat denatured salmon sperm DNA (100 µg/ml); and 4x SSC (1x SSC = 8.77g NaCl, 4.67 Na_3 citrate, and 0.01 ml of 0.2N HCl). The solution was added to a sealable plastic bag containing the membrane, air bubbles removed, the plastic bag was sealed and incubated for 16-24 h at 42°C in a shaking water bath. Heat denatured radioactive probe was added, the plastic bag was resealed as before and hybridization was allowed to proceed at 42°C for 24 h in a shaking water bath. After hybridization, the membranes were washed as follows: a) 3 times in 2x SSC containing 0.1% SDS, 10 min per wash at room temperature; b) 2-3 times in 0.2x SSC containing 0.1% SDS, 30 min per wash at 68°C. The filters were air dried and subjected to autoradiography at -70°C using an intensifying screen. The films were developed after an appropriate exposure time, as instructed by the manufacturer.

h) Homoserine utilization and plant inoculation

Utilization of homoserine as a sole carbon source and stem-inoculation of sensitive host cultivars were done as previously described (section B. 1).

Testing for hypersensitivity (HR) was by inoculation of Nicotiana tabacum cv. White Burley (Klement, 1982).

Results

a) P. syringae pv. pisi and pv. syringae plasmids

All strains which reacted with P. syringae pv. pisi antiserum contained at least two plasmids which ranged from 45 to 80 kb, except for non-pathogenic strains 3940 and 3941 which were plasmidless (Fig 27). In P. syringae pv. syringae, strains L, N, O, 1212, 3319 and 33379 contained one plasmid of about 50 kb; all other strains tested had no plasmid (Fig 28 and Table 33).

b) Plasmid transfer

Because pathogenicity was correlated with the presence of plasmids in strains of P. syringae pv. pisi, attempts were made to transfer the plasmids to plasmidless recipients. Strain DAR 58730 was used as the donor and its two plasmids were named pDAR58730a (c. 55 kb) and pDAR58730b (c. 50 kb). Strain DAR 58730 is non-fluorescent on King's B (KB) medium (King *et al.*, 1954) and this proved useful in distinguishing donor from transconjugants because all recipients were fluorescent. Before plasmid transfer could be attempted, the plasmids had to be labelled with a Tn5 insertion and then R68.45 introduced as described in materials and methods. When the plasmidless strains 3940 Rif^r and 3941 Rif^r of P. syringae pv. pisi were used as recipients, no transconjugants were obtained. To establish that the problem was not with the first and second matings (Tn5 labelling and R68.45 introduction, respectively), several hundred colonies containing both Tn5 and R68.45 were recovered and used as donors with strains 3940 Rif^r and 3941 Rif^r as recipients but again, no transconjugants were obtained. Plasmid extraction from the donors followed by the transformation procedure, was attempted but no transformants were obtained. When other P. syringae pv. pisi strains (Table 33) were used as donors in conjugations, no transconjugants resulted.

Figure 27. Agarose gel electrophoresis of plasmid DNA from several strains of P. syringae pv. pisi. Plasmid bands are identified from top to bottom. Lane 1, strain 3575; lane 2, strain 5310; lane 3, strain 815; lane 4, strain 1217; lane 5, strain 3940; lane 6, strain 3941; lane 7, strain 5316; lane 8, strain DAR 33369; lane 9, strain DAR 30566; lane 10, strain DAR 58730; lane 11, strain 5055; lane 12, strain 2452; lane 13, strain UQM 551. The migrations of the molecular weight standards are shown at the right of the gel; E. coli (C600) pNJ5000 (44 kb) (Saint *et al.*, 1990); the sizes of plasmids from Agrobacterium rhizogenes (K84) pAgK84 (47) (Slota and Farrand, 1982); E. coli (HB101) pPH1JI (55) (Hirsch and Beringer, 1984); P. putida (UCC22) pTDN1 (79) (Saint *et al.*, 1990). Chr, chromosomal DNA.

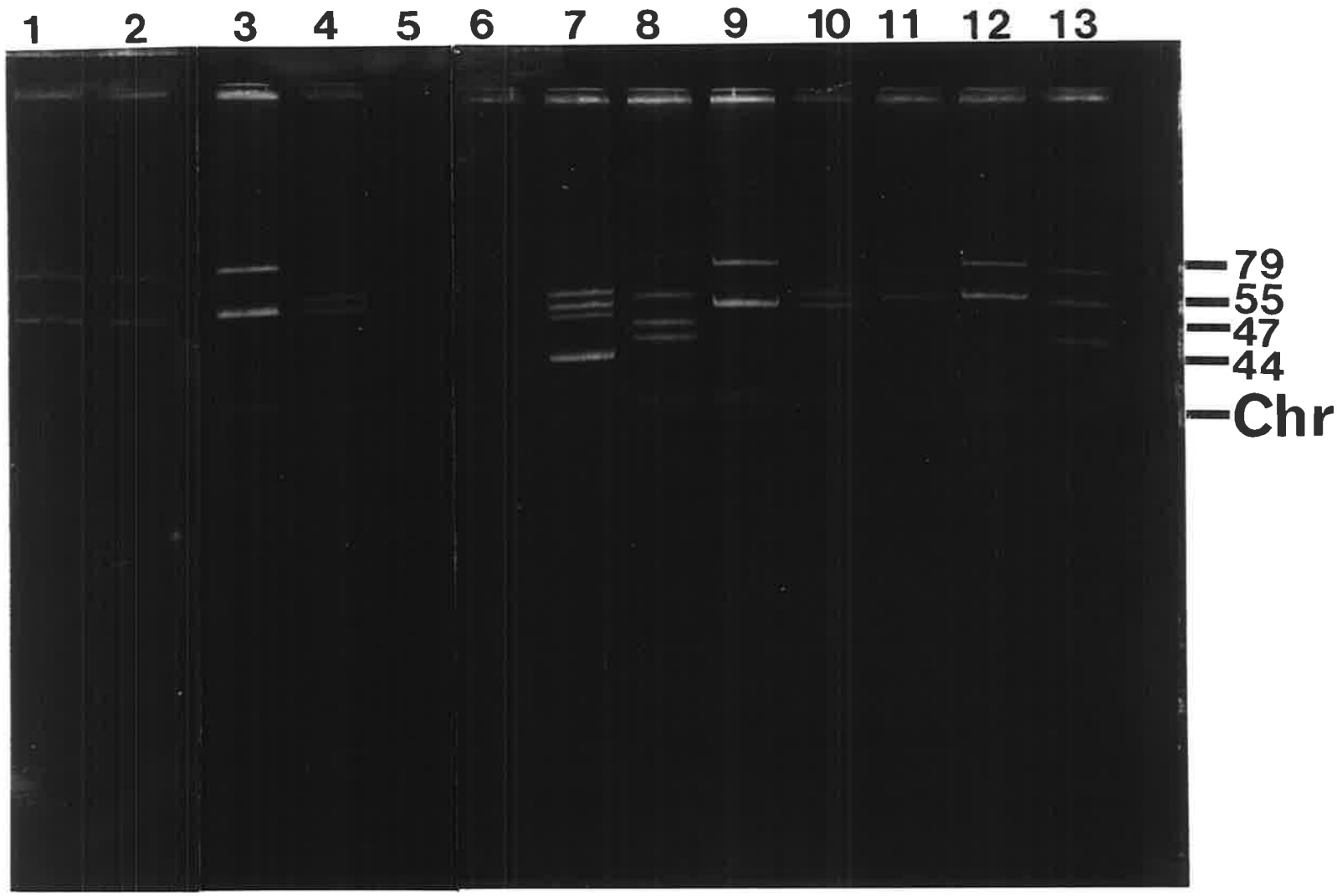

1
2
3
4
5
6
7
8
9
10
11
12
13
79
55
47
44
Chr

Figure 28. Agarose gel electrophoresis of plasmid DNA from several strains of P. syringae pv. syringae. Lane 1, strain DAR 33379; lane 2, strain 3319; lane 3, strain 1212; lane 4, strain L; lane 5, strain N; lane 6, strain O. The migrations of the molecular weight standard, λ DNA (50 kb), is indicated to the right of the gel.

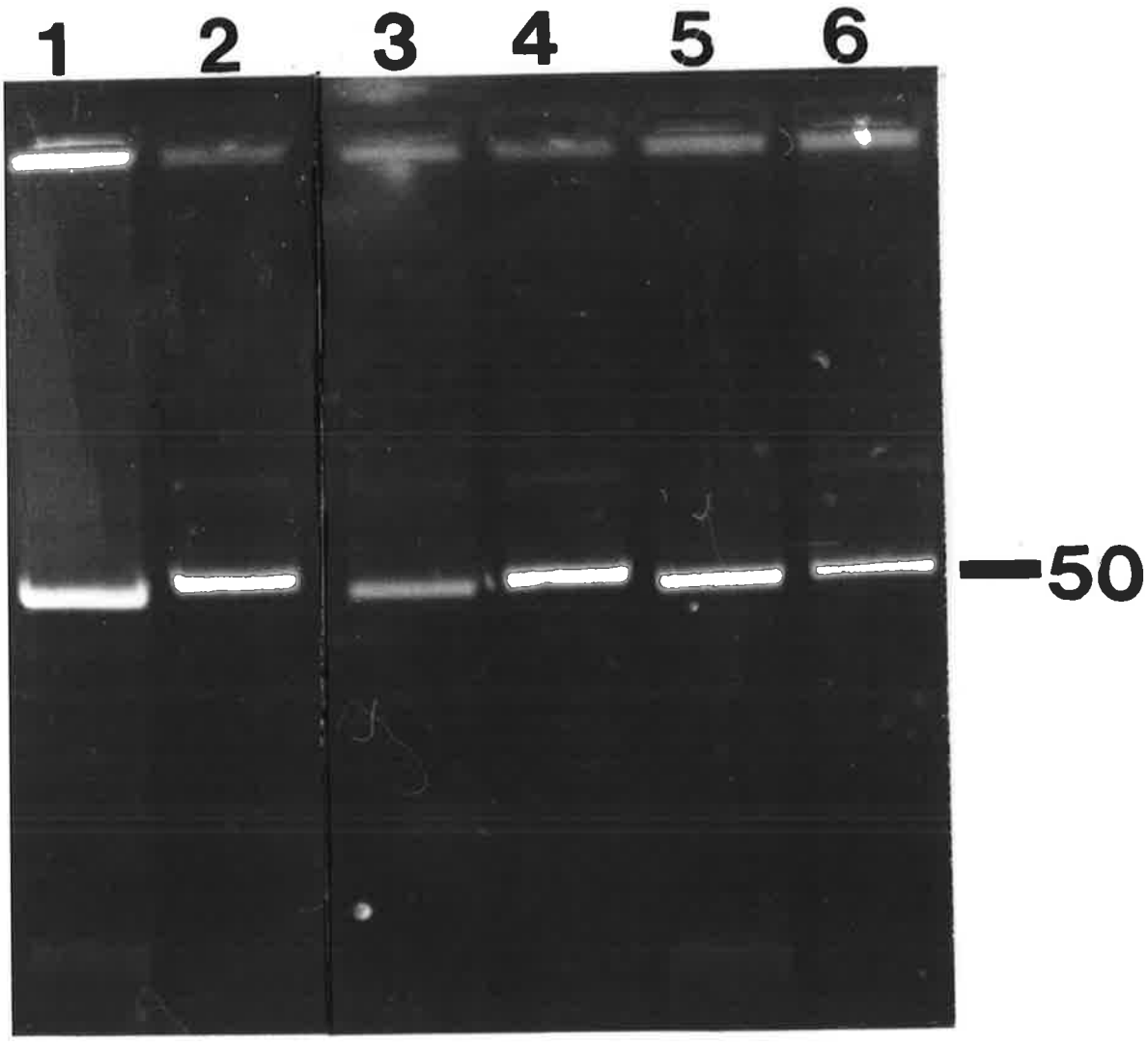

1
2
3
4
5
6
50

Table 33. Characteristics of 13 strains of P. syringae pv. pisi and 25 strains of P. syringae pv. syringae.

Strains	Utilization of homoserine	Pea stem inoculation of cultivars Rovar & Blue-Prussian	Pathovar*	Presence of plasmid(s)
815, 1217 2452, 3575 5055, 5310 5316, DAR30566 DAR 33369, DAR 58730, UQM 551	+	WS	pisi	+
3940, 3941	-	-	pisi	-
3319**, L, N, O	-	N	syringae	+
1212, DAR33379	+	N	syringae	+
2796,DAR26811** DAR41320**, DN20**, DN28**	-	N	syringae	-
3576, 5319, P54	+	N	syringae	-
DAR35712,UQM227	-	N	syringae	-
3543, P47, P52 DAR33368	+	-	syringae	-
3939**, P53**, P55**, P60, UQM213	-	-	syringae	-

WS = Water soaked (pv. pisi pathogenic character).

N = Extensive necrosis and collapse (pv. syringae pathogenic character).

* = The pathovar designation is based on pathological and serological reactions (section B. 1, 2).

** = Serologically do not react with antisera to pv. syringae or pv. pisi.

As strains 3940 and 3941 were the only plasmidless strains of P. syringae pv. pisi available, rifampicin resistant mutants of the five non-pathogenic, homoserine negative strains of P. syringae pv. syringae listed in Table 33 were used as recipients in conjugations with strain DAR 58730 as donor. Several transconjugants were obtained when strain UQM 213 Rif^r was the recipient. Km^r, Rif^r colonies were purified, tested for fluorescence on KB medium and plasmid content determined. Most strains contained only plasmid R68.45. However, when Km^r, Rif^r colonies were screened for Tc sensitivity and plasmids extracted, two different kinds of putative transconjugant were recovered, apparently corresponding to transfer of plasmid pDAR58730a : : Tn5 and pDAR58730b : : Tn5 (Fig 29). This was confirmed by probing the plasmids with the HindIII internal fragment of Tn5 (Fig 29). Further confirmation was obtained by digesting plasmids from both kinds of transconjugant with HindIII followed by probing with the HindIII internal fragment of Tn5 (Fig 30). Transconjugants containing both pDAR58730a : : Tn5 and pDAR58730b : : Tn5 were never observed. All transconjugants reacted with antiserum to P. syringae pv. syringae, not with that to P. syringae pv. pisi.

In a repeated attempt to transfer these plasmids to P. syringae pv. pisi strains 3940 and 3941, Cm^r mutants of these strains were selected and used as recipients in crosses with donors containing either pDAR58730a : : Tn5 or pDAR58730b : : Tn5, with and without R68.45. Selective medium contained chloramphenicol and kanamycin. No transconjugants were detected. Similarly, there were no transformants when tranformation of strains 3940 and 3941 by plasmid DNA of pDAR58730a : : Tn5 and pDAR58730b : : Tn5 was attempted.

c) Plasmid banding patterns

HindIII-digested total plasmid DNA of donor strain DAR 58730 gave reproducible banding patterns in separate preparations. Typical banding patterns produced by electrophoretic separation are shown in Fig 30.

All transconjugants containing pDAR58730a : : Tn5 or pDAR58730b : : Tn5 were digested and their banding patterns determined (Fig 30). The patterns vary depending on

Figure 29. Tn5-labelling of plasmids in P. syringae pv. pisi strain DAR 58730. (A) Agarose gel electrophoresis of plasmid DNA in the donor strain DAR 58730, recipient strain UQM 213 (rifampicin resistant) and seven transconjugants. (B) Hybridization of DNAs shown in panel A, with a HindIII internal fragment of Tn5. Bands are identified from top to bottom. Lane 1, UQM 213, contains only chromosomal DNA; lane 2, strain S17.1 (containing Tn5); lane 3, DAR 58730, pDAR58730a and pDAR58730b and chromosomal DNA; lane 4 to 7, pDAR58730b : : Tn5 and chromosomal DNA; lane 8 to 10, pDAR58730a : : Tn5 and chromosomal DNA.

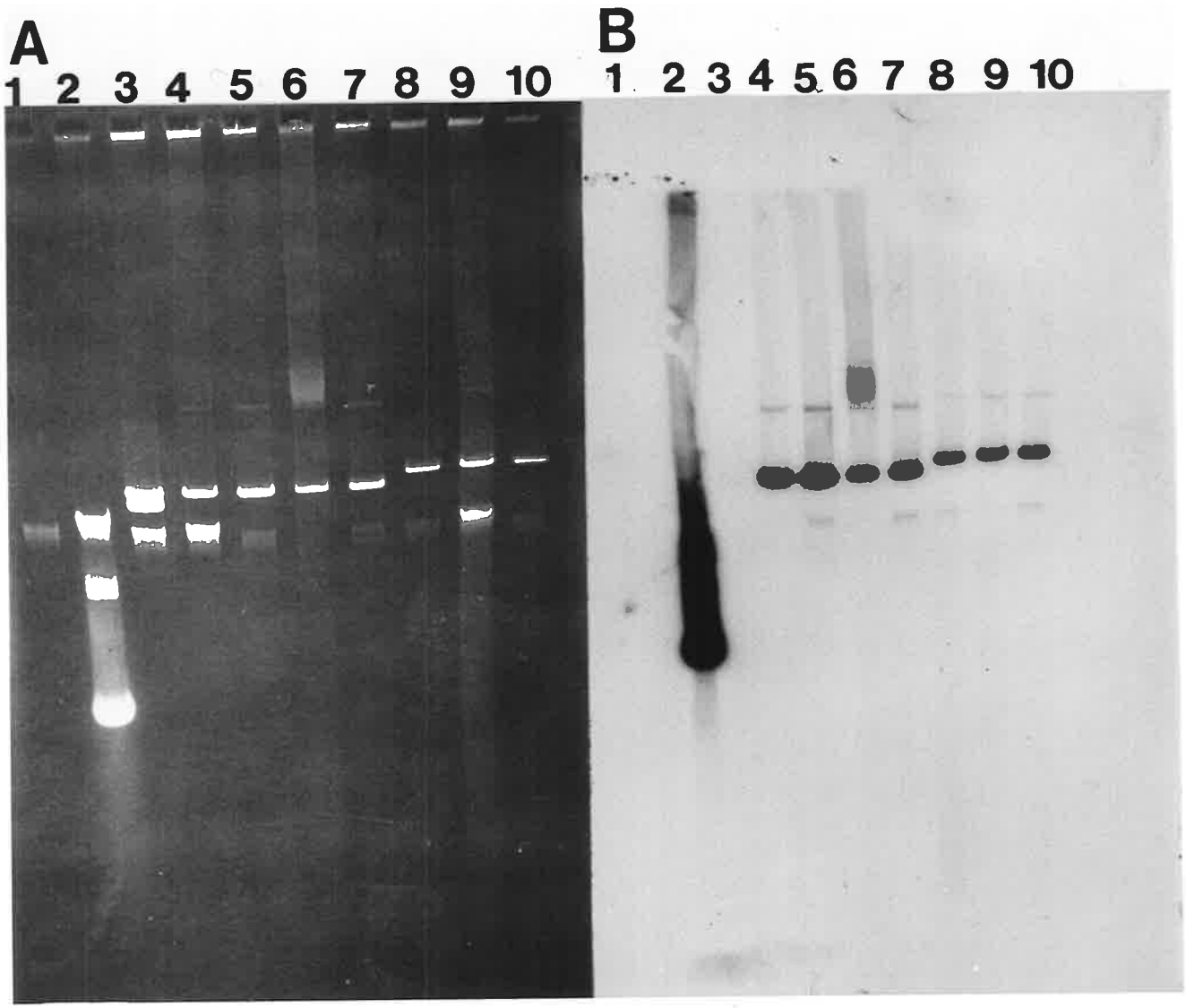
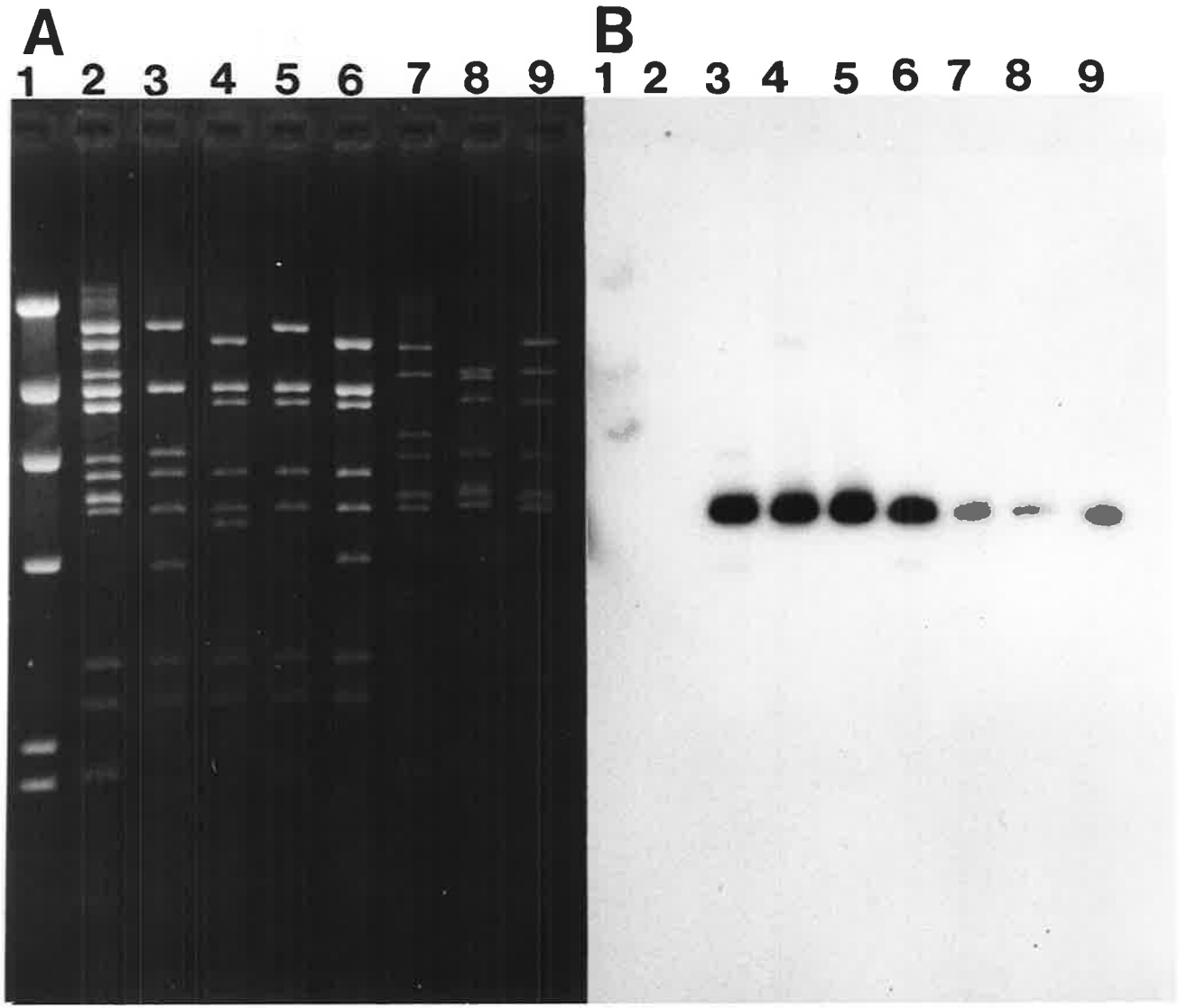

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

Figure 30. (A) Fragment banding patterns produced by cleavage of plasmids in strain DAR 58730 (P. syringae pv. pisi) with restriction endonuclease HindIII, followed by separation on a 0.7% agarose gel. (B) Hybridization of DNAs shown in panel A, with a HindIII internal fragment of Tn5. Bands are identified from top to bottom. Lane 1, λ DNA HindIII fragments, size marker (kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56; lane 2, DAR 58730; lane 3 to 6, pDAR58730b : : Tn5; lane 7 to 9, pDAR58730a : : Tn5.


A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9

where Tn5 has inserted. Based on the disappearance of fragments present in strain DAR 58730 and the appearance of new fragments, it is possible to allocate the fragments in strain DAR 58730 to the two separate plasmids. Fragment banding patterns of the two separate plasmids (pDAR58730a and pDAR58730b) are indicated in Fig 31. Fragment sizes were estimated against a λ digest standard and the two plasmids are estimated to be 56.8 kb and 49.4 kb for pDAR58730a and pDAR58730b respectively.

d) Homoserine utilization and plant inoculation

Twenty eight plasmid transconjugants were tested for utilization of homoserine as a sole carbon source. All were negative.

Pea stems (cultivars Rovar and Blue-Prussian) were inoculated with the parent strains DAR 58730, and UQM 213 Rif^r and with 28 plasmid transconjugants. Strain DAR 58730 produced typical water soaked lesions and strain UQM 213 Rif^r was non-pathogenic. All transconjugants tested produced reactions which were different from those produced by both parents. The reactions varied, with some transconjugants producing localised necrosis at the site of inoculation while others caused a more extensive reaction (Fig 32). The type of reaction induced was not correlated with the type of plasmid present in the transconjugant.

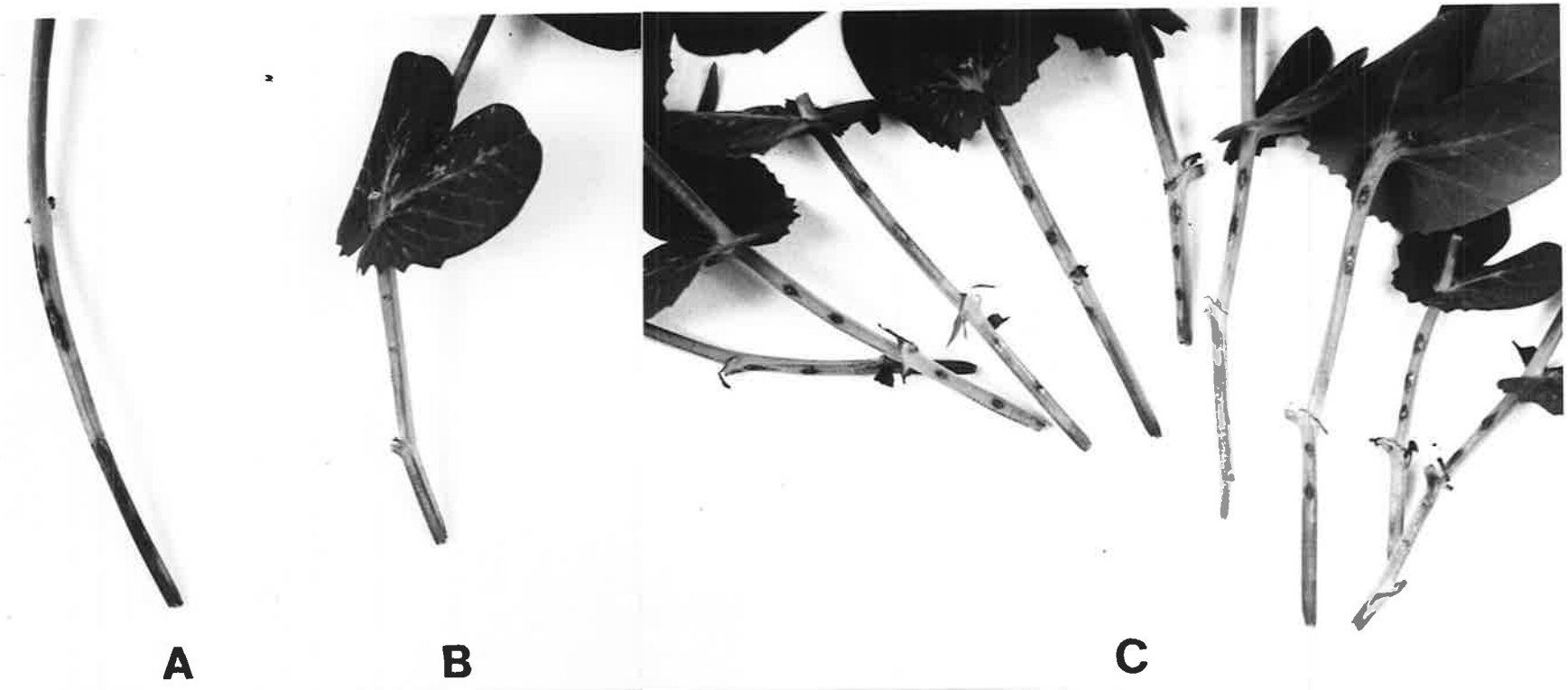
Twenty eight plasmid transconjugants were inoculated into tobacco leaves using bacterial suspensions of about 10^8 cfu/ml. All transconjugants elicited the HR reaction as did the donor strain DAR 58730. Strain UQM 213 Rif^r was HR negative.

Figure 31. Diagrammatic representation of fragment banding patterns produced by HindIII digestion of total plasmid DNA from strain DAR 58730 (pDAR58730a and pDAR58730b), P. syringae pv. pisi, and its two separate plasmids.

Strain DAR 58730

pDAR58730a pDAR58730b			**pDAR58730a**	**pDAR58730b**
Size (kb)		Fragment number		
19.5	—	1		—
17.5	—	2	—	
11.7	—	3	—	
9.40	—	4		—
8.00	—	5 a, b	—	—
6.60	—	6	—	
6.28	—	7		—
5.63	—	8	—	
5.30	—	9	—	
3.35	—	10		—
2.83	—	11		—
2.04	—	12	—	

Figure 32. Inoculation of pea seedlings. (A) Donor (P. syringae pv. pisi), strain DAR 58730, a typical water-soaked lesion spreading from the site of inoculation. (B) Recipient (P. syringae pv. syringae), strain UQM 213 (rifampicin resistant), a null response, only the inoculation wounds are visible. (C) Transconjugants, a typical localized, necrotic lesion can be seen at the site of inoculation.


A
B
C

Discussion

The evidence is strong that plasmids in P. syringae pv. pisi carry genes for pathogenicity. First, there is circumstantial evidence that strains containing plasmids are pathogenic, those without plasmid are not. Secondly, when the plasmids are transferred individually to a non-pathogenic strain, that strain becomes pathogenic; finally, on a nonhost plant, the same strain is converted from HR negative to HR positive following plasmid transfer.

It is perhaps unfortunate, that the converted strain is serologically identified as pv. syringae, not pv. pisi, indicating that it must have a different chromosomal background from P. syringae pv. pisi. As one might expect, P. syringae pv. syringae containing a plasmid from P. syringae pv. pisi does not give the typical pathogenic response of P. syringae pv. pisi. Presumably that will only be achieved when a competent, plasmidless P. syringae pv. pisi recipient strain is available. We are trying to construct such a strain by eliminating the plasmids from P. syringae pv. pisi. Another possible reason why the typical P. syringae pv. pisi response was not demonstrated by the transconjugants is that none contained the two plasmids present in the donor strain; transconjugants contained one or other of the plasmids, not both. It may be necessary to use a different selective marker on one of the plasmids before the two can be combined in one cell. So, on two counts, the ideal transconjugant was not achieved but this cannot detract from the hard evidence that plasmid transfer converted a non-pathogenic strain to a pathogenic one. It is perhaps surprising that both plasmids conferred the pathogenic trait. Clearly more work is required to determine the relationship between the two plasmids. Another possible line of investigation is the nature of pathovar difference. The pathogenic response on inoculated pea stems by the transconjugants was similar to, if not identical with that induced by pathogenic P. syringae pv. syringae, not by pathogenic P. syringae pv. pisi. What is the role of the chromosomal background in symptom expression?

Another character, utilization of homoserine, reported to be characteristic of P. syringae pv. pisi (Hildebrand, 1973) does not appear to be important and is probably not

plasmid encoded, although circumstantial evidence suggested otherwise i.e. the inability of the plasmidless strains 3940 and 3941 to catabolize homoserine.

The inability to transfer plasmids, even R68.45, into strains 3940 and 3941 either by conjugation or transformation is presumably due to efficient restriction. Presumably also this is not present in strain UQM 213 which appeared to be an efficient recipient although transfer efficiency could not be measured by the methods used. It might prove useful in other genetic studies on P. syringae.

In contrast to our results with P. syringae pv. pisi, there seems to be no strong evidence to link pathogenicity with plasmids in P. syringae pv. syringae. Although all pathogenic strains had a plasmid, several pathogens had none. It is possible of course, that in the latter, the plasmid had integrated into the chromosome or that a pathogenic plasmid is present but is so large that it was sheared and destroyed during plasmid extraction. It may be worth further investigation, especially as all non-pathogenic strains lacked plasmids.

Two other aspects of this research are notable. First, the value of pSUP5011 in such studies. The presence of mobilization genes in Tn5 combined with transfer genes on R68.45 makes this a very powerful system for plasmid transfer or for gene transfer in general. Frequently Tn5 must have inserted into the chromosome and then transferred to the recipient, presumably accompanied by part of the chromosome. Such strains were much more common than P. syringae pv. pisi plasmid transconjugants, so much so, that it made the latter difficult to find. Quite inexplicably, plasmid transconjugants never contained R68.45 and they could be located by screening for Tc susceptibility. Conversely, colonies containing R68.45 never had a P. syringae pv. pisi plasmid. It almost looks as if an incompatibility factor is involved.

Finally, the HindIII fragments allocated to the two different plasmids must be considered tentative. More definitive tests are needed to confirm or refute the results, although with only one doublet, there seems to be little basis for confusion.

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Appendix

Publications

1- Identification and seasonal population changes of ice nucleation active bacteria in a pea field in South Australia.

Mazarei, M., and Kerr, A.

Australasian Plant Pathology (1987) 16: 13-15

2- Distinguishing pathovars of *Pseudomonas syringae* on peas: nutritional, pathogenicity and serological tests.

Mazarei, M., and Kerr, A.

Plant Pathology (1990) 39: 278-285

3- Plasmids in *Pseudomonas syringae* pv. *pisi* carry genes for pathogenicity.

Mazarei, M., and Kerr, A.

Plant Pathology (1991) (in press)

Mazarei, M. & Kerr, A. (1987). Identification and seasonal population changes of ice nucleation active bacteria in a pea field in South Australia. *Australasian Plant Pathology*, *16*(1), 13-15.

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