STUDIES ON PLANT PATHOGENIC BACTERIA

I. CULTURAL AND BIOCHEMICAL CHARACTERS

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Summary

Studies on 119 plant pathogenic bacterial strains belonging to 22 species are described. These include organisms isolated in Australia and type species obtained from abroad.

The collection was maintained by storing the cultures under paraffin oil and in a lyophilized state and both procedures proved to be satisfactory for the survival of most bacteria. Various selective and indicator media were employed and an assessment was made of King's agar and Hartley's glycerol agar for the enhancement of fluorescin and pyocyanin production by *Pseudomonas*. The ability to liquefy a pectin medium was shown to correlate with the ability to decompose plant tissues and both tests were useful for differentiation of *Erwinia* from coliforms and paracolons. Biochemical reactions showed great variation within species and appeared to be of limited value in classification.

I. Introduction

The pathogenicity test is the main criterion for the identification of bacteria suspected of being the aetiological agents of a plant disease. This involves reproduction of lesions following artificial infection of suitable hosts under greenhouse conditions. Occasionally, pathogenicity tests may be performed under controlled laboratory conditions on a part of a plant, such as bean pod, lemon fruit, or on slices of tissue, e.g. potato, carrot. The difficulty of choosing susceptible varieties of the host and suitable conditions indicates the necessity to use additional and practical means of identification of pathogenic bacteria.

This work is concerned with the bacteriological investigation of a number of plant pathogenic species, most of which are endemic in Australia. The study is divided into two parts: in Part I emphasis is placed on methods of maintaining the organisms and on evaluation of cultural and biochemical techniques in classification while Part II is concerned with methods of production of potent antisera and antigenic analysis of various species.

II. Materials and Methods

(*a*) *Cultures*

Type cultures of plant pathogenic bacteria were obtained through the courtesy of overseas workers and local strains were contributed by colleagues or were isolated by the authors of this paper. The suitability of various media for the cultivation

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of these organisms was determined. Meat infusion agar, Burkholder's potato extract agar (Dowson 1949) with 0·6 per cent. glucose, and brain–heart agar (Difco), which contains 0·2 per cent. glucose, were tried.

The cultures used in routine work were maintained on brain–heart agar under paraffin oil at 4°C and subcultures were made approximately every 4 months. Preservation of cultures was achieved by lyophilization prepared by emulsifying growth from a solid medium in 10 per cent. blood in nutrient broth and drying under vacuum in ampoules in a "Dryvac" apparatus as described by Gray (1948, 1954). The ampoules were stored at room temperature.

(*b*) *Selective Media*

The media used as differential substrates were sheep blood agar, deoxycholate agar (Leifson 1935), and papain tellurite agar. Various media were tested for the enhancement of fluorescin and pyocyanin production and these included the following:

(i) Meat infusion broth plus 10 per cent. carrot extract or 5 per cent. glycerol.
(ii) Peptone water with 5 per cent. glycerol (4 parts) plus serum (1 part).
(iii) Sullivan's (1905) three-component and complete medium.
(iv) Hartley's broth (Mackie and McCartney 1953) which was prepared with dehydrated trypsin instead of pancreatic extract. Glycerol was added at 1, 2, 3, 4, and 5 per cent. concentration.
(v) Hartley's broth with 5 per cent. glycerol (4 parts) plus serum (1 part).
(vi) Hartley's glycerol agar which contained 1·5 per cent. Davis' agar plus 1 per cent. glycerol.
(vii) King's agar—medium B of King, Ward, and Raney (1954) which contained proteose peptone No. 3 plus 1 per cent. glycerol.

The ability to liquefy pectate gel was compared with the ability to decompose plant tissues. Pectate gel was prepared according to directions given by Sabet and Dowson (1951) and the brand of sodium pectate used was as recommended by Rudd Jones (1946). Burkholder's potato extract medium (Dowson 1949) was used for the cultivation of some organisms prior to the inoculation of pectate gel; this conditioning procedure was suggested by Potter and McCoy (1952). Potato and carrot slices for tissue-decomposition tests were cut into plugs under aseptic conditions and inserted into tubes containing sterile meat infusion broth. The cultures for the production of fluorescin, fermentation of pectate gel, and decomposition of vegetable slices were kept under observation at 25°C for 28 days.

(*c*) *Biochemical Tests*

In the biochemical tests, carbohydrate fermentations were observed in a synthetic base as recommended by Dowson (1949). Hydrogen sulphide production was recorded with lead acetate paper using Hartley's agar. These reactions and gelatin liquefaction were observed for a period of 28 days.

Other tests were done according to directions by Wilson and Miles (1946), some with slight modifications. Indole production was determined after 7 days incubation

using xylol for extraction. The Voges-Proskauer test was performed by O'Meara's method. Nitrate reduction was observed in a medium with 0·2 per cent. potassium nitrate and the cultures were spot-tested after 1, 2, and 5 days incubation. Citrate utilization was demonstrated in Koser's liquid medium.

(*d*) *Plant Pathogenicity Tests*

Inoculated French bean pods were placed in individual "moist chambers" consisting of glass tubes lined on the bottom with moist cotton wool.

TABLE 1

LIST OF SPECIES EXAMINED

Species	No. of Strains	Species	No. of Strains
Agrobacterium tumefaciens	3	*Ps. pisi*	4
Corynebacterium fascians	1	*Ps. solanacearum*	9
C. flaccumfaciens	6	*Ps. syringae*	18
C. michiganense	3	*Ps. viridiflava*	3
Erwinia aroideae	2	*Xanthomonas albilineans*	1
E. atroseptica	3	*X. campestris*	4
E. carotovora	3	*X. carotae*	2
Pseudomonas aeruginosa	1	*X. incanae*	1
Ps. barkeri	1	*X. juglandis*	2
Ps. cerasi	2	*X. phaseoli*	8
Ps. medicaginis var. *phaseolicola*	40	*X. vesicatoria*	2

III. RESULTS

(*a*) *Cultures*

Table 1 shows that 22 different plant pathogenic species which were represented by 119 strains were studied. Brain–heart agar proved to be a suitable medium for the cultivation of these bacteria. Potato extract agar gave a more luxurious growth but tended to promote the mucoid phase which masks the specific antigens in serological tests and therefore was not used.

In all, 38 representative strains belonging to 12 bacterial species were examined for longevity under paraffin oil after a period of 1 year. All except *Pseudomonas solanacearum* survived under those conditions.

The 22 pathogenic species represented by 52 strains were tested for survival when lyophilized under vacuum. The maximum time of preservation was over 5 years and the minimum about 2 years. Of the 62 ampoules tested viable organisms were recovered from 77 per cent. of them. A variability in the survival time of some strains within certain species was noticed. *Ps. solanacearum* again did not survive the conditions of the test, namely 3 years of the preservation period.

Lyophilization of most plant pathogenic bacteria proved to be satisfactory and for routine work storage under paraffin oil is recommended for its simplicity.

(*b*) *Cultural Characters*

(i) *Sheep Blood Agar.*—Most species grew well on this medium but none exhibited haemolytic activity.

(ii) *Deoxycholate Agar.*—This medium allowed good or moderate growth of *Erwinia* within 1–2 days and the intensity of colour of the colonies indicated the active and less-active lactose fermenters. *Agrobacterium* grew well, *Xanthomonas* showed variability and some strains failed to develop, *Pseudomonas* gave good growth except some strains of *Ps. solanacearum* which were inhibited. Gram-positive bacteria are suppressed on this substrate and consequently *Corynebacterium* species failed to grow.

Deoxycholate agar is recommended as a complementary medium for the isolation of most Gram-negative plant pathogenic bacteria from tissues overgrown with contaminants and should be used together with a non-selective medium.

(iii) *Papain Tellurite Agar.*—This medium failed to support the growth of *Corynebacterium* plant pathogens.

(iv) *Fluorescin and Pyocyanin Production.*—Some pseudomonads in our collection lost their ability to produce pigment on brain–heart or nutrient agar medium, also some fresh isolates failed to show colour and therefore various media were tested for the enhancement of fluorescin production.

The following 28 *Pseudomonas* strains, 27 of them plant pathogens, were used in this investigation: *Ps. aeruginosa* (1 strain), *Ps. barkeri* (1), *Ps. cerasi* (2), *Ps. medicaginis* var. *phaseolicola* (5), *Ps. pisi* (2), *Ps. solanacearum* (4), *Ps. syringae* (10), *Ps. viridiflava* (2), and *Ps. fluorescens* (1).

King's agar and Hartley's glycerol agar proved to be the most suitable media for fluorescin production but occasionally pigment was deeper on the first substrate. None of the five *Ps. medicaginis* var. *phaseolicola* strains showed pigment on Hartley's agar but all produced it on Hartley's glycerol agar and on King's agar which contains glycerol. Some strains of *Ps. solanacearum* and one *Ps. viridiflava* did not form pigment in any of the media tested.

The colour of *Ps. aeruginosa* was deeper and a more characteristic blue-green on Hartley's glycerol agar than on King's agar. Eight additional strains of *Ps. aeruginosa* were isolated from the human intestinal tract and again five gave a more characteristic colour on Hartley's glycerol agar.

All pseudomonads were tested for pyocyanin in Hartley's broth with 1 per cent. glycerol and in King's liquid medium by chloroform extraction. An improved technique was the incubation of liquid cultures at 30°C for 48 hr on a shaker. The oxygenated conditions of the medium greatly promoted production of pyocyanin giving a spectacular deep blue alkaline chloroform phase and a brick-red acid phase.

The results of the tests indicated that of the plant pathogenic pseudomonads *Ps. aeruginosa* was the only culture to form pyocyanin.

In conclusion, King's agar proved to be the medium of choice for the enhancement of fluorescin production while Hartley's glycerol agar was slightly less efficient for the demonstration of fluorescin but superior for pyocyanin. These

colourless agar media were preferred to the liquid as the opacity of liquid cultures tended to obscure the colour.

(v) *Activity on Pectate Gel Medium.*—Results in Table 2 demonstrate that of the phytopathogens tested all *Erwinia* strains of the soft-rot group liquefied the gel in 2 days; the pH of the medium fell slightly.

Some members of the genus *Xanthomonas*, namely *X. campestris*, *X. carotae*, and *X. vesicatoria*, also decomposed the gel, at times after prolonged incubation, and the pH of the medium did not change. The remaining strains of *Xanthomonas*, *Pseudomonas*, and *Corynebacterium* and also strains of *Bacillus* and *Pseudomonas* of faecal origin did not liquefy the gel and the pH rose slightly.

TABLE 2

ACTIVITY OF BACTERIA* ON PECTATE GEL AND VEGETABLE SLICES

Species	No. of Strains Tested	Liquefaction of Pectate Gel		Decomposition of Vegetable Slices			
				Potato		Carrot	
		Degree	Time (days)	Degree	Time (days)	Degree	Time (days)
Erwinia aroideae	1	+++	2	+++	2	+++	2
E. atroseptica	2	+++		+++		+++	
E. carotovora	3	+++		+++		+++	
Xanthomonas campestris	3	++,+	3–5	++,+	15–30	++,+	15–30
X. carotae	2	++		++		++	
X. vesicatoria	1	+	20	−		−	
Pseudomonas solanacearum	1	−		+	15	−	
Ps. syringae	1	−		+	30	−	
Aerobacter spp.	2	−		+	15–30	+	20–40
Paracolobactrum spp.	5	−		+	15–30	+	20–40

*The following organisms were inactive: *Agrobacterium tumefaciens* (1 strain), *Corynebacterium flaccumfaciens* (3), *C. michiganense* (3), *Pseudomonas barkeri* (1), *Ps. cerasi* (2), *Ps. medicaginis* var. *phaseolicola* (4), *Ps. pisi* (2), *Ps. solanacearum* (1), *Ps. syringae* (7), *Ps. viridiflava* (1), *Xanthomonas albilineans* (1), *X. incanae* (2), *X. juglandis* (2), *X. phaseoli* (3), *Ps. aeruginosa* (1), *Aerobacter aerogenes* (2), *A. cloacae* (1), *Aerobacter* spp. (13), *Escherichia* spp. (12), *Paracolobactrum* spp. (42), *Ps. aeruginosa*, faecal (2), *Ps. fluorescens* (1), *Bacillus* spp. (2).

Pectate gel was used as a substrate for coliform and paracolon strains. Of these, type cultures of *Aerobacter* (*A. aerogenes* I and II, *A. cloacae*) and our own isolates from human faecal material including 15 *A. aerogenes* I and II strains and 47 paracolons (*Paracolobactrum aerogenoides* I and II, *P. intermedium* I and II, and irregular strains) (Mushin 1949) did not decompose the medium.

Representative strains of the five genera of phytopathogenic bacteria were grown on Burkholder's potato extract medium prior to cultivation on pectate gel. This conditioning did not enhance the pectolytic activity.

TABLE 3

BIOCHEMICAL REACTIONS

A = acid; (g) = gas; V = variable

Species	No. of Strains Tested	Glucose	Lactose	Sucrose	Maltose	Xylose	Salicin	Mannitol	Glycerol	Gelatin	Citrate	Indole	Hydrogen sulphide	Nitrate–nitrite
Agrobacterium tumefaciens	3	A	—	A	A	A	A	V	A	—	V	—	+	—
Corynebacterium fascians	1	A	—	—	A	—	—	—	—	—	—	—	+	—
C. flaccumfaciens	6	V	V	V	V	V	V	V	V	V	—	—	V	—
C. michiganense	3	V	—	—	—	V	—	—	—	—	—	—	+	—
Erwinia aroideae	2	A	A	A	A	—	A	A	A	—	+	—	++	+
E. atroseptica	3	A (g)	A (g)	A (g)	V	A (g)	A (g)	A (g)	V	V	+	—	++	+
E. carotovora	3	A (g)	A (g)	A (g)	—	A (g)	A (g)	A (g)	A (g)	V	+	—	++	+
Pseudomonas aeruginosa	1	A	—	—	—	A	—	—	A	+	+	—	—	+
Ps. barkeri	1	A	A	—	A	A	—	A	A	—	+	—	—	—
Ps. cerasi	2	A	A	A	—	A	—	A	A	—	+	—	—	—
Ps. medicaginis var. *phaseolicola*	38	A	—	A	—	A	—	V	A	—	+	—	—	—
Ps. pisi	2	A	—	A	—	A	—	A	A	—	+	—	V	—
Ps. solanacearum	2	A	V	A	V	A	—	V	A	—	+	—	+	V
Ps. syringae	18	A	—	A	—	V	—	A	V	V	+	—	V	—
Ps. viridiflava	2	V	—	—	V	—	—	—	—	+	+	—	—	—
Xanthomonas albilineans	1	A	—	A	A	A	—	A	—	—	+	—	+	—
X. campestris	3	A	A	A	A	A	—	V	V	+	+	—	++	—
X. carotae	1	A	A	A	—	A	—	—	—	—	+	—	++	—
X. incanae	1	A	A	—	—	A	—	A	A	+	+	—	++	—
X. juglandis	2	A	V	V	A	V	—	—	V	—	+	—	++	—
X. phaseoli	4	V	V	V	V	V	—	—	—	V	V	—	++	—
X. vesicatoria	2	A	A	A	A	—	—	A	—	V	V	—	++	—

With the use of pectate gel a striking separation was obtained for the *Erwinia* soft-rot group from the morphologically, culturally, and biochemically closely related coliform and paracolon strains.

(vi) *Decomposition of Vegetable Slices.*—It was of interest to find out whether the results of pectin liquefaction correlated with the ability to macerate healthy plant tissue. As shown in Table 2, *Erwinia* strains were rapid in tissue decomposition (2 days) while *X. campestris*, *X. carotae*, and one strain of *Ps. solanacearum* and *Ps. syringae* were much slower. All other phytopathogenic strains did not cause any loss of coherence in tissues.

The results of pectate gel liquefaction and vegetable tissue decomposition correlated well in *Erwinia*, while *X. carotae* and *X. campestris* acted more slowly on vegetable slices than on the gel. A few other phytopathogenic strains with feeble activities were irregular in decomposition of gel or digestion of tissues.

Of the 15 *Aerobacter*, 12 *Escherichia*, and 47 *Paracolobactrum* strains tested, two *A. aerogenes* type I and five paracolon strains (four *P. aerogenoides* type I and one type II) softened the tissues, especially potato, after a prolonged incubation of 15–40 days.

Thus the correlation of pectin gel liquefaction and tissue decomposition is more marked for *Erwinia*. A feeble and delayed activity was found with some *Aerobacter* and paracolon strains.

(*c*) *Biochemical Reactions*

The results of experiments listed in Table 3 allow certain conclusions to be drawn.

Fermentation of carbohydrates was usually delayed and often took place after one or several weeks with the production of acid only, except for *Erwinia* species, most of which evolved acid or acid and gas after 1 or 2 days incubation. Some strains of *Ps. syringae* produced acid after 2 days incubation. *Pseudomonas* and *Xanthomonas* consistently gave negative results in salicin but variable readings were recorded with other carbohydrates for the species tested.

Other biochemical tests in Table 3 showed that all cultures were negative for indole. Hydrogen sulphide production was strongly positive for *Erwinia* and *Xanthomonas*, slight for *Agrobacterium*, and variable for other genera. Growth in citrate medium was consistently positive for *Erwinia* and *Pseudomonas*, negative for *Corynebacterium*, and variable for other genera. Reduction of nitrate to nitrite was positive for *Erwinia*, variable for *Pseudomonas*, and negative for remaining species.

Erwinia strains—soft-rot group—were studied in more detail. As already pointed out, these species were the most active biochemically. In accordance with the classification in "Bergey's Manual" (Breed, Murray, and Smith 1957) a distinction was made between dulcitol-positive strains of *E. carotovora* and dulcitol-negative *E. atroseptica* and *E. aroideae*. The separation of the last two species on gas production proved to be unreliable as some stock strains of *E. atroseptica* were anaerogenic.

Elrod's (1941) differentiation of *Erwinia* on maltose and sorbitol fermentation did not correlate with the breakdown of dulcitol.

A comparison was made between *Erwinia* and the coliform–paracolon group on the basis of "IMViC" reactions (I = indole, M = methyl red, Vi = Voges-Proskauer, C = citrate):

	I	M	Vi	C
E. aroideae	–	–	+	+
E. atroseptica	–	+	–	+
E. carotovora	–	Variable	Variable	+

It can be seen that *Erwinia* species resemble biochemically the organisms of *Aerobacter–Paracolobactrum aerogenoides* and intermediate groups; however, the prompt gel liquefaction and rapid maceration of tissues provided a distinct line of demarcation.

TABLE 4

POD-INOCULATION TESTS ON FRENCH BEANS

Species	No. of Strains Tested	Type of Lesion	Average Size of Lesion (mm)
Corynebacterium flaccumfaciens	6	None	0
Pseudomonas barkeri	1	None	0
Ps. medicaginis var. *phaseolicola*	38	Water-soaked*	3·7
Ps. pisi	2	None	0
Ps. syringae	4	Necrotic	2
Ps. syringae	5	Brown	2·2
Xanthomonas phaseoli	3	Water-soaked*	2
Ps. pyocyanea	1	Brown	2·5
Ps. fluorescens	1	Slight brown	1

*Positive result.

A biochemical variability of strains of the same species was frequently observed. This and the fact that different techniques are frequently used in the bacteriological tests makes it impractical to attempt a comparison of the present results with those listed in Dowson's (1949, 1957), Elliott's (1951), and Bergey's (1957) manuals. With a few exceptions most biochemical tests are of little value in the classification of plant pathogenic bacteria.

(*d*) *Plant Pathogenicity Tests*

These tests are critical in the diagnostic procedure. However, many of our strains were imported from abroad on condition that no pathogenicity experiments would be carried out under greenhouse or field conditions. Accordingly, many tests could not be performed.

The potential bean pathogens in our collection were used for the inoculation of French bean pods. As shown in Table 4 all *Ps. medicaginis* var. *phaseolicola*

strains gave characteristic water-soaked "halo blight" lesions after 5–7 days incubation. This test was simple and reliable. *Ps. syringae* produced a different type of lesion and *Ps. aeruginosa* of faecal origin gave lesions similar to those of *Ps. syringae*. Only "halo blight" lesions were considered of diagnostic value.

IV. Discussion

The organisms used in this study were best preserved in a lyophilized state (Gray 1948, 1954) using thick suspensions prepared in 10 per cent. blood in nutrient broth. Storage of cultures under paraffin oil was convenient for routine work. There is variability in the longevity of various strains, for instance *Ps. solanacearum* did not survive one year's storage while Kelman and Jensen (1951) reported viability after 3 years under similar conditions.

Various studies on the demonstration of fluorescin in cultures have been reported and listed by Seleen and Stark (1943). Sullivan (1905) pointed out that the presence of both sulphur and phosphorus was essential for fluorescin formation, and mineral requirements were further investigated by various workers (Burton, Campbell, and Eagles 1948). In this work enriched media with the addition of glycerol were found best.

In decomposition of plant tissues the protopectinases are concerned with the breakdown of cementing material in middle lamellae (Elrod 1942; Sabet and Dowson 1951). The chemical character and macerating ability of pectolytic enzymes secreted by soft-rot bacteria were studied recently (Echandi, van Gundy, and Walker 1957). Some workers did not consider the secretion of pectic enzymes in pure culture to be an indication of bacterial invasiveness (Elrod 1942; Wood 1955). Rudd Jones (1946) found a correlation but pointed out that these two activities are not necessarily the same. The pectin media used by various workers (Elrod 1942; Rudd Jones 1946; Sabet and Dowson 1951; Jacobelli 1953) differed in the origin and type of pectin and in the composition and methods of preparation of media and some critical factor may account for the conflicting results. Tests on synthetic media under controlled conditions would give additional information.

The present results pointed clearly to a correlation between the rapid liquefaction of the pectate gel used and the destruction of plant tissues by *Erwinia* soft-rot bacteria which Dowson (1957) recently classified as *Pectobacterium*. Each of these properties can be used as a demarcation between *Erwinia* and the closely related *Aerobacter*–paracolon group. The rudimentary ability of some coliform and paracolon strains to soften plant tissue due to pectolytic enzymes suggests an interesting line of investigation. More active variants could be selectively isolated to effect a more rapid tissue maceration.

Biochemical variability of various strains of plant pathogenic bacteria showed the limitation of these reactions in the identification of species. Various studies were made on their minimal nutritive requirements (Starr and Mandel 1950) but this did not show a clear distinction between genera and is not practical for routine work.

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