DIFFERENTIATION OF FLUORESCENT PSEUDOMONADS BY THEIR EFFECT ON MILK AGAR

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APART from some phytopathogens, the fluorescent group of pseudomonads comprises *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida*. *P. aeruginosa* is the commonest and most clinically significant species. The other two species are occasionally associated with serious infections (Gilardi, 1972 and 1976; Martin, Maker and Washington, 1973) but, more significantly, they may be confused with *P. aeruginosa* in the laboratory (Falkiner, Keane and Taylor, 1974).

Brown and Scott-Foster (1970) devised a simple milk medium to distinguish between *P. aeruginosa* and *P. fluorescens*. Colonies of *P. aeruginosa* were surrounded by a clear zone due to hydrolysis of the casein, and formed a green pigment that diffused into the medium. We report our experience of this medium for the differentiation of fluorescent pseudomonads.

MATERIALS AND METHODS

Bacterial strains. A study of pseudomonas infections in the seven hospitals served by this laboratory was started in 1971; 3800 isolates of *P. aeruginosa* were collected in 7 years. Eighteen strains of *P. aeruginosa* that did not produce pyocyanin on Diagnostic Sensitivity Test Agar (DST, Oxoid) and Cetrimide Agar (Pseudosel, BBL) were included in the present study because only these strains require further identification. Forty-six isolates of *P. putida* and 21 of *P. fluorescens* were collected from the same seven hospitals and one reference strain of *P. fluorescens*, strain NCTC10038, was included in this study.

Culture media and methods. Antibiotic sensitivities were determined by a modified Kirby-Bauer method and Multodiscs (Oxoid) on DST agar. The ability of strains to grow at 42°C was determined by incubating a nutrient-broth culture, freshly-seeded from a colony on solid medium, at 42°C in a waterbath overnight and subculturing twice at 24-h intervals into nutrient broth held at 42°C.

Lecithinase production was indicated by the development of a zone of turbidity around colonies on Nutrient Agar (Oxoid) containing 10% Egg Yolk Emulsion (Oxoid) within 5 days. Hydrolysis of gelatin was indicated by the release of charcoal from Charcoal-Gelatin Discs (Oxoid) in heavily-seeded Nutrient Broth No. 2 (Oxoid) after incubation at 37°C for 72 h. Ammonium Salt Sugars (Difco) containing ethanol, glucose and mannitol were used to confirm the identity of the fluorescent pseudomonads (King and Phillips, 1978).

Fluorescein production was demonstrated by fluorescence on DST and Pseudosel Agar under an ultraviolet lamp and pyocyanin production was detected in King's "A" medium (*Pseudomonas* Agar P, Difco; King, Ward and Raney, 1954).

Preparation and use of milk medium. The milk agar was prepared as described by Brown and Scott-Foster (1970) except that Dairy Bawn Defatted Milk Granules (Mitchelstown Creamery) were used instead of Marvel (Cadbury); the formulations of these products were identical (manufacturers' specifications). The plates were seeded in the normal manner and incubated at 37°C for 24 h and then at room temperature for another 48 h. The procedure was repeated with incubation at 37°C for 72 h, and also with a modified milk medium that contained 25% nutrient broth. The plates were examined at 24-h intervals and the results scored as ++, + or ±, for clearing around colonies (casein hydrolysis) and + or ± for pigment production.

Received 15 Nov. 1978; accepted 8 Jan. 1979.

RESULTS

Identification of the fluorescent pseudomonads

All the isolates were gram-negative, oxidase-positive bacilli that grew on cetrimide agar and fluoresced under ultraviolet illumination. The 18 strains of *P. aeruginosa* produced pyocyanin only on King's "A" medium and most of them had the characteristic odour of trimethylamine. Most of them were resistant to kanamycin and sensitive to carbenicillin. Conversely *P. fluorescens* and *P. putida* were sensitive to kanamycin and resistant to carbenicillin (table I). This difference is the first indication in the routine laboratory whether a fluorescent pseudomonad is *P. aeruginosa* or one of the other two species; only five isolates of *P. aeruginosa* from two patients out of the total of more than 3800 were sensitive to kanamycin and resistant to carbenicillin and they produced pyocyanin.

The results of the tests used to differentiate the fluorescent pseudomonads are shown in table I. The Computer Trials Laboratory, Central Public Health Laboratory, Colindale kindly confirmed the identity of some of the strains.

TABLE I
Results of tests used to differentiate between the fluorescent pseudomonads

Test	Results obtained with		
	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	<i>P. putida</i>
Antimicrobial susceptibilities to			
carbenicillin	S*	R	R
polymixin	S	S	S
gentamicin	S	S	S
kanamycin	R	S	S
Production of pigment other than			
fluorescein on King's "A" medium	+	—	—
Growth at 42°C	+	—	—
Production of lecithinase	—	+	—
Hydrolysis of gelatin	V	+	—
Fermentation of ammonium salt sugars			
ethanol	+	—	V
glucose	+	+	+
mannitol	+	+	—

S = Sensitive; R = resistant; + = positive reaction; — = negative reaction; V = variable reaction.

* 85% of *P. aeruginosa* were sensitive to carbenicillin; 98% were sensitive to gentamicin.

Hydrolysis of casein by fluorescent pseudomonads

The results obtained with the milk medium incubated at 37°C for 72 h are shown in table II. Most (72%) of the *P. aeruginosa* strains produced pigment in 72 h although pigmentation was poor when compared with a control strain of *P. aeruginosa* and with 80 well-pigmented strains that were tested in a previous study (unpublished data). In no case was pigmentation strong enough to warrant a ++ score. Many (59%) of the *P. fluorescens* strains also produced the yellow pigment fluorescein; it was difficult to distinguish this from pigmentation produced by some strains of *P. aeruginosa* that produced only a small amount of pyocyanin. Approximately the same proportions of the *P. fluorescens* (82%) and *P. aeruginosa* (83%) strains hydrolysed casein. *P. putida* did not produce obvious amounts of fluorescein or hydrolyse casein.

Brown and Scott-Foster (1970) found that the addition of 25% nutrient broth to the milk in the medium gave improved hydrolysis and pigmentation after 24 h. They also noted an improvement in pigmentation when the plates were incubated at 37°C for 24 h and then 20°C for

TABLE II

The hydrolysis of casein and pigment production by fluorescent pseudomonads on milk-agar medium

Graded ($\pm$, +, ++) positive reaction at 24, 48 and 72 h at 37°C	Number of strains (%) of the given species that gave the stated reaction					
	<i>P. aeruginosa</i> (18 strains)		<i>P. fluorescens</i> (22 strains)		<i>P. putida</i> (46 strains)	
	casein hydrolysis	pigment production	casein hydrolysis	pigment production	casein hydrolysis	pigment production
24 hours	$\pm$	7 } (67%)	3 } (50%)	5 } (45%)	3 } (27%)	0
	+	5 }	6 }	5 }	3 }	0
	++	0	0	0	0	0
48 hours	$\pm$	3 }	0	1 }	3 }	0
	+	7 } (78%)	10 } (56%)	13 } (68%)	7 } (45%)	0
	++	4 }	0	1 }	0	0
72 hours	$\pm$	0	0	2 }	2 }	0
	+	5 } (83%)	13 } (72%)	8 } (82%)	11 } (59%)	0
	++	10 }	0	8 }	0	0

24 h. In the present study however neither the addition of 25% broth nor incubation of the plates at room temperature after the initial incubation at 37°C significantly affected casein hydrolysis or pigmentation. Green pigment produced by *P. aeruginosa* rarely diffused into the milk medium either in this study or previously when 80 strains of normally pigmented *P. aeruginosa* were tested.

DISCUSSION

In general, *P. aeruginosa* is readily identified by its characteristic odour and green pigmentation on routine laboratory media. However, the strains that are poorly pigmented on ordinary media require further identification, and for epidemiological reasons it is necessary to distinguish between these organisms and *P. fluorescens* and *P. putida*. According to King and Phillips (1978) *P. fluorescens* does not hydrolyse casein, but this statement was the result of a transcription error in their report (Professor I. Phillips, personal communication); *P. fluorescens* strains did hydrolyse casein in their tests. Gilardi (1976) found that all *P. fluorescens* strains and 60% of *P. aeruginosa* strains hydrolysed casein. Our results also suggest that hydrolysis of casein is not a suitable characteristic for differentiation between *P. aeruginosa* and *P. fluorescens*. Furthermore we do not consider that pyocyanin production is a reliable characteristic, except on King's "A" medium; it is particularly unreliable in poorly pigmented strains because pigmentation is affected by environmental conditions, especially aeration.

The marked discrepancy between our findings and those of Brown and Scott-Foster (1970) may be explained by the small numbers and origin of their test strains, which were mostly reference strains. The strains used in the present study were, with one exception, clinical isolates.

We conclude that the scheme of Phillips (1969) should be used for the routine identification of strains of *P. aeruginosa* that are not obvious pigment producers. The distinction between *P. fluorescens* and *P. putida* is less straightforward but the important differences are the production of gelatinase and lecithinase and the hydrolysis of casein by *P. fluorescens*.

SUMMARY

Eighty-six clinical isolates of fluorescent pseudomonads that did not produce pyocyanin on Diagnostic Sensitivity Test Agar or Cetrimide Agar were identified on the basis of their antibiotic sensitivity, production of pigment on King's "A" medium, growth at 42°C, production of lecithinase and hydrolysis of gelatin. The identity of the strains was confirmed in tests

with the ammonium salt sugars ethanol, glucose and mannitol. These tests were adequate for distinguishing between the three important fluorescent pseudomonads.

The detection of casein hydrolysis on milk agar was assessed as a rapid method of distinguishing *P. aeruginosa* from the other species of fluorescent pseudomonads but proved unhelpful when compared with, or included in, a small set of tests. Most strains of *P. aeruginosa* and *P. fluorescens* hydrolysed casein.

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