A simple diagnostic milk medium for *Pseudomonas aeruginosa*

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SYNOPSIS An agar medium containing 10% defatted milk has been tested as a diagnostic medium for *Pseudomonas aeruginosa* and, in particular, for differentiating between *P. aeruginosa* and *P. fluorescens*. Only *P. aeruginosa* colonies gave clear zones due to hydrolysis of casein together with diffused green pigment. Results on milk agar correlated well with the pattern of results from a variety of conventional tests used to identify this organism. Pigment production of *P. aeruginosa* on milk agar was better than on special media commonly used to enhance this characteristic. Routine diagnosis of *P. aeruginosa* is recommended by streaking on a solid medium containing 10% defatted milk granules, 25% nutrient broth, and 2% agar, and examining for clear zones and pigment after 24 hours' incubation.

The genus *Pseudomonas* is a group of aerobic, Gram-negative, motile rods that are catalase- and oxidase-positive as well as attacking sugars oxidatively without producing gas. In addition diffusible yellow or green pigments may be produced. Identification of this genus (the pseudomonads) depends on the pattern of results from a series of tests to establish these characters (Cowan and Steel, 1965). In addition, only some pseudomonads are pigmented, and in pigmented species there are some non-pigmented strains. Many methods for the enhancement of pigment production have been described and analysed (Colwell, 1964), and, although together they may be useful, none is specific on its own.

Several tests have been proposed as specific for *P. aeruginosa* (Haynes, 1951; Gaby and Free, 1953; 1958), but it is generally agreed that in order to distinguish conclusively between the so-called fluorescent aerobic pseudomonads, ie, between *P. aeruginosa*, *P. fluorescens*, and *P. putida*, a study of such tests as those already quoted, and others similar, is necessary. Unless *P. aeruginosa* produces pyocyanine (or pyorubin) pigments its identification is often difficult and

may necessitate the use of a whole battery of tests (Rhodes, 1961; Colwell, 1964). Phillips (1969) has proposed a series of nine tests for the identification of *P. aeruginosa* from clinical material. Stanier, Palleroni, and Doudoroff (1966) have shown that the determination of the nutritional spectra is probably the most useful method available at the moment for practical taxonomic purposes, but that even this represents only a first approximation towards characterizing the phenotypes of the aerobic pseudomonads.

Routine use of an agar milk medium has proved valuable in confirming the presence of *P. aeruginosa*, colonies of which showed a clear zone due to hydrolysis of casein, together with green pigment that diffused into the medium. This paper reports on a number of strains of the three species within the fluorescent aerobic pseudomonad cluster grown on several such milk media, with a view to its use as a diagnostic medium for *P. aeruginosa*.

Materials and Methods

ORGANISMS

Table I lists the organisms used.

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Original Source and Description

<i>Pseudomonas aeruginosa</i>			<i>Pseudomonas fluorescens</i>	<i>Pseudomonas putida</i>
CST	651	Path. lab. faeces sample	NCIB 3756	Lab. stock strain—source unknown
CST	652	Path. lab. faeces sample	NCIB 8248	NCIB 9034
CST	653	Path. lab. infected ear	NCIB 8251	
CST	654	Path. lab. faeces sample	NCIB 8248	
CST	655	Path. lab.	NCIB 8249	
OSU	64	From Dr R. G. Eagon ¹	NCIB 8729	
NCTC ²	6750		NCIB 8865	
NCTC	7244		NCIB 8729	
NCTC	8203		NCIB 9033	
NCTC	1999		NCIB 9046	
NCTC	6751 ³		NCIB 9494	
NCTC	5940 ⁴			
NCTC	950 ⁴			
NCTC	8505 ⁴			
CST	656	Path. lab. Post mortem-infant		
NCIB ⁴	5940			
NCIB	8295			
NCIB	8626			
CST	657	Contaminated distilled water Bath University		

Table I *Organisms used in diagnostic tests*¹Kindly supplied by Dr R. G. Eagon, University of Georgia, Athens, GA, USA²National Collection of Type Cultures, Central Public Health Laboratory, Colindale Ave, London, NW9, England³These organisms were kindly given by Dr S. P. Lapage, Curator of the National Collection of Type Cultures, as examples of *P. aeruginosa* which are poor pigment producers⁴National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland**MEDIA**

Yeast extract broth was used throughout this study (Rhodes, 1959). When solid media were required for plating, 2% w/v New Zealand agar was dissolved in the yeast extract broth.

A 20% solution of either defatted skim milk powder (Oxoid)¹ or Marvel (Cadburys)² defatted milk granules was prepared, and autoclaved for five minutes at 115°C. Equal volumes of sterilised 4% solution Bacto agar (Difco)³ and milk solution were mixed at approximately 60° and 15 ml plates immediately poured. The plates were oven dried at 37°C for one hour before use.

GRAM REACTION

Microscopic examination of a modified Gram-stained smear (Preston and Morrell, 1962) of a culture grown on yeast extract agar was performed to study the Gram reaction.

MOTILITY

Motility was evaluated by microscopy of a hanging drop.

CATALASE

Catalase was tested for by adding a few drops of 10 vol hydrogen peroxide to a colony on yeast

extract agar. A rapid and ebullient evolution of gas (oxygen) was taken as evidence of the presence of catalase.

OXIDASE TEST

The Rogers (1963) modification of the Kovacs (1956) method was used with colonies grown on yeast extract agar.

OXIDATION/FERMENTATION TEST

The method of Hugh and Leifson (1953) was used.

OTHER TESTS

Other tests to distinguish between species in the fluorescent cluster of the aerobic pseudomonads (Stanier *et al.*, 1966) were as follows.

Growth temperatures

Growth was investigated at temperatures of 42, 37, 22, and 4°C, by inoculating 5 ml volumes of yeast extract broth maintained at the stated temperature to within $\pm 0.5^\circ\text{C}$ in a water bath. Tubes were examined daily for seven days to determine if growth had occurred.

Gelatin hydrolysis

Nutrient gelatin (Oxoid) was inoculated with a wire stab in 20 ml volumes in McCartney bottles. The containers were incubated at 22° for seven days. Those showing growth down the line of inoculation and no liquefaction were recorded as negative, those showing growth with liquefaction of the medium were recorded as positive, and those showing no growth were recorded accordingly.

Production of pyocyanine

Pyocyanine production on King's medium A, (King, Ward, and Raney, 1954) and the Tween medium of Wahba and Darell (1955) was tested by streaking 18-hour cultures on to 20-ml plates and incubating for 48 hours at 37° and 22°C.

Gluconate utilization

The method of Haynes (1951) was used, modified by the use of Clinistix⁴, to detect the presence of the oxidized product, 2-keto-gluconate, as reducing substances, shown to be absent by testing the medium before inoculation (Cowan and Steel, 1965). Slime production was also tested for by the general appearance of the medium after five days' growth, at 37°C, and by the 'reverse swirl test' (Haynes, 1951).

¹Oxo Ltd, Thames House, Queen Street Place, London, EC4²Cadbury Bros Ltd, Bournville³Difco Laboratories, Detroit 1, Michigan, USA⁴Ames & Co. Ltd, Stoke Poges, Bucks.

Test	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	<i>P. putida</i>
Growth at 4°C	—	+	v
Growth at 22°C	+	+	+
Growth at 37°C	+	+	—
Growth at 42°C	+	—	—
Gelatin hydrolysis	+	v	—
Production of pyocyanine	+	—	—
Conversion of gluconate to 2-keto-gluconate	+	+	v
Slime production	+	v	v
Utilization of acetamide	+	—	v
Utilization of geraniol	+	—	—
Utilization of trehalose	—	+	—
Utilization of inositol	—	+	—
Utilization of sucrose and levan formation	—	+	—
Utilization of benzylamine	—	—	+
Utilization of creatine	—	—	+

Table II *Differentiation between P. aeruginosa, P. fluorescens, and P. putida on the basis of the tests described*

+ = more than 90% of strains positive
 — = less than 10% of strains positive
 v = variable

Utilization of various carbon sources

Stanier *et al* (1966) have stated that the nutritional spectra of this group probably forms one of the most satisfactory taxonomic methods for differentiating between *P. aeruginosa* and *P. fluorescens*. Accordingly we have selected those characters that give the greatest degree of differentiation. Using the method of Stainer *et al* (1966), the ability of the organisms to utilize acetamide, geraniol, trehalose, inositol, sucrose (levan formation), benzylamine, and creatine was tested.

The reactions to each of these tests are summarized in Table II.

Growth on skim milk agar

The main purpose of this study was to determine if growth, pigment production, and caseinase production on milk agar could be used to differentiate between *P. aeruginosa* and other related fluorescent aerobic pseudomonads. Accordingly, milk agar plates were streaked with the organisms listed, and incubated at 37° for 48 hours. Growth, pigment production, and hydrolysis of the milk, as shown by clear transparent zones surrounding the areas of growth, were all recorded separately.

One disadvantage of the simple milk agar is that it takes 48 hours for full pigmentation and hydrolysis to show. Varying amounts of Oxoid nutrient broth no. 2 were added to the milk to determine if the incubation time could be reduced.

Colony counts of a suspension of *P. aeruginosa* were performed in quintuplicate on agar plates prepared from 100% nutrient agar, 75% nutrient agar + 25% milk agar, 50% nutrient agar + 50%

milk agar, 25% nutrient agar + 75% milk agar, and 100% milk agar. Twenty-ml plates were poured and oven dried for two hours at 37°C, and 0.5 ml aliquots of an appropriate dilution of a 24-hour broth culture of *P. aeruginosa* NCTC 6750 in sterile nutrient broth were spread on the surface with a wire spreader. Colonies were counted after 48 hours' incubation at 37°C.

Results

IDENTIFICATION

Cowan and Steel (1965) define the genus *Pseudomonas* in the following terms: Gram-negative rods, motile, aerobic, catalase positive, oxidase positive, attack sugars by oxidation, but do not produce gas, and diffusible yellow-green pigments may be produced. Their scheme of identification has been followed, with the addition of tests designed to distinguish between *P. aeruginosa* and *P. fluorescens* with greater precision.

All of the organisms tested conformed to this description. The results of the other tests were interpreted in a manner similar to that of Cowan and Steel (1965), and, on this basis, the three species under investigation were separated (Table II).

GROWTH TEMPERATURE

Growth, where it occurred, was nearly always clearly visible, but comparison was always made with a tube of inoculated yeast extract broth incubated at the same temperature for seven days. Where growth was doubtful or scant, replicate tube was re-inoculated, and incubated for a further seven days.

Growth tests at 22°C, in addition to the three other usual temperatures, were carried out as a control for gelatin liquefaction, since reading of these results is easier if the gelatin is not allowed to melt.

Where no growth was present, particularly at 4°C when growth is to be expected to proceed much more slowly, tubes were returned to the water bath for a further seven days before negative was finally recorded.

GELATIN HYDROLYSIS

Gelatin hydrolysis, as evidenced by liquefaction of the nutrient gelatin around the area of growth down the stab, was easy to see when it occurred.

PRODUCTION OF PYOCYANINE

Pyocyanine, the blue-green pigment that is characteristic of most strains of *P. aeruginosa*, was enhanced on various special media. Both King's A medium (King *et al*, 1954) and the Tween

Fig. Appearance of (left) *P. aeruginosa*, and (right) *P. fluorescens* after incubation of milk agar.

80 medium of Wahba and Darell (1965) are reported to give good pigment production, with otherwise non-pigmented varieties. All but three of the strains of *P. aeruginosa* tested produced pyocyanine equally well on both these media.

GLUCONATE UTILIZATION

The utilization of gluconate, and its conversion to 2-keto gluconate with the production of slime, has been used as a method for differentiation between *P. aeruginosa* and *P. fluorescens* (Haynes, 1951). Although all strains of *P. aeruginosa* produce both slime and keto-gluconate from this source, we have found that production of slime is not a reliable guide on its own. The presence of reducing compounds in the medium after growth in addition to the slime has been found to give far more reliable results than the measurement of slime production alone.

UTILIZATION OF VARIOUS CARBON SOURCES

The determination of the nutritional spectra has been advocated by Stanier *et al* (1966) as a method for differentiating between members of the genus *Pseudomonas*. The specificity of this method has been substantiated in this study, but the method is tedious and not well suited for day-to-day routine use. It will only work reliably when several different carbon sources are used to determine the pattern, and also if the method described is carried out exactly with due care and attention to all details.

GROWTH ON MILK AGAR

The main purpose of this study was to ascertain if this simple medium might have a use in distinguishing between *P. aeruginosa* and related species, especially *P. fluorescens*. All the strains shown to be *P. aeruginosa* as a result of the previous tests gave a typical reaction on milk agar when incubated for 48 hours at 37°C. Pigment production was as good, if not better

than on those media specifically designed to demonstrate pyocyanine pigment. It was bright green, and diffused throughout the whole of the plate. In addition, the presence of extracellular proteinase was demonstrated by the hydrolysis of milk protein as shown by a clear transparent zone surrounding the colonies (Figure). Best results were obtained if the plates were only lightly streaked, and well isolated colonies were obtained, when a clear zone could be seen surrounding the individual colonies. On a heavily inoculated plate, the whole of the plate may be cleared by some potent proteinase producers. The best pigment production is obtained by incubating the plates for 24 hours at 37°C followed by 24 hours at 20°C, when many apparently weakly pigmented strains show much enhanced pigment production. All of the *P. fluorescens* spp. examined grew on the milk agar, but failed to produce any hydrolysis as demonstrated by clear zones (Figure). Clearly, any extracellular proteinase they produce is not a caseinase. In addition, nearly all are completely non-pigmented, but a few strains produce yellow pigments after prolonged incubation at low temperatures. *P. putida* produced no hydrolysis or pigment.

Initially in this study the milk powder used to prepare this medium was skim milk powder. This is not easy to sterilize as it will readily caramelize if overheated, and on some occasions does not reconstitute easily, particularly at the double-strength concentration necessary for the preparation of the agar. Subsequently Cadbury's Marvel granules were used, and this was found easier to use. The latter product was not stated to be thermophil free. Repeated testing of samples has, however, failed to reveal any such organism in this product.

INCREASE IN GROWTH RATE BY INCORPORATION OF NUTRIENT BROTH INTO THE MILK AGAR

The addition of less than 10% broth produced no detectable difference in the results. Concentrations of broth in excess of 25% tended to mask the hydrolysis, and in some instances to suppress pigmentation. At 25% broth concentration in the medium both hydrolysis and pigmentation were clearly visible after 24 hours. Therefore if speed is of the utmost importance, then 25% broth in the milk will give the desired result; however, if time is no great concern, then the plain milk agar gives clearer results.

The colony counts showed no significant variation in the number of colonies per plate ($\pm 3.8\%$; $p = 0.01$) irrespective of the composition of the agar on which they were grown, the only difference being that not all colonies had completely hydrolysed the milk after 48 hours' incubation on agar containing 50% or more milk. Prolonged incubation up to a week in no case caused an

Strain <i>P. aeruginosa</i>	Growth at 4°C	Growth at 42°C	Gelatin	Pyocyanin	Acetamide	Geraniol	Trehalose	Inositol	Sucrose	Benzylamine	Creatine	Milk
CST 651	—	+	G	P	+	+	—	—	—	—	—	G,P,H
CST 652	—	+	L	P	+	+	—	—	—	—	—	G,P,H
CST 653	—	+	L	P	+	+	—	—	—	—	—	G,P,H
CST 654	—	+	L	P	+	+	—	—	—	—	—	G,P,H
CST 655	—	+	L	P	+	+	—	—	—	—	—	G,P,H
CST 656	—	+	L	P	+	+	—	—	—	—	—	G,P,H
CST 657	—	+	L	P	+	+	—	—	—	—	—	G,P,H
OSU 64	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCIB 3756	—	+	L	—	+	+	—	—	—	—	—	G,P,H
NCIB 5940	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCIB 8248	—	+	L	—	+	+	—	—	—	—	—	G,P,H
NCIB 8295	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCIB 8626	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 1999	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 6750	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 6751	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 7244	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 8203	—	+	L	P	+	+	—	—	—	—	—	G,P,H
NCTC 8505	—	+	L	—	+	+	—	—	—	—	—	G,P,H
<i>Intermediate strains</i>												
NCTC 950	—	+ ?	L ?	—	+	—	+	—	—	—	—	G,H
NCTC 5940	—	+ ?	L ?	—	+	—	+	—	—	—	—	G,H
<i>P. fluorescens</i>												
NCIB 8249	+	—	—	—	—	—	+	+	+	—	—	G
NCIB 8251	+	—	—	—	—	—	+	+	+	—	—	G
NCIB 8729	+	—	L	—	—	—	+	+	+	—	—	G
NCIB 8865	+	—	L	—	—	—	+	+	+	—	—	G
NCIB 8986	+	—	L	—	—	—	+	+	+	—	—	G
NCIB 9033	+	—	L	—	—	—	+	+	+	—	—	G
NCIB 9046	+	—	L	—	—	—	+	+	+	—	—	G
NCIB 9494	+	—	—	—	—	—	+	+	+	—	—	G
NCTC 8248	—	+	L	—	—	+	+	—	—	—	—	G
<i>P. putida</i>												
Lab. strain	+	—	—	—	+	—	—	—	—	+	+	G
NCIB 9034	—	—	—	—	—	—	—	—	—	+	+	G

Table III Results of tests for differentiation between the fluorescent aerobic pseudomonads

+ = Positive — = Negative G = Growth L = Liquefaction ? = slight reaction P = Pigment H = Hydrolysis

increase in the number of colonies, but allowed the medium surrounding every colony to clear.

The individual results are summarized in Table III.

Discussion

From the results presented it can be seen that the pattern of biochemical and other tests for the strains of *P. aeruginosa*, *P. fluorescens*, and *P. putida* tested follow the pattern shown in Table II. In addition it can be seen that where these tests are all positive for *P. aeruginosa* then the growth on the milk agar shows both green pigment and proteinase, confirming the view that these two features alone on this medium are equally reliable as the whole series of biochemical tests for the identification of *P. aeruginosa*.

Likewise it can be shown that when growth is not accompanied by pigment or hydrolysis then the organism is not *P. aeruginosa*, and this is shown by the tests performed on a number of species of *P. fluorescens* and *P. putida*. An interesting point emerges, in that the strains NCIB 3756 and 8248 were received as *P. fluorescens* yet both produced green pigment and hydrolysed the

casein in the milk agar. Thus on the basis of this one test these two strains should be renamed *P. aeruginosa*. This is supported by the other biochemical tests, which show the typical *P. aeruginosa* pattern. In addition, phage typing of these two strains gave results that were consistent with their being *P. aeruginosa*. The remainder of the strains of *P. fluorescens* gave typical results on both biochemical tests and reaction on milk agar. The results for gelatin hydrolysis from *P. fluorescens* agree with those of Cowan and Steel (1965), Wahba and Darell (1965), and Rhodes (1959), in that this character was variable: eight out of 11 strains used in this study hydrolysed gelatin. This is in contrast to the findings of Stanier *et al.*, who observed gelatin liquefaction in all their strains of *P. fluorescens*. This difference is most probably attributable to small differences in the composition in the nutrient gelatin medium and in the length of the incubation period.

Strains NCTC 950, 5940, 6751, and 8505 were supplied as being examples of poorly pigmented varieties of *P. aeruginosa*. They provide interesting examples of what seem to be in some respects atypical strains of this organism. As can be clearly seen from the results they fall into two main groups. Strains 6751 and 8505, apart from

a low production of pigment on normal media, according to all the other biochemical tests showed them to be normal *P. aeruginosa* strains; this was confirmed by the results of the growth on the milk agar, both hydrolysis of the casein and pigment production being observed. In the case of the other two strains, 950 and 5940, the position was not so straightforward. Neither produced pigment on any of the solid media, including the milk agar. They did, however, show some signs of hydrolysis of the casein, and this increased on incubation for a further 48 hours, but no pigment was demonstrable. The results of the biochemical tests likewise showed that these two strains did not conform precisely to the *P. aeruginosa* or the *P. fluorescens* patterns, but in fact have some characters common to both, and these can be regarded as intermediate *fluorescens/aeruginosa* types. This is supported by the results of the milk agar media previously discussed. Indeed, in view of the recently demonstrated high transformation frequency within the genus *Pseudomonas* (Khan and Sen, 1967), the existence of such intermediate strains is not surprising. In either case, the milk agar has shown itself to be as reliable as the more conventional biochemical tests.

We wish to thank Dr S. P. Lapage, of the National Collection of Type Cultures, for kindly supplying the poorly pigmented strains of *P. aeruginosa*, and Dr M. T. Parker, of Central Public Health Laboratory, Colindale, London, NW9, for kindly carrying out the phage typing. We also wish to thank The Medical Research Council for a grant which supported part of this work.

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Errata

In Table II in the paper entitled, 'Comparison of quick and slow thaw methods of producing cryoprecipitate antihemophilic factor from fresh and 24-hour-old blood' A. L. Bloom (*J. clin. Path.*, **22**, 447-452) the P values for the supernatant have been printed under the wrong headings. The correct table 'Factor VIII content of cryoprecipitate and supernatant plasma', is printed below.

	Fresh Blood		Twenty-four Hour Blood	
	Quick-Thaw (A)	Slow-Thaw (B)	Quick-Thaw (C)	Slow-Thaw (D)
Number of samples	101	67	102	76
Factor VIII in cryoprecipitate (units) Mean $\pm$ SD	83 $\pm$ 32	112 $\pm$ 44	53 $\pm$ 25	72 $\pm$ 30
Factor VIII in supernatant (units) Mean $\pm$ SD	42 $\pm$ 21	30 $\pm$ 21	31 $\pm$ 19	25 $\pm$ 12
Statistical significance	A v B	C v D	A v C	B v D
Supernatant	P = <0.001 P = <0.001			
Cryoprecipitate	P = <0.001 P = <0.001 P = <0.001 P = <0.001			

In Table IIa of the paper by Davis *et al.*, *J. Clin. Path.*, 1969, **22**, 634, the figures for *Proteus mirabilis* under the columns for tetracycline should read: S: 9.7%, 'S': 0.9% and R: 89.4%; the mean percentages of total should therefore read: S: 30.8%; 'S': 6.1%; R: 63.1%.