Use of an improved cetrimide agar medium and other culture methods for *Pseudomonas aeruginosa*

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SYNOPSIS In a comparison of two selective media for *Ps. aeruginosa* containing 0.03% cetrimide, stronger fluorescence was obtained from growth on the medium prepared with King's medium B as the base (CTA 2); although *Ps. aeruginosa* was not isolated more frequently from burns on this medium than from cetrimide agar made with a Lemco base (CTA 1), results were easier to assess and there were fewer cases of doubtful fluorescence on CTA 2 than on CTA 1.

Pyocyanin production on CTA 1 was better than on CTA 2, but for demonstration of pyocyanin production Wahba and Darrell's medium was preferable and showed the pigment in 455/497 (92%) of fluorescent strains of *Ps. aeruginosa* from burns.

A series of tests on 99 strains of *Ps. aeruginosa* or presumptive *Ps. aeruginosa*, on 30 strains of other species of *Pseudomonas*, and on 68 strains of other species of Gram-negative rods showed the value of growth on cetrimide agar and on triphenyl tetrazolium chloride medium, of slime production in gluconate broth, of gas production from nitrate, and of growth at 42°C. as diagnostic aids in the recognition of *Ps. aeruginosa*.

A selective medium containing 0.03% cetrimide in Lemco agar (Lowbury and Collins, 1955) has for many years been used here and found effective in the isolation of *Pseudomonas aeruginosa* (*Ps. pyocyanea*) from burns and other sources; with the aid of ultra-violet irradiation, fluorescein is easily detected in the majority of cultures of *Ps. aeruginosa* on this medium and on certain other solid media (Lowbury, 1951; Lowbury, Lilly, and Wilkins, 1963). Many fluorescent cultures also show the presence of pyocyanin by varying degrees of blue-green pigmentation. A few strains of *Ps. aeruginosa* show relatively poor fluorescence, and non-fluorescent strains, which are in other respects indistinguishable from *Ps. aeruginosa*, are very occasionally isolated.

King, Ward, and Raney (1954) have described two media, one of which (medium A) enhances the production of pyocyanin by *Ps. aeruginosa*, while the other (medium B) enhances the production of fluorescein. An improved medium for pyocyanin production (modified Sierra medium) has been developed by Wahba and Darrell (1965). In this paper we describe the improvement of cetrimide agar by the use of King's medium B as the base; we compare the frequency of isolation of *Ps. aeruginosa* from natural sources (burns) on the new and the

old cetrimide agar, and also assess the value of pyocyanin production in the detection of *Ps. aeruginosa* from the same sources. A comparison of 99 strains of *Ps. aeruginosa* and presumptive *Ps. aeruginosa* with other pseudomonads and species of Gram-negative rods is described.

MATERIALS AND METHODS

CULTURE MEDIA Two cetrimide agar media were prepared.

Cetrimide agar with Lemco base (CTA 1) This was a slightly modified form of the medium described by Lowbury and Collins (1955) and contained the following ingredients:—Peptone (Oxoid), 10 g.; Lab-Lemco (beef-extract), 10 g.; sodium chloride, 5 g.; agar (New Zealand), 15 g.; distilled water, 1,000 ml. The pH was adjusted to 7.4. This basal medium was autoclaved at 121°C. for 15 minutes and stored. To prepare the selective medium it was melted in the steamer and cooled to 45°C.; 15 ml. of a Seitz-filtered 2% solution of cetrimide (B.P.) was then added, to give a concentration of 0.03% in the medium.

Cetrimide agar with modified King's base (CTA 2) The basal medium was prepared as follows:—Proteose peptone no. 3 (Difco), 20 g.; New Zealand agar, 15 g.; glycerol, 10 g.; distilled water, 1,000 ml. The medium was adjusted to pH 7.2. It was autoclaved for 15 minutes at 121°C. and stored. To 100 ml. of the melted solution were

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added 1 ml. of a 15% solution of K_2HPO_4 (anhydrous) and 1 ml. of a 15% solution of $MgSO_4 \cdot 7H_2O$; these solutions were prepared with distilled water and sterilized by Seitz filtration. A Seitz-filtered 2% solution of cetrimide (B.P.) was added to the basal medium to give a final concentration of 0.03% cetrimide for the selective medium.

Wahba and Darrell's (1965) modified Sierra medium for enhancement of pyocyanin production (P.A.) was prepared as follows (Wahba, personal communication):—Bacto peptone (Difco), 10 g.; NaCl, 5 g.; $CaCl_2 \cdot 1H_2O$, 0.1 g.; agar (Davis), 18.0 g.; water (distilled), 1,000 ml. This basal medium was adjusted to pH 7.4. Tween 80 and $MgSO_4 \cdot 7H_2O$ solutions were separately autoclaved and added to the basal medium to give a final concentration of 1.0% and 0.1% respectively.

EXPERIMENTS 1 A comparison was made of cetrimide agar with Lemco base (CTA 1) and cetrimide agar with a slightly modified King's base (CTA 2) in the isolation of *Ps. aeruginosa* from 1,780 consecutive swabs from burns. The swabs were taken and set up in culture as described elsewhere (Lowbury, 1960). For the comparison of cetrimide media, subcultures were plated on to them from cooked meat broth cultures which had been incubated at 37°C. overnight. Plates of cetrimide agar were inoculated by transferring a loopful of each liquid culture to a marked square on the solid medium ('spot plate'); up to 25 cultures could be inoculated on one spot plate.

Cultures were examined after approximately 18 and 42 hours' incubation at 37°C. for growth on the selective medium and for pigment production; they were also examined for characteristic fluorescence under ultraviolet irradiation in a viewing cabinet (Lowbury *et al.*, 1963). Growth on cetrimide agar with production of characteristic fluorescence was taken as presumptive evidence that the culture contained *Ps. aeruginosa*.

All cultures were also inoculated on spot plates of Wahba and Darrell's medium for pyocyanin production, six strains per plate; cultures showing poor or doubtful blue pigment production after 48 hours' incubation were inoculated on slopes of Wahba and Darrell's medium and were extracted after 48 hours' incubation by grinding the medium with a small volume of chloroform; the separated layer of chloroform was examined for blue pigment.

2 A comparison was made of CTA 1, CTA 2, and PA medium in the isolation of *Ps. aeruginosa* by direct inoculation of material from selected burn swabs. Swabs taken from burns were extracted first by rubbing thoroughly in a small volume (0.2 ml.) of nutrient broth. These extracts were inoculated by wire loop on spot plates of CTA 1 and CTA 2 and on slopes of Wahba and Darrell's medium (PA). Cultures on CTA 1 and CTA 2 were examined as described above; cultures on PA were examined after 48 hours' incubation at 37°C. for blue pigment; a proportion of these tubes showing varying degrees of blue pigmentation or non-detectable pigment were extracted with chloroform and examined for chloroform-soluble pigment.

3 A series of tests was made on 99 strains of presumptive *Ps. aeruginosa* (so described on the basis of character-

istic growth and fluorescence on 0.03% cetrimide agar), 55 strains of known genera or species of other Gram-negative rods, and 30 strains of known species of *Pseudomonas*. The tests were as follows:—(1) Morphology (Gram stain); (2) growth on cetrimide agar (CTA 1); (3) fluorescence on CTA 1; (4) pyocyanin production (on Wahba and Darrell's medium); (5) oxidase production (Kovacs, 1956); (6) nitrate reduction with production of gas, by the Griess-Ilosvay method (Wilson and Miles, 1964); (7) growth at 42°C. in nutrient broth; (8) deamidation of acetamide (Bühlmann, Vischer, and Bruhin, 1961); (9) glucose oxidation/fermentation (Hugh and Leifson, 1953); (10) arginine dihydrolase (Thornley, 1960); (11) gelatin liquefaction (in nutrient gelatin stabs, examined after 24 hours' incubation at 37°C.); (12) egg yolk reaction (Klinge, 1960); (13) gluconate oxidation and slime production (Haynes, 1951); a loopful of an overnight broth culture was inoculated into a medium prepared for the gluconate oxidation test (Cruickshank, 1965). The test for slime production was made by touching the top of the culture with a wire loop and drawing the loop back to the mouth of the tube; slime was shown by the presence of a mucoid 'string'; and (14) growth in triphenyl tetrazolium chloride medium (Selenka, 1958).

RESULTS

Table I shows a comparison of the two cetrimide agar media for the isolation of *Ps. aeruginosa* from cooked meat broth cultures of burn swabs. When *Ps. aeruginosa* was isolated it appeared on both media, but in all except 12 cultures fluorescence was stronger on the medium made with King's base (CTA 2). The medium made with Lemco base, on the other hand, showed better pyocyanin production; growth on CTA 2 had a vivid lemon-yellow colour, while that on CTA 1 was often dark or bluish green, and the presence of pyocyanin reduced the intensity of fluorescence under ultraviolet irradiation.

TABLE I
ISOLATION OF *PSEUDOMONAS AERUGINOSA* FROM BURNS
ON TWO CETRIMIDE AGAR MEDIA

Tests	Results (Number of Swabs)
Total swabs	1,780
<i>Ps. aeruginosa</i> isolated	
CTA 1 and CTA 2	497
CTA 1 only	0
CTA 2 only	0
Fluorescence under ultra-violet light	
Stronger on CTA 2	485
Stronger on CTA 1	0
Equal to CTA 1 and CTA 2	12
Doubtful fluorescence	
On CTA 1	4
On CTA 2	0
Pyocyanin in PA medium	455

Of the 497 strains of fluorescent pseudomonas isolated in these cultures, 455 (92%) showed blue pigment in Wahba and Darrell's medium. Although it was often possible to recognize pyocyanin production in CTA 1, many strains which showed good pigmentation in Wahba and Darrell's medium did not show obvious blue-green or dark green pigment on CTA 1. Pyocyanin was not seen in cultures on PA from any specimen which did not show fluorescent growth on CTA 1 or CTA 2.

Table II shows the results of parallel culture of 158 selected swabs from burns on CTA 1, CTA 2, and PA media. From 84 of these *Ps. aeruginosa* was isolated on solid medium; the majority of these (54) showed *Ps. aeruginosa* on all three media, but from eight specimens the organism was detected only on one medium, and from 22 it was detected only on two of the media. Although fluorescence was almost always better on CTA 2 than on CTA 1, there were seven specimens which showed fluorescent growth on CTA 1 but not on CTA 2; and although fluorescein production was shown in subculture from liquid medium to be a more general property of these strains than pyocyanin production (see Table I), on direct inoculation four specimens were detected by pyocyanin production when fluorescein production was not detected, in each case a mixed growth of *Ps. aeruginosa* and of a *Proteus* which was able to grow on CTA 1.

TABLE II

ISOLATION OF *PSEUDOMONAS AERUGINOSA* ON TWO CETRIMIDE AGAR MEDIA AND ON A MEDIUM FOR PYOCYANIN PRODUCTION (PA) BY DIRECT INOCULATION OF SWAB EXTRACTS

Tests	Results (Number of Swabs)
<i>Ps. aeruginosa</i> isolated on direct inoculation of solid media	84
<i>Ps. aeruginosa</i> isolated:	
On CTA 1, CTA 2, and PA	54
Only on CTA 1	0
Only on CTA 2	4
Only on PA	4
Only on CTA 1 and CTA 2	7
Only on CTA 1 and PA	7
Only on CTA 2 and PA	8

Table III shows the results of examining 99 strains of *Ps. aeruginosa* or presumptive *Ps. aeruginosa*, 30 strains of other pseudomonads, and 68 strains of other genera and species of aerobic Gram-negative rods for growth on CTA 1. Plates were inoculated with loopfuls of undiluted 24-hour broth cultures; they were incubated at approximately 22°C. when the strains had been shown in previous tests to grow poorly at 37°C. on blood agar; those which grew

TABLE III
GROWTH OF *PSEUDOMONAS AERUGINOSA* AND OTHER GRAM-NEGATIVE RODS ON CETRIMIDE AGAR (CTA 1)

Temperature of Incubation ¹	Growth of <i>Ps. aeruginosa</i> or Presumptive <i>Ps. aeruginosa</i>			Growth of Other Pseudomonads			Growth of Other Gram-negative Rods		
	++ ³	+or±	—	++	+or±	—	++	+or±	—
37°C.	99	0	0	0	2	4	0	26	37
Approximately 22°C.	—	—	—	1 ²	18	5	0	0	5
Total	99	0	0	1	20	9	0	26	42

¹Strains which grew poorly at 37°C. on blood agar were grown on CTA 1 at approximately 22°C. (room temperature); other strains were grown at 37°C.

²*Ps. putida*

³++ = heavy growth; + = moderate growth; ± = scanty growth

well on blood agar at 37°C. were grown on CTA 1 at 37°C. The following strains of pseudomonas were tested:—*Ps. aeruginosa* (96), *Ps. aeruginosa* var pyorubin (2), *Ps. polycolor* (grouped here with *Ps. aeruginosa*) (1), *Ps. fluorescens* (8), *Ps. putida* (1), *Ps. marginalis* (1), *Ps. pastinaceae* (1), *Ps. viridiflava* (1), *Ps. syringae* (2), *Ps. ovalis* (1), *Ps. astrofaciens* (1), *Ps. fragi* (1), *Ps. denitrificans* (1), *Ps. oxidans* (5), *Ps. alcaligenes* (1) and *Ps. species* (3). Of the other Gram-negative rods the following were tested: *Proteus mirabilis* (16), *Providence (Proteus inconstans)* (5), *Escherichia coli* (9), *Enterobacter aerogenes* (6), *Klebsiella aerogenes* (1), *Cloaca sp.* (11), *Achromobacter anitratus* (7), *Alcaligenes sp.* (7), and *Comamonas sp.* (6).

All the strains of *Ps. aeruginosa* showed a heavy growth after 18 hours' incubation on CTA 1. Of the other strains of pseudomonas, only *Ps. putida* grew heavily on the selective medium, but this and most of the other strains of pseudomonas did not grow well at 37°C. and were tested at 22°C. Most of the other species of Gram-negative rods failed to grow on CTA 1, and none gave a heavy growth on CTA 1, though moderate or scanty growth was obtained from 12 strains of *Proteus mirabilis*, five strains of *Providence (Proteus inconstans)*, and two strains of *Comamonas*. When no growth was found after 18 hours, plates were reincubated for six days and re-examined.

Table IV shows the results of examining these strains by a number of tests which have been found useful in the study of *Pseudomonas* species. Most strains of presumptive *Ps. aeruginosa* showed several features which usually differentiated them and other pseudomonads from the majority of other Gram-negative rods (oxidase reaction, oxidative metabolism of glucose, positive arginine dihydrolase reaction, and acetamide deamidation); they also showed certain features which differentiated them from most other

TABLE IV
MISCELLANEOUS TESTS ON *PS. AERUGINOSA*
AND OTHER GRAM-NEGATIVE RODS

Test	Ps. aeruginosa or Presumptive aeruginosa ¹		Other Pseudomonads		Other Gram-negative Rods	
	+	-	+	-	+	-
Fluorescence on CTA 1	98	1	9	21	0	68
Pyocyanin production (on Wahba and Darrell's medium)	87	12	0	30	0	68
Oxidase (Kovacs)	99	0	28	2 ²	13 ³	55
Gas from nitrate	90	9	1	29	0	68
Growth at 42°C.	98	1	5	25	59	9
Acetamide deamidation	98	1	17	13	5 ⁴	63
Hugh and Leifson's test	99	0	29	1	19 ⁵	49
Arginine dihydrolase	99	0	24	6	14 ⁶	54
Gelatin liquefaction	90	9	11	19	26	42
Egg yolk reaction	8(±) 91	11	19	6(±) 62		
Gluconate oxidation	95	5	17	13	11	57
Slime production	98	1 ⁵	0	30	0	68
Growth in triphenyl tetrazolium chloride	97	2	7(4 ±) 23	6 ⁷	62	

¹Gram-negative rods growing and giving characteristic yellow, green, or blue green fluorescence on CTA 1 or CTA 2 at 37°C.; atypical strains showing purple fluorescence are also included.

²*Ps. syringae*

³Six *Comamonas* and seven *Alcaligenes*.

⁴Providence.

⁵Seven *A. anitratus*, six *Comamonas* (±), and six *Alcaligenes* (±).

⁶Eleven *Cloaca* and three *E. coli*.

⁷*Cloaca*.

⁸A strain which produced no pyocyanin or fluorescein, but showed atypical purple fluorescence; gluconate oxidation, growth on tetrazolium chloride medium, and gelatin liquefaction were negative, but their reactions were characteristic of *Ps. aeruginosa*.

pseudomonads as well (slime production, pyocyanin production, and production of gas from nitrate), or from other pseudomonads though not from some of the Enterobacteriaceae (growth at 42°C.).

Slime was present in gluconate broth cultures of all but one of the strains of *Ps. aeruginosa* (or presumptive *Ps. aeruginosa*) and not found in growth of any of the other pseudomonads or other Gram-negative rods. The test was of interest both on account of its diagnostic value and because of the probable importance of slime as a factor in the pathogenicity of *Ps. aeruginosa* (Liu, Abe, and Bates, 1961).

All but one of the strains of *Ps. aeruginosa* (a pyorubin producer) showed fluorescent pigment on CTA 1 and CTA 2; five of these strains showed an atypical purple fluorescence which has been found in extremely few of the thousands of strains seen in the routine examination of the laboratory.

DISCUSSION

In these studies *Ps. aeruginosa* usually fluoresced much more brilliantly on cetrimide agar made with King's base (CTA 2) than on Lemco-based cetrimide agar (CTA 1) (Lowbury and Collins, 1955); although

pyocyanin, a pigment produced only by *Ps. aeruginosa*, was more abundant in growth on CTA 1 than on CTA 2, this advantage was outweighed by the better production on CTA 2 of fluorescein, a pigment produced by a larger proportion of strains of *Ps. aeruginosa* (virtually all). If the hands of those familiar with both media, *Ps. aeruginosa* was isolated as often on CTA 1 as on CTA 2, but those with less experience of CTA 1 might have been in doubt about some of the strains showing weaker fluorescence on this medium. It detection of both fluorescein and pyocyanin is desired, special media should be used for each pigment. For fluorescein, King's medium B (King *et al.*, 1954) is highly effective, and can be converted into a selective medium by inclusion of 0.03% cetrimide. Wahba and Darrell's (1965) medium is equally effective for the detection of pyocyanin; the presence of Tween 80 in this medium, however, makes it incompatible with the selective agent, cetrimide. Blue pigment can be found in the medium even when *Ps. aeruginosa* is heavily outnumbered by other bacteria (occasional strains of which may grow moderately well on CTA media and prevent the recognition of fluorescent pigment).

Attempts to improve the fluorescence under ultra-violet irradiation of *Ps. aeruginosa* on blood agar by using King's base and some modifications of it were fairly successful, but the colonial appearance of various other bacteria, including *Staphylococcus aureus*, was less recognizable than on standard blood agar media. The method was therefore not considered practicable and no further investigation was made of its use.

A range of tests on many strains of presumptive *Ps. aeruginosa*, i.e., Gram-negative rods growing and fluorescing on cetrimide agar, and of other known species of *Pseudomonas*, *Klebsiella*, *Escherichia*, *Proteus*, *Enterobacter*, and other Gram-negative rods has confirmed the value of growth on cetrimide agar and detection of fluorescence in the recognition of *Ps. aeruginosa* isolated from clinical material. A few other pseudomonads (*Ps. fluorescens*, *Ps. putida*, *Ps. marginalis*, *Ps. pastinaceae*, *Ps. astrofaciens*) also gave fluorescent growth on CTA media at 22°C., but grew poorly, if at all, at 37°C., and failed to grow at 42°C.; *Ps. aeruginosa* could be differentiated from them by other tests (notably slime production in virtually all and pyocyanin production in most of the strains tested).

Studies with electronic computers have shown that the species *Ps. aeruginosa* forms a fairly well defined 'taxonomic cluster' (Colwell and Liston, 1961; Colwell, 1964). In this study we have not attempted to match our strains with the median *Ps. aeruginosa* defined by computer analysis, but have examined

these strains with a small series of tests that have particular value in differentiating *Ps. aeruginosa*; from this we were able to assess the value and limitations of fluorescent growth on cetrinide agar as a presumptive test and showed which additional tests are desirable for confirmation and for improvement of diagnosis of *Ps. aeruginosa*.

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