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A NEW BACTERIAL LEAF SPOT OF ANTIRRHINUM SEEDLINGS CAUSED BY A SUBSPECIES OF PSEUDOMONAS FLUORESCENS MIGULA, 1895

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SUMMARY

A new bacterial leaf spot of Antirrhinum majus **L. is described. The pathogenicity of the associated organism is established and a description of morphological, cultural and biochemical characters is given. This organism is considered to be a subspecies of** Pseudomonas fluorescens **Migula, 1895, and the name** Pseudomonas fluorescens **var.** antirrhinastri **var. nov. is proposed.**

I. INTRODUCTION

In May 1964, punnets containing Antirrhinum seedlings blighted by a leaf spot were received from a nursery at Southport, near Brisbane. In severe cases, the condition resulted in the death of the seedlings. The loss from this disease was extremely high, 80% of some plantings being unsaleable. The seedlings were grown in aluminium foil punnets on raised concrete slabs. A perforated plastic cover approximately 8 ft high covered the whole seedling area. Seeds were sown twice weekly and always well separated from previous plantings. Heavy losses occurred through the months from May to October. A similar disease has been reported from other nurseries. A disease of this type had not been recorded previously in Queensland and detailed investigations were therefore commenced.

II. DESCRIPTION OF THE DISEASE

The first symptoms on the true leaves are small watersoaked spots which may enlarge to depressed irregular lesions up to 5 mm in diameter (Figure 1). The small watersoaked spots quickly dry to a tan colour; as they age, the centre becomes light brown and surrounded by a narrow tan margin. The larger lesions are usually irregular in shape, having a tan centre surrounded by an irregular dark green margin varying in width from 1 to 2 mm. Margins of adjacent spots

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frequently coalesce, forming large lesions. These often envelop the whole leaf, which then dries out to a tan colour and dies. Lesions are present on both surfaces of the leaves. Infection may take place marginally or at any other point on the leaf and under conditions of favourable humidity the disease spreads rapidly, resulting in early death of the seedling. No lesions have been noticed on any other part of the plant.

Fig. 1.—Natural infection of Antirrhinum seedlings.

Infected seedlings were planted out in the open in an experimental plot where many of the seedlings died due to the rapid spread of the disease. Those that survived grew quickly away from the disease, which was restricted to the lower leaves. There was no evidence of the disease spreading up the plant to the flowering head. The margins of the lesions on the variety Black Prince were mainly green and purple rather than green and tan as on the tetraploid seedlings.

III. ISOLATION AND PATHOGENICITY OF THE CAUSAL ORGANISM

When infected leaf tissue was lightly teased in a drop of water and examined microscopically, a heavy ooze of motile bacteria was observed streaming from the tissue.

Affected leaves were washed in tap water and small pieces of tissue were removed from the outer margins of lesions and macerated in a few drops of beef extract broth (B.E.B.). The resulting suspension was streaked onto potato dextrose agar (P.D.A.), which was then incubated for 2-3 days at 27°C. The plates were examined and the bacterial colonies found to be predominantly of one

type. A large number of isolations from diseased leaves of Antirrhinum seedlings were carried out throughout the winter months. Bacterial colonies closely resembling the original isolate were consistently obtained.

A 48-hour B.E.B. culture derived from a single colony growing on P.D.A. was used for pathogenicity tests. A small amount of carborundum was added to the broth culture, which was sprayed by means of an atomizer onto the Antirrhinum seedlings at the 4-6-leaf stage. The control plants were sprayed with sterile B.E.B. plus approximately the same amount of carborundum. The plants were kept in a moist chamber for 4 days and then transferred to a glass-house. Six to 7 days after inoculation, watersoaked lesions closely resembling the natural infection were observed on the leaves (Figure 2). Lesions did not appear on the control plants. Isolations from inoculated plants were carried out and the bacteria obtained were proved to be identical with the organism used for inoculation. Eight isolates were proved pathogenic on Antirrhinum and the organism re-isolated. Seedlings of tetraploid and Black Prince semi-dwarf variety were used for pathogenicity tests.

Fig. 2.—Antirrhinum seedlings spray-inoculated with the seedling organism.

IV. HOST RANGE

Pathogenicity tests were carried out on a number of genera belonging to the family Scrophulariaceae and on a number of plants belonging to other families.

In the Scrophulariaceae, adult plants of *Calceolaria crenatiflora* Cav., *Pentstemon* sp., *Angelonia* sp., *Allophyton* sp. and *Hebe raolii* (Hook.f.) Ckn. & Allan and seedlings of *Calceolaria crenatiflora* Cav., *Pentstemon* sp., *Torenia* sp. and *Linaria* sp. were tested. Plants in other families inoculated included seedlings of *Brassica oleracea* L. cv. All Seasons, *Nicotiana tabacum* L. cv. White Burley, and *Cucumis sativus* L. cv. Marketer, pods of *Phaseolus vulgaris* L. cv. Windsor Long Pod, Redlands Green Leaf and Brown Beauty, stems of *Vicia faba* L. and the fruit of Lisbon and Meyer lemon, Tahiti lime and Late Valencia orange.

All foliage tests were performed by using a hand atomizer as described previously. The inoculated plants were placed in a moist chamber for 4 days at near saturation. They were then removed and kept at 68°C, with fairly high humidity. Bean pods, broad bean stems and citrus fruit were inoculated by pricking a drop of a B.E.B. culture into the pods, stems and fruit. Sterile B.E.B. was pricked into the controls. Where the pathogenicity test produced lesions, the organism was re-isolated and inoculated onto Antirrhinum seedlings. A test was considered positive only if the re-isolation proved pathogenic.

The Antirrhinum organism proved pathogenic on the adult plants of *Calceolaria crenatiflora* Cav., *Pentstemon* sp. and *Angelonia* sp., and on seedlings of *Calceolaria crenatiflora* Cav. and *Pentstemon* sp. Although a positive pathogenicity test was obtained on these hosts, the symptoms, consisting of very small tan lesions, were very mild in comparison with the severe symptoms found on Antirrhinum. The organism was not pathogenic to the non-Scrophulariaceae tested.

Pathogenicity tests were carried out on Antirrhinum plants which were past the seedling stage but had not developed flower buds. The organism was not pathogenic and so appears to be a disease of seedlings and not of the mature plant.

V. SOURCE OF ORGANISM

The seedling leaf spot of Antirrhinum had not been noticed previously in Queensland by nurserymen. It was claimed that the first occurrence of the disease was after planting a new batch of seed. The disease was first noticed on the variety Black Prince, the seed of which had been imported from Europe. This variety was not planted again and the nurseryman reverted to planting tetraploid Antirrhinum seed, the seedlings of which subsequently became infected. The nursery uses U.C. potting mix, which is partly steam-sterilized at 140°F.

Tests were carried out on the remaining seed of the variety Black Prince in an attempt to determine if the bacteria are seed-borne.

The seeds were sampled to a workable sample of approximately 500 seeds. Of these, 200 were surface-sterilized—30 sec in 95% alcohol, 2 min in mercuric chloride—followed by four washes in sterile water. The seeds were plated out onto P.D.A., 8 seeds per plate, and incubated until germinated. There was no sign of the organism on the plates.

Seeds from the remainder of the sample were surface-sterilized as above and ground up in B.E.B. The suspension was streaked onto P.D.A. plates and incubated at 27°C for 7 days. There was no sign of bacterial growth in that period.

Between May and November, Antirrhinum seedlings were raised in the glasshouse from the unsterilized suspected seed on a number of occasions without any development of the bacterial leaf spot.

VI. DESCRIPTION OF CAUSAL ORGANISM

The procedure adopted for determining the morphology and biochemical reactions of the organism is found in Appendix A. Six pathogenic isolates plus a re-isolate from spray-inoculated Antirrhinum seedlings were examined. The characteristics of the organisms were as follows:—

Morphology.—The organism consisted of Gram-negative, non-sporing, encapsulated rods with rounded ends, occurring singly, in pairs and in chains. The rods ranged in size from 3·0–1·6 x 0·9–0·6μ, the majority of the rods being about 1·8 x 0·8μ.

Motility.—The rods were motile and 1–4 polar flagella were observed (Figure 3).

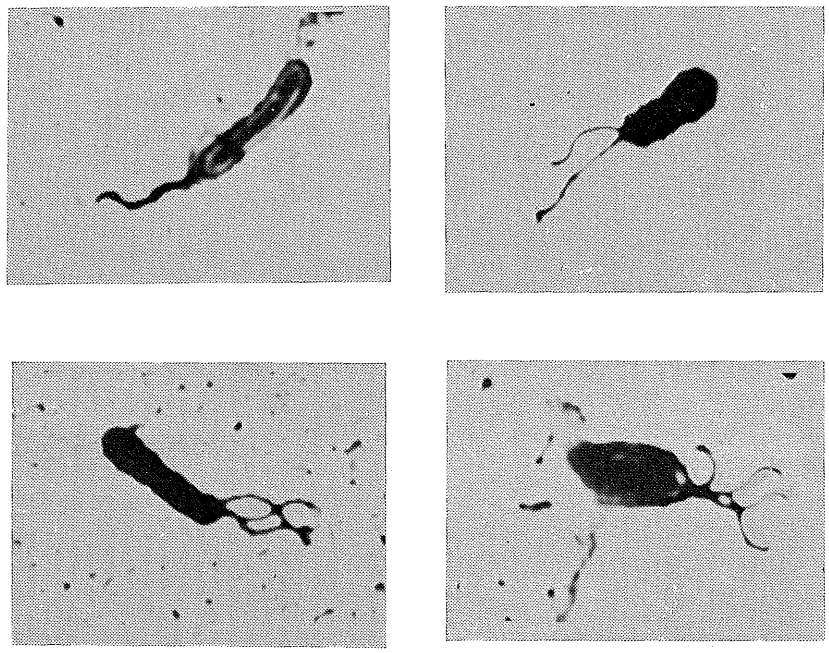

Fig. 3.—Flagella of the Antirrhinum seedling organism (X4900). (Photographs by W. Manley).

Cultural characteristics.—After 48 hr, inoculated B.E.B. was uniformly turbid. No pellicle or fluorescence was observed after 14 days. After 96 hr on P.D.A., colonies were circular, creamy white, shining, smooth, margin slightly irregular, convex or slightly umbonate, opaque, approximately 2 mm in diameter. The central area of the colony darkened as it aged, the colony as a whole becoming more creamy in colour and larger, up to 4 mm in diameter.

On B.E.A. after 5 days colonies were circular, off-white, shining, smooth, margin entire, raised, translucent in direct light and opaque in reflected light, and up to 3 mm in diameter.

After 48 hr on peptone yeast extract agar (P.Y.E.A.), colonies were circular, greyish white, shining, smooth, margin entire, raised, translucent in transmitted light, opaque in reflected light and up to 1 mm in diameter.

On cooked potato slants, growth was creamy, smooth, shining, margin entire, and growth restricted to the slope. There was no discoloration of the potato tissue but the growth darkened to a creamy brown as it aged.

Temperature relations.—The organism grew at 33°C but not at 33·5°C with slight growth at 1°C. The optimum growth was between 23 and 25°C.

Biochemical reactions.—Acid but no gas was produced from glucose, sucrose, arabinose, raffinose, xylose, fructose, mannose, galactose, ribose, glycerol, sorbitol and mannitol. Acid was not produced from lactose, rhamnose, cellobiose, maltose, aesculin, inulin, salicin, starch, erythritol and dulcitol. Metabolism of glucose was oxidative. Litmus milk was alkaline after 3 days. After 7 days, clearing of the milk had commenced, and within 28 days complete peptonization took place. The litmus was reduced. Clearing around the colonies took place after 4 days on gelatin agar plates and the liquefaction of the nutrient gelatin stabs was stratiform to a depth of approximately 12 mm in 28 days. Chitin was not hydrolysed. Indole was not produced. Hydrogen sulphide was not produced. Nitrite was not produced from nitrate. Ammonia was produced from peptone. Starch was not hydrolysed. 1% tributyrin was hydrolysed. Alginate was not hydrolysed. The oxidase test was negative. The methyl red test was negative. The Voges-Proskauer reaction was negative. Asparagine was utilized as a sole source of carbon and nitrogen. Malonate was not utilized. All cultures could utilize the sodium salts of citric, acetic, succinic and formic acid but not lactic, oxalic and tartaric acid. A positive reaction in Simmons' citrate was produced in 24 hr. There was slight hydrolysis of sodium hippurate. The catalase test was positive. All isolates were urease negative in 6 days. Pectate was not hydrolysed. A green fluorescent pigment occurred in the agar and liquid fluorescent media. The isolates tested could not tolerate 6% or 4% NaCl. The isolates were freeze-dried in a suspending medium composed of 6% peptone and 6% sodium gluconate in distilled water and sterilized using a bacteriological filter. The cultures were stored at approximately 10°C and were viable after 12 months.

VII. DISCUSSION

The results of the morphological, cultural and biochemical tests place the organism isolated from the Antirrhinum seedlings in the genus *Pseudomonas* Migula, 1894, as described in the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith 1957).

Takimoto (1920) reported a bacterial leaf and stem spot of Antirrhinum from Japan. In 1943, Dowson proposed that this organism should be placed in the genus *Xanthomonas* and it is now accepted as *Xanthomonas antirrhini,* (Takimoto, 1920) Dowson, 1943. Simonet (1925) reported a bacterial disease of Antirrhinum caused by a rather long *Bacterium* which he claimed had not previously been reported. It produced lesions on both leaves and stems and in this respect resembles *X. antirrhini.* Valder (1963) recorded a bacterial leaf blight of Antirrhinum seedlings in New South Wales. The causal organism was identified as *Pseudomonas* sp. by the Commonwealth Mycological Institute and it is possible that this is the same disease as occurs in Queensland.

The description of the Queensland organism does not fit that of any previously described species of *Pseudomonas* as listed in Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith 1957). Described species with reactions close to it are *Pseudomonas lachrymans* (Smith and Bryan, 1915) Carsner, 1918; *P. maculicola* (McCulloch, 1911) Stevens, 1913; and *P. syringae* van Hall, 1902. Characteristics of these three organisms differing from those of the Antirrhinum organism are shown in the table below.

Organism	Varying Characteristics	Corresponding Characters of Antirrhinum Organism
P. lachrymans	Weak indole reaction No acid from raffinose and glycerol Optimum temperature 25–27°C Maximum temperature 35°C	Negative indole reaction Acid from raffinose and glycerol Optimum temperature 23–25°C Growth at 33°C but not at 33·5°C
P. maculicola	Weak indole reaction Slight growth in 4% NaCl Maximum temperature 29°C	Negative indole reaction No growth in 4% NaCl Growth at 33°C but not at 33·5°C
P. syringae	No acid from raffinose Lactic acid utilized Acetic and formic acid not utilized Slight growth in 4% NaCl 1 or 2 flagella	Acid from raffinose Lactic acid not utilized Acetic and formic acid utilized No growth in 4% NaCl 1–4 flagella observed

As previously stated, the organism from Antirrhinum seedlings was not pathogenic on seedlings of cabbage and cucumber, fruit of Lisbon and Meyer lemons, Tahiti lime and Late Valencia orange or bean pods.

A comparison of the pathogenicity of *Pseudomonas syringae* isolated from bacterial brown spot of bean and the Antirrhinum organism was carried out on Brown Beauty bean pods and fruit of Tahiti lime. As illustrated in Figures 4

and 5, the organism from Antirrhinum was non-pathogenic, whereas *P. syringae* gave characteristic symptoms on these hosts. Antirrhinum seedlings were inoculated, as described previously, with *P. syringae* and the Antirrhinum seedling organism. *P. syringae* was non-pathogenic whereas the other organism produced the usual symptoms on the seedlings. Under the present system of classification, these pathogenicity tests and the difference in biochemical characteristics separate this organism from *P. syringae*.

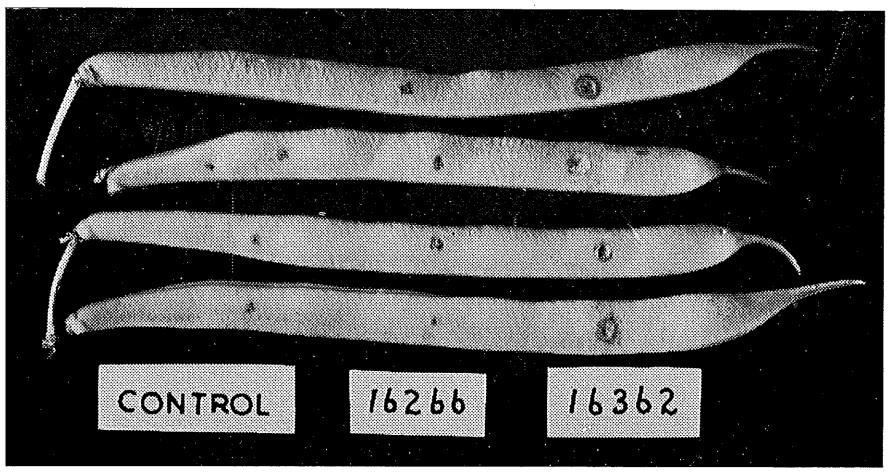

Fig. 4. A comparison of inoculations of the Antirrhinum seedling organism (16266) and *Pseudomonas syringae* (16362) into Brown Beauty bean pods.

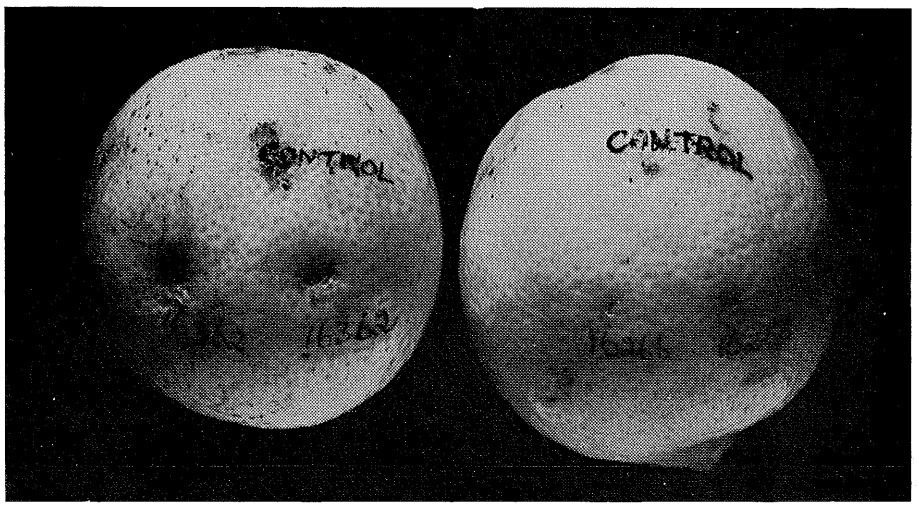

Fig. 5—Tahiti lime fruit inoculated with the Antirrhinum seedling organism (16266) and *Pseudomonas syringae* (16362). Fruit on left inoculated with 16362 and fruit on right inoculated with 16266.

As a result of this work, the organism causing the disease of Antirrhinum seedlings in Queensland was thought to be a *Pseudomonas,* apparently distinct from the point of view of pathogenicity from any previously described species of this genus. The data were processed in the computer by Professor R. R. Colwell, Georgetown University, Washington, D.C., U.S.A., with 70 strains of *Pseudomonas, Xanthomonas* and *Flavobacterium* species. The Antirrhinum isolates joined the phenom of *P. fluorescens* at the >60% level. It is therefore proposed that these strains constitute a new subspecies of *P. fluorescens* to be designated *P. fluorescens* var. *antirrhinastri.* These component strains formed a >98% single linkage group. They are unusual in that they are all oxidase negative.

Strain 16266 is designated as the type culture. Subcultures have been lodged with The National Collection of Plant Pathogens, Harpenden, England.

VIII. ACKNOWLEDGEMENTS

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APPENDIX A

Single colonies from a 96-hr P.D.A. culture were inoculated into B.E.B. After 48 hours' incubation, these broth cultures were used for biochemical tests. Two drops of inoculum from a sterile pasteur pipette were used to inoculate all liquid tests except in the case of the carbon and nitrogen free media, where 1 drop of inoculum was used. All cultures were incubated at 27°C unless otherwise stated. All tests were carried out in duplicate.

Gram reaction.—Smears were made from 96-hr-old cultures grown on P.D.A. and the Gram reaction was determined by the technique recommended by Skerman (1959).

Morphology.—Size and shape of the organism were determined from smears from a 4-day-old culture on P.D.A. and stained with crystal violet (Society of American Bacteriologists 1957).

Capsule stain used was a modification of Hiss's method (Mackie and McCartney 1945), basic fuchsin being substituted for gentian violet.

Motility and flagella stain.—Motility was determined by the hanging-drop method on culture grown for 96 hr on P.D.A. and flagella were stained by Gray's method (Society of American Bacteriologists 1957), Leifson's method (Skerman 1959), and the Rhodes (1958) modification of Fontana's silver-plating method. The last method gave the most satisfactory results.

Colony characteristics.—Media used to describe colony characteristics were:—

Potato dextrose agar (potato 200 g, dextrose 20 g, granular agar 20 g, tap water 1 l, pH 6·9).

Beef extract agar ("Oxoid Lab. Lemco" 0·5%, NaCl 0·5%, "Difco" bacto-peptone 1%, tap water 500 ml, pH 7·2).

Peptone yeast extract agar (peptone 10 g, NaCl 5 g, "Difco" yeast extract 1 g, agar 15 g, tap water 1 l, pH 7·4).

Temperature range of organism.—Uniformly streaked P.D.A. slopes were incubated in a multiple-temperature incubator over the temperature range of 1°C—45·5°C. Tubes of B.E.B. inoculated with a single drop of a 48-hr-old culture from a sterile pasteur pipette were also incubated in the multiple-temperature incubator over the same temperature range. The turbidity of the broth cultures was compared every day for 7 days, using a colorimeter. Maximum temperature was determined in a thermostatically controlled water-bath.

Action on carbon compounds.—The synthetic media of Ayres, Rupp, and Johnson (Society of American Bacteriologists 1957) was used as the basal media into which the carbon compounds were incorporated. Bromo-cresol purple was used as the indicator. The carbon compounds were made up as 10% solutions, steamed for 1 hr on 3 successive days and then added aseptically to the basal media to a final concentration of 1% for glucose, lactose and sucrose and 0·5% for the remaining carbon compounds. The carbon sources tested were: glucose, lactose, sucrose, L-arabinose, raffinose, rhamnose, xylose, fructose, cellobiose, mannose, galactose, maltose, ribose, aesculin, inulin, salicin, starch, glycerol, erythritol, sorbitol, dulcitol, mannitol. Durham tubes were included in the tube for observation of any gas production. Cultures were examined for acid and gas production after 48 hr, 5 days and at 7-day intervals up to 28 days.

Determination of oxidative and fermentative metabolism of glucose.—The method used to determine oxidative or fermentative metabolism of glucose was that described by Hugh and Leifson (1953).

Action on litmus milk.—"Baltimore Biological Laboratory" litmus milk was used in this test.

Liquefaction of gelatin.—Two methods were used for this test. Smith's modification of the Frazier method (Society of American Bacteriologists 1957) was used, and tubes of "Baltimore Biological Laboratory" nutrient gelatin were stab-inoculated and incubated at 22°C for 28 days.

Chitin hydrolysis.—Chitin agar (Skerman 1959) plate method was used.

Indole production.—1% "Difco" bacto-tryptone was used as the source of tryptophan and the production of indole was determined by the Ehrlich-Böhme method (Society of American Bacteriologists 1957).

Production of hydrogen sulphide.—Peptone-cystine-sulphate media (Skerman 1959) was used and the production of H_2S was determined by the lead acetate paper method (Skerman 1959).

Nitrate reduction.—The media used was "Difco" bacto-peptone 10g, KNO_3 2g, distilled water 1l. The pH was adjusted to 7·4 and the media was dispensed in narrow tubes. After inoculation, the surface of one tube was covered with sterile paraffin and the other was left open. Testing for nitrate reduction was carried out after 48 hr, each following day for 7 days and then weekly. The method used was the starch-iodide spot test (Skerman 1959). A zinc filing was added at the conclusion of each spot test to check for the presence of nitrate.

Ammonia production.—Cultures were grown in 1% peptone water (Skerman 1959). Nessler's reagent was used to detect the presence of ammonia after 2 days' and 7 days' incubation.

Starch hydrolysis.—The starch agar plate method (Skerman 1959) was used. After 5 days' incubation, the plates were flooded with Lugol's iodine (Dowson 1957).

Alginate hydrolysis.—Alginate agar (Skerman) was used.

Lipolysis.—Cultures were grown on B.E.A. + 1% tributyrin. Clear zones around the colonies indicated lipolysis.

Oxidase test.—48-hr cultures grown on P.D.A. were used for the oxidase test described by Kovacs (1956).

Methyl red test and Voges-Proskauer reaction.—Cultures were grown in glucose phosphate peptone water (Skerman 1959) and after 4 days the methyl red test and the Voges-Proskauer reaction (Skerman 1959) were carried out.

Utilization of asparagine as the sole source of carbon and nitrogen.—This was determined by the method described by Lewis (1930). The tubes were acid washed, then washed ten times in tap water followed by 10 rinses in distilled water. Growth was recorded after four serial transfers into fresh media.

Utilization of malonate.—Leifson's (1933) malonate broth, modified by the addition of 0·1% yeast extract and 0·025% glucose, was used.

Utilization of organic acids.—The methods of Lewis (1930) and Dye (1962) were used. The sodium salts of acetic, lactic, succinic, tartaric, citric, oxalic, and formic acids were used.

Simmons' citrate.—The citrate agar medium described by Simmons (1926) was used.

Catalase production.—A loopful of growth from a 48-hr P.D.A. culture was transferred into 4 drops of hydrogen peroxide and examined for the production of gas bubbles.

Hippurate hydrolysis.—The hydrolysis of hippurate was determined in a peptone media (Ayres and Rupp 1922) and the ferric-chloride test (Ayres and Rupp 1922) for the presence of benzoic acid.

Urease production.—Slopes of urease media (Christensen 1964) were streaked and examined for colour change every day up to 6 days.

Pectolytic activity.—The pectate media (Starr 1947) was dispensed in narrow tubes and inoculated by stabbing deeply into the media.

Fluorescence.—Two media were used to demonstrate pigment production. These were a liquid medium (Georgia and Poe 1931) and an agar medium containing proteose peptone 1·0%; bacto agar 1·5%; glycerol C.P. 1·0%; K_2HPO_4 (anhydrous) 0·15%; $MgSO_4$. $7H_2O$ 0·15%; pH adjusted 7·2.

Tolerance of NaCl.—The ability of the organism to tolerate 4% and 6% NaCl was observed in B.E.B. + final concentration of NaCl at 4% and 6%. Tubes were inoculated with 1 drop of broth culture from a sterile pipette. Tubes were examined for growth after 48 hr, 5 days and at 7-day intervals to 28 days.

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