Characteristics and Identification of Oxidative *Pseudomonads* Isolated from Diseased Fish

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SUMMARY

Several characteristics of 20 strains of oxidative pseudomonads, all but one of which were isolated from freshwater fish, were determined. All strains oxidized glucose and gluconate, produced NH_3 from 1% peptone, grew at 6, 12, 20 and 30° and produced cytochrome oxidase. Lipase was produced by 17 of the strains, while 15 produced gelatinase, and 16 produced fluorescent pigment. These and other characteristics used to identify fish-spoilage pseudomonads are also useful for identification of fish-pathogenic pseudomonads. Species determinations from the present data were not attempted. However, from previously published data on speciation of *Pseudomonas*, most of the 20 strains appeared to be closely related to or identical with *P. fluorescens*.

INTRODUCTION

Pseudomonads, which usually oxidize carbohydrates and produce fluorescent pigment, have been reported as causing diseases in warm-water and cold-water fish (Wolf, 1937; Rucker & Whipple, 1951; Rucker, Earp & Ordal, 1954; Schäperclaus, 1954; Seaman, 1951). The identification and separation of these organisms from the closely related aeromonads is desirable for diagnosis of the diseases and proper control measures. Current methods used for identification of these pseudomonads lack the precision needed for separation of closely related species or strains isolated from diseased fish. Recent studies on the taxonomy of the genus *Pseudomonas* have shown the inadequacies of the present classification (Rhodes, 1959; Colwell & Liston, 1961). Often speciation within the genus has been a matter of personal opinion, or has been based on particular tests considered important by the investigator. This situation is by no means limited to pseudomonads, since one has only to look to the closely related aeromonads to find the same disagreement on speciation (Ewing, Hugh & Johnson, 1961; Eddy, 1960, 1962). Sneath (1957*a, b*) stated that many schemes of taxonomy are catalogues rather than true classifications, and he discussed a computer method for comparing organisms for overall similarity. This method has been applied to several groups of bacteria including those in the genus *Pseudomonas* (Rhodes, 1961; Colwell & Liston, 1961; Lysenko, 1961). In our opinion the computer analysis method while having limitations is a sound and objective approach, which promises improvement in bacterial classification. The present work was undertaken for two reasons. First, it was desirable to determine the degree of homogeneity of characteristics of the oxidative pseudo-

monads isolated from diseased fish, and secondly it was hoped that, by the use of some of their characteristics, better methods of identification of fish-pathogenic pseudomonads could be established.

METHODS

Selection of cultures. The cultures used in this study (Table 1) were Gram-negative, polarly flagellate rods. With the exception of the two ATCC strains, all cultures came from this laboratory's collection, and all except no. 80 were isolated from fish, most of which were diseased. Culture no. 80 was included because it was isolated from a cold-blooded vertebrate. Characteristics of many of the cultures had been previously studied but not reported. To obtain the characteristics of the strains near the time of isolation, lyophilized cultures were used except in the case of the two ATCC strains and the most recent isolates P-1, P-2, P-3. To ensure purity, all test strains were streaked twice before use.

Table 1. *Strains of pseudomonads used*

Designation	Original source or source of isolation
<i>P. fluorescens</i> nos. 11250, 12633	American Type Culture Collection
1, 2, 3	Dr Troutman, Ohio
7	Quitman, Texas
11	Brook trout (<i>Salvelinus fontinalis</i>), Berlin, N.H.
21	<i>Pseudomonas</i> no. 28, Dr R. R. Rucker, State of Washington
22b	Pisgah Forest Hatchery, North Carolina
37	Adult brook trout (<i>S. fontinalis</i>)
49	Brook trout fingerling (<i>S. fontinalis</i>), Leetown, West Va.
50, 51	<i>Pseudomonas</i> Dr R. R. Rucker, State of Washington
66	Dr Prevost, Canada
80	Purple salamander (<i>Ambystoma</i> sp.), Leetown, West Va.
81	Black dace (<i>Rhinichthys atratulus</i>), Leetown, West Va.
86, 87, 88	Spirit Lake, Iowa
P-1	Fathead minnow (<i>Pimephales promelas</i>), Tennessee
P-2, P-3	Brook trout brood stock (<i>S. fontinalis</i>), Spring Run Trout Hatchery, Petersburg, West Virginia

Characters examined. Unless otherwise stated, all the following tests were repeated, all media steam sterilized, and all cultures incubated at 20–22° for 1 week.

1. Cell form and Gram reaction of 18–24 hr nutrient agar cultures.
2. Flagellation; Novel's (1939) staining method was used.
3. Hydrogen sulphide in Motility Sulfide Medium (Difco; Difco Laboratories, Detroit, Michigan, U.S.A.).
4. Nitrate reduction in nutrient broth containing 0.1 % (w/v) KNO₃; zinc powder (ZoBell, 1932) was used to detect false negatives. Tested after 1, 2, 7 days.
5. MR-VP in MR-VP (Difco) Broth. VP test performed after 48 hr and 1 week.
6. Gelatinase by Smith's modification of Frazier's technique in the *Manual of Microbiological Methods* (1957).
7. Lecithinase in egg yolk agar; method and medium as in the *Manual of Microbiological Methods* (1957).
8. Levan production on nutrient agar containing 4 % (w/v) sucrose (Klinge, 1960). A thick slimy growth was considered positive for levan production.

9. Ammonia production as indicated by increased alkalinity of 1 % (w/v) peptone broth containing pH indicator.

10. Indole production in 1 % (w/v) tryptone broth by the method of Kovacs, as in the *Manual of Microbiological Methods* (1957).

11. Hydrolysis of starch in nutrient agar containing 0.2 % (w/v) soluble starch. Medium flooded with Lugol's iodine solution after incubation.

12. Oxidation of glucose in O/F Basal Medium (Difco) containing 1 % (w/v) glucose. This commercial medium is almost identical with the medium of Hugh & Leifson (1953).

13. Production of fluorescent pigment was tested on *Pseudomonas* F Agar (Difco) slopes. This commercial medium is designed to enhance fluorescein production by *Pseudomonas*. Fluorescence was tested by exposing inoculated slopes to ultraviolet radiation after 24–48 hr incubation. Lyophilized cultures and the same cultures which had been kept for 10 years in stock culture were tested.

14. *Growth at different temperatures.* All cultures were tested for ability to grow at 0, 6, 12, 20, 30, 37 and 42°. The preparation of inocula was a modification of Klinge's (1960) method in that, for each culture tested, a 1/100 dilution, in 0.85 % (w/v) sterile saline, was made from a 24 hr turbid broth culture. After shaking, 0.1 ml. of the dilution was inoculated into each tube of nutrient broth, which contained 5 ml. broth. Inoculated and control tubes were placed in covered pans of water at the indicated temperatures. Glycerol was added to the pan water at 0° to keep the water from freezing. Temperatures were checked at least twice a day during the 2-week incubation period and did not fluctuate more than 0.5–1.0°. It took 30–45 min. from the time the first tubes of a series were inoculated until all tubes of that series had reached proper temperature. Results were recorded daily for the first week and three times during the second week. Growth was measured by visually comparing turbidity of inoculated tubes with uninoculated controls. Tubes were not removed from the incubator for more than 5 min. to record results. Ability to grow at 6, 12, 20 and 30° was determined once, while ability to grow at 0, 37 and 42° was determined twice.

15. Lipase was demonstrated by: (a) clearing of 1 % (v/v) tributyrin in nutrient agar; (b) change in colour of pH indicator in Rhodes (1959) medium substituting peanut oil for olive oil.

16. The presence of cytochrome oxidase was examined by the method of Ewing & Johnson (1960).

17. The oxidation of gluconate was determined in the medium of Haynes (1951) with the exception that 2 % (w/v) peptone was substituted for tryptone. The basal medium was autoclaved, and 0.5 ml. of a 40.0 % (w/v) Seitz-sterilized potassium gluconate solution was added to a final concentration of 4 % (w/v). The inoculated medium was shaken twice by hand during incubation. Presence of reducing compounds was tested with Benedict's qualitative reagent.

RESULTS

Of the 20 pseudomonads isolated from fish and one salamander, 15 of the strains showed positive reactions for 12 of the 22 applied tests (Fig. 1). Reactions of the strains to the other tests listed in Fig. 1 ranged from 11 to none positive. Detailed

results of all individual strain reactions are given in Table 2. Flagellar stains indicated most strains were lophotrichous but a few appeared monotrichous. Also the oxidative carbohydrate metabolism of the test strains was confirmed in O/F Basal Medium (Difco) containing 1% glucose. Although the two ATCC strains were similar in their reactions to the test strains, they did differ in lipase production and gluconate oxidation. Also, neither produced gelatinase, which, according to *Bergey's Manual* (1957), is typical for *Pseudomonas fluorescens*. It was also found that, when *Pseudomonas* F Agar (Difco) was used, fluorescence was not lost after repeated subcultivation.

Fig. 1. Number of *Pseudomonas* strains from fish and one salamander having positive reactions with the applied tests.

DISCUSSION

The pseudomonads isolated from diseased fish used in the present study represent a fairly homogeneous group, especially in those characteristics normally used for identification, namely polar flagellation, fluorescence, carbohydrate metabolism and cytochrome oxidase reaction. These and other criteria were used by Shewan, Hobbs & Hodgkiss (1960*a, b*) to identify and group the very important pseudomonad spoilage organisms of marine fish. The scheme of Bullock (1961) used to identify fish pathogens also incorporates some of these characteristics for *Pseudomonas* identification. However, in this scheme a peptone basal medium + carbohydrate was used to determine carbohydrate metabolism and the more definite O/F Basal Medium (Difco) + carbohydrates is now preferred. A further comparison of fish-pathogenic and fish-spoilage pseudomonads would place the pathogens studied into groups I and II of Shewan *et al.* (1960*a*) and, since 16 of the 20 strains

Table 2. *Characteristics of pseudomonad test strains*

All strains oxidized glucose, were cytochrome oxidase positive and produced NH_3 from 1 % peptone. All strains were MR-VP, amylase, H_2S , and indole negative.

Character determined	Culture number (see Table 1)																	
	1, 2 3, P-2	7	11	21	22b	37	49	50, 51	66	80	81	86	87	88	P-1	P-3	ATCC 11250	ATCC 12633
Gluconate oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Lipase																		
Peanut oil	+	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-
Tributyrin	+	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-
Growth at: 0°	+	-	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-
6°, 12°, 20°, 30°	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
42°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fluorescence	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	-	-
Lecithinase	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Levan	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
Nitrate reduction	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	-	+

produced fluorescent pigment, mainly in group I. Organisms producing an alkaline or no reaction in the medium of Hugh & Leifson (1953) and corresponding to groups III and IV (Shewan *et al.* 1960*a*) are rarely encountered as pathogens of fish and are of no practical importance in this field.

While speciation was not an aim of this work it may be possible on the basis of growth at various temperatures more closely to define our strains. The ability to grow at 42° is considered an important feature in the separation of *Pseudomonas aeruginosa* from other *Pseudomonas* species (Haynes, 1951; Klinge, 1960; Haynes & Rhodes, 1962). Haynes & Rhodes (1962) recommended three successive transfers for determining growth at 42°. This was not used in the present work, but since after two attempts no strain was found to grow at 42° and only two strains grew at 37°, it is unlikely that *P. aeruginosa* plays an important role in diseases of freshwater fish. We are mainly concerned with psychrophilic pseudomonads, which are identical with or closely resemble *P. fluorescens*.

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