1789

INFLUENCE OF LOW INCUBATION TEMPERATURE ON CLASSIFICATION OF *PSEUDOMONAS GENICULATA*

Received for publication January 15, 1962

ABSTRACT

FRANK, HILMER A. (Eastern Regional Research Laboratory, Philadelphia, Pa.). Influence of low incubation temperature on classification of *Pseudomonas geniculata*. J. Bacteriol. **84**:68–71. 1962.—Biochemical and physiological properties of psychrophilic *Pseudomonas geniculata* isolates were determined at 8 C and at 27 C. No qualitative differences in taxonomically significant properties were observed as a result of incubation temperature. With sufficient incubation time, classification of psychrophilic strains would not be impaired by low temperatures.

Classification of the pseudomonads is sometimes inconclusive and unsatisfactory (Gaby, 1955; Rhodes, 1959), frequently because species determination is based on a series of negative characters (Shewan, Hodgkiss, and Liston, 1954). Several important properties, notably pigment production, litmus milk reaction, gelatin liquefaction, and acid production from carbohydrates, are extremely variable or may be lost during repeated subculture (Ayres, 1960; Brown and Weidemann, 1958; Gaby, 1955; Rhodes, 1959; Shewan et al., 1954). Determination of flagellar distribution and number may also be inaccurate and unreliable because of the precision required for executing and interpreting stained preparations (Colwell and Liston, 1961; Gaby, 1955; Thornley, 1960). Several workers have suggested criteria which they believed more suitable for classifying pseudomonads (Gaby and Free, 1958; Haynes, 1951; Moore and Picket, 1960; Thornley, 1960).

Using an electronic computer and giving equal weight to every property tested, Colewell and Liston (1961) examined a large number of pseudomonads and reported that *Pseudomonas* includes four distinct groups of bacteria. Most psychrophilic bacteria, regardless of source, belong to *Pseudomonas* (Brown and Weidemann, 1958; Ingraham and Stokes, 1959). Psychrophilic *Pseudomonas* strains have been isolated from refrigerated meats (Ayres, 1960; Brown and Weidemann, 1958; Wolin, Evans, and Nivens, 1957), dairy products (Thomas, 1958), maple sap and maple-tree tapholes (Naghski, Reed, and Willits, 1957; Sheneman and Costilow, 1959; Willits, Frank, and Bell, 1961). *P. geniculata* is probably the most commonly reported species among psychrophilic bacteria isolated.

The purpose of this study was to examine the effect of low incubation temperature on taxonomically useful properties of psychrophilic *P. geniculata* strains. Brown (1957) reported that nutritional requirements of a psychrophilic pseudomonad were identical at 0 C and at 20 C. Alford (1960) observed false negative reactions in *Pseudomonas* and *Achromobacter* when incubation was carried out at the temperature of most rapid growth. He reported that in some strains gelatin liquefaction, lipolysis, citrate utilization, fermentation of litmus milk, and oxidative fermentation of xylose and arabinose were inhibited by slight elevation of incubation temperature.

MATERIALS AND METHODS

Organisms. Fifteen strains of psychrophilic bacteria, isolated from maple sap and maple-tree tapholes and previously identified as *P. geniculata* (Willits et al., 1961), were employed. Stock cultures were maintained on nutrient agar.

Procedures. Air incubators, set at 8 C and at 27 C, were used. Inocula for tests consisted of one or two drops of faintly turbid aqueous suspensions made from young cultures grown on Difco Tryptone Glucose Extract (TGE) Agar. The incubation periods were 3 days at 27 C and 2 weeks at 8 C. Media and methods were as described in the *Manual of Microbiological Methods* (Society of

[1] Present address: Department of Food Processing and Utilization, University of Hawaii, Honolulu.

American Bacteriologists, 1957) or as noted below. Flagella were examined by electron microscopy and by staining (Leifson, 1951). Pigment production was tested in several media, including the liquid asparagine medium of Georgia and Poe (1931). Lipolytic activity was tested on spirit blue agar (Starr, 1941). Hydrogen sulfide production was tested in Kligler Iron Agar (Difco). Gelatin hydrolysis was tested on an agar-containing medium (p. 55, *Manual of Microbiological Methods*). Procedures described by Gaby and Free (1958) for testing gluconate oxidation (method of Haynes), cytochrome oxidase, and oxidase (method of Kovacs) were employed using organisms grown at both temperatures. Acid production from carbohydrates was tested by the method of Rhodes (1959), employing filter-sterilized carbohydrates, heat-sterilized nutrient broth, and bromcresol purple indicator. Since these organisms are obligately aerobic, carbohydrate breakdown was assumed to be oxidative and, therefore, examination for fermentative acid production was deemed unnecessary. Oxidative metabolism was indicated by acid production, initially at the surface, and gradually progressing toward the bottom of the tube. Utilization of several carbohydrates as sole added carbon source for growth was tested in Koser's chemically defined basal medium as described by Rhodes (1959). Antibiotic sensitivity was determined on dried TGE agar, using Difco sensitivity discs. Optimal growth temperature was determined from the incubation time needed for maximal turbidity in nutrient broth.

RESULTS

Psychrophilic strains of *P. geniculata* can grow over a temperature range of −2 to 36 C, most rapidly at 25 to 30 C (Table 1). Several strains grow at temperatures up to 40 C. From observations made after incubation at 27 C, *P. geniculata* can be said to have the following characteristics: obligately aerobic; mono- or, more commonly, lophotrichous flagella; growth at pH 4.0; growth in presence of 5% sodium chloride; catalase positive; gelatin hydrolyzed; fluorescent pigment produced; alkaline reaction in litmus milk; gluconate oxidized; oxidase and cytochrome oxidase positive; acetate, lactic acid, succinate, L-malate, or citrate utilized as sole carbon source for growth; resistant to 18 chemotherapeutic agents tested (bacitracin, chloramphenicol, erythromycin, nitrofurazone, nitrofurantoin, isoniazid magnamycin, nystatin, novobiocin, oleandomycin, *p*-aminosalicylic acid, penicillin, polymyxin B, ristocetin, spiramycin, sulfamerazine, vancomycin, and viomycin); may or may not be lipase positive, produce acid oxidatively from arabinose, galactose, glucose, mannose, rhamnose, and xylose, or be resistant to 7 drugs tested (sulfamethoxypyridazine, sulfisomidine, sulfisoxazole, sulfathiazole, sulfamethizole, streptomycin, and dihydrostreptomycin); does not reduce nitrate to nitrite, produce indole or hydrogen sulfide, break down urea or hydrolyze starch; is negative to methyl red and Voges-Proskauer; does not produce acid oxidatively from adonitol, cellobiose, dextrin, dulcitol, erythritol, fructose, glycerin, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, salicin, sorbitol, starch, sucrose, or trehalose; does not utilize formate, oxalic acid, or tartaric acid as sole carbon source for growth; sensitive to eight drugs tested (chlortetracycline, kanamycin, methenamine mandelate, neomycin, sulfadiazine, oxytetracycline, tetracycline, and Triple Sulfa).

Except for growth at pH 4.0, an identical list of properties could be drawn up from observations made at 8 C. There were only slight qualitative differences between results observed at the two temperatures. At 8 C, fewer strains were lipase positive, and fewer produced acid from several carbohydrates (Table 1). On the other hand, a few strains were more resistant to several antibiotics at 8 C than at 27 C.

Some quantitative differences were found between results obtained at the two temperatures. For example, pigment production and growth (turbidity) in the presence of added sodium chloride were always greater at 8 C. Lipase activity and oxidative acid production were reduced by incubation at 8 C. Colony-forming ability on TGE agar was essentially the same at both temperatures.

DISCUSSION

These results show that a low incubation temperature does not affect qualitative estimation of many biochemical and physiological properties of psychrophilic *P. geniculata* strains. Classification could be accomplished equally well from taxonomically significant properties observed at low or at moderate temperatures.

According to *Bergey's Manual* (Breed, Murray,

TABLE 1. *Effect of incubation temperature on various properties of 15 strains of Pseudomonas geniculata*

Property tested	Number of strains positive	
	27 C	8 C
Obligately aerobic	15	15
Polar flagella, 1 to 12 per cell	15	15
Growth at pH 4.0	15	7
Growth in presence of 5% sodium chloride	15	15
Catalase positive	15	15
Gelatin hydrolyzed	15	15
Fluorescent pigment produced in liquid Georgia and Poe medium	15	15
Lipase positive	12	9
Litmus milk alkaline	15	15
Gluconate oxidized	15	15
Oxidase positive	15	15
Cytochrome oxidase positive	15	15
Nitrite produced from nitrate	0	0
Methyl red positive	0	0
Voges-Proskauer positive	0	0
Indole produced	0	0
Hydrogen sulfide produced	0	0
Urease positive	0	0
Starch hydrolyzed	0	0
Acids produced oxidatively from: adonitol, cellobiose, dextrin, dulcitol, erythritol, fructose, glycerin, inositol, inulin, lactose, maltose, mannitol, melezitose, melibiose, raffinose, salicin, sorbitol, sorbose, starch, sucrose, or trehalose	0	0
Acids produced oxidatively from:		
Arabinose	12	6
Galactose	7	6
Glucose	6	3
Mannose	11	10
Rhamnose	2	2
Xylose	13	8
Utilized as sole carbon source for growth:		
Formate, oxalic acid, or tartaric acid	0	0
Acetate, lactic acid, succinate, L-malate, or citrate	15	15
Resistant to: bacitracin, chloramphenicol, erythromycin, nitrofurazone, nitrofurantoin, isoniazid, magnamycin, nystatin, novobiocin, oleandomycin, *p*-aminosalicylic acid, penicillin, polymyxin B, ristocetin, spiramycin, sulfamerazine, vancomycin, and viomycin	15	15
Resistant to: chlortetracycline, kanamycin, methenamine mandelate, neomycin, sulfadiazine, oxytetracycline, tetracycline, and triple sulfa	0	0
Resistant to:		
Sulfamethoxypyridazine	1	5
Sulfisomidine	1	3
Sulfisoxazole	1	2
Sulfathiazole	1	1
Sulfamethizole	1	3
Streptomycin	2	1
Dihydrostreptomycin	2	3
Growth in nutrient broth over −2 to 36 C range	15	
Growth in nutrient broth at 40 C	6	
Optimal growth temperature 25 to 30 C	15	

and Smith, 1957), these isolates are properly classified as *P. geniculata*. Nevertheless, this is not entirely satisfactory, since *P. geniculata* differs from *P. fluorescens* in nitrate reduction (by the latter species). Actually, separation of these two species is not warranted. Rhodes (1959), who studied a large number of isolates, observed nitrate reduction in only 25% of *P. fluorescens* strains examined. Rhodes (1959) and Colwell and Liston (1961) suggested that *P. geniculata* be included in *P. fluorescens*. Any contemplated revision of *Pseudomonas* taxonomy should give serious consideration to this suggestion.

ACKNOWLEDGMENTS

I wish to thank R. A. Bell for technical assistance, R. J. Carroll for the electron micrographs, and C. O. Willits and A. E. Wasserman for advice and criticism in preparation of this manuscript.

LITERATURE CITED

ALFORD, J. A. 1960. Effect of incubation temperature on biochemical tests in the genera *Pseudomonas* and *Achromobacter*. J. Bacteriol. **79**:591–593.

AYRES, J. C. 1960. Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. Food Research **25**:1–18.

BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH. 1957. Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.

BROWN, A. D. 1957. Some general properties of a psychrophilic pseudomonad: the effects of temperature on some of these properties and the utilization of glucose by this organism and *Pseudomonas aeruginosa*. J. Gen. Microbiol. **17**:640–648.

BROWN, A. D., AND J. F. WEIDEMANN. 1958. The taxonomy of the psychrophilic meat-spoilage bacteria: a reassessment. J. Appl. Bacteriol. **21**:11–17.

COLWELL, R. R., AND J. LISTON. 1961. Taxonomic relationships among the pseudomonads. J. Bacteriol. **82**:1–14.

GABY, W. L. 1955. Taxonomic problems relating to the identification of species within the genus *Pseudomonas*. Intern. Bull. Bacteriol. Nomenclature and Taxonomy **5**:153–160.

GABY, W. L., AND E. FREE. 1958. Differential diagnosis of *Pseudomonas*-like microorganisms in the clinical laboratory. J. Bacteriol. **76**:442–444.

GEORGIA, F. R., AND C. F. POE. 1931. Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. J. Bacteriol. **22**:349–361.

HAYNES, W. C. 1951. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. **5**:939–950.

INGRAHAM, J. L., AND J. L. STOKES. 1959. Psychrophilic bacteria. Bacteriol. Rev. **23**:97–108.

LEIFSON, E. 1951. Staining, shape, and arrangement of bacterial flagella. J. Bacteriol. **62**:377–389.

MOORE, H. B., AND M. J. PICKETT. 1960. The *Pseudomonas-Achromobacter* group. Can. J. Microbiol. **6**:35–42.

NAGHSKI, J., L. L. REED, AND C. O. WILLITS. 1957. Maple sirup. X. Effect of controlled fermentation of maple sap on the color and flavor of maple sirup. Food Research **22**:176–181.

RHODES, M. E. 1959. The characterization of *Pseudomonas fluorescens*. J. Gen. Microbiol. **21**:221–263.

SHENEMAN, J. M., AND R. N. COSTILOW. 1959. Identification of microorganisms from maple tree tapholes. Food Research **24**:146–151.

SHEWAN, J. M., H. HODGKISS, AND J. LISTON. 1954. A method for the rapid differentiation of certain non-pathogenic, asporogenous bacilli. Nature **173**:208–209.

SOCIETY OF AMERICAN BACTERIOLOGISTS. 1957. Manual of microbiological methods. McGraw-Hill Book Co., New York.

STARR, M. P. 1941. Spirit blue agar: a medium for the detection of lipolytic microorganisms. Science **93**:333–334.

THOMAS, S. B. 1958. Psychrophilic micro-organisms in milk and dairy products. Dairy Sci. Abstr. **20**:356–369, 468.

THORNLEY, M. J. 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23**:37–52.

WILLITS, C. O., H. A. FRANK, AND R. A. BELL. 1961. Maple sirup. XIX. Flavor and color through controlled fermentation of maple sap. Food Technol. **15**:473–474.

WOLIN, E. F., J. B. EVANS, AND C. F. NIVENS, JR. 1957. The microbiology of fresh and irradiated beef. Food Research **22**:682–686.