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SOME CHARACTERISTICS OF AN OLEATE-REQUIRING, HEMOLYTIC *PEDIOCOCCUS*

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ABSTRACT

DEIBEL, R. H. (St. James Hospital, Chicago Heights, Ill.), J. H. SILLIKER, AND P. T. FAGAN. Some characteristics of an oleate-requiring, hemolytic *Pediococcus*. J. Bacteriol. 88:1078-1083. 1964. —A gram-positive, catalase-negative, tetrad-forming coccus, isolated from a bronchial aspirate, is described. The organism was classified as a member of the genus *Pediococcus* on the basis of morphological and physiological characteristics. It differed from previously described members of this genus in that it evidenced a requirement for oleic acid, even in complex culture media. This isolate produced hemolysis on blood-agar formulated with certain specimens of human blood and not with others. It was demonstrated that hemolysis required oleate supplementation and that the level of this fatty acid required for hemolysis is greater than that necessary for growth of the organism. A cell-free hemolysin could not be demonstrated in the culture supernatant fluid of oleate-grown cultures. The organism was isolated from a patient suffering from tuberculosis; however, attempts to isolate similar organisms from other tuberculous patients as well as from routine throat and sputum specimens were negative. No pathogenicity was manifested when laboratory animals were inoculated with the hemolytic *Pediococcus*.

The genus *Pediococcus* consists of homofermentative, gram-positive cocci that generally tend to form tetrads. Strains of the various species may or may not possess an enzyme which has catalase-like activity (Felton, Evans, and Niven, 1953); however, iron-porphyrin compounds have not been detected in these organisms (Deibel and Evans, 1960).

Deibel and Niven (1960) described the characteristics of a group of tetrad-forming, catalase-negative cocci which were isolated from meat-curing brines. These organisms were similar, if not identical, to *Aerococcus viridans* (Williams, Hirch, and Cowan, 1953) and *Gaffkya homari* (Hitchner and Snieszko, 1947; Aaronson, 1956). The physiological and morphological characteristics of all these organisms were sufficiently similar to justify placing them in one species; because of their close relationship to the pediococci, the species *Pediococcus homari* was proposed.

It is the purpose of this communication to describe a gram-positive, tetrad-forming, catalase-negative coccus, isolated from the human respiratory tract, which is physiologically similar to *P. homari*. This strain possesses a number of unique characteristics, including the ability to form hydrogen sulfide from cysteine, a requirement for oleate in complex media, and the possession of a peculiar hemolytic principle which requires oleate for activity in cultures grown on blood-agar.

MATERIALS AND METHODS

Isolation of organism and source of other strains. In the routine culture of a bronchioscopic aspirate, moderate numbers of the organism to be described (*Pediococcus* sp., strain SG1) occurred in apparently pure culture. Colonies were picked and grown in APT broth (Difco). After 24 hr of incubation, the broth cultures were streak plated, and the organism was reisolated to insure purity. For comparative purposes, strains of *P. cerevisiae* and *P. homari* were obtained from the stock culture collection of the American Meat Institute Foundation, Chicago, Ill.

Physiological methods. The routine physiological methods employed in this study were described previously (Deibel and Niven, 1960). All media were supplemented with 0.1% Tween 80.

Media. All strains were maintained by daily transfer in APT broth. In studies concerned with the effect of oleate on growth, the APT medium was compounded according to the formula of the supplier, with the omission of the Tween 80. Blood-agar was prepared with Blood

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Agar Base (BBL). After sterilization and cooling, 5.0 ml of blood were added per 100 ml of medium.

Blood collection. Human blood was drawn by use of Vacutainer tubes (Becton, Dickinson and Co., Rutherford, N.J.) containing A.C.D. solution as an anticoagulant. This solution has the following composition: citric acid, 0.44%; sodium citrate, 1.32%; and glucose, 1.47%. The tubes used contained 1 ml of A.C.D. solution, and the vacuum in the tube was so adjusted as to draw 6 ml of blood.

Determination of hemolysin. Pediococcus sp., strain SG1, was cultured for 24 hr at 37 C in Veal Infusion Broth (BBL) with and without Tween 40 (0.01%) and oleic acid (2×10^{-4}%). The cultures were centrifuged, and the clear supernatant fluids were tested for soluble hemolysin, by use of human red blood cells that had been washed three times with saline. A 2-ml amount of the packed cells was suspended in 100 ml of saline. To test for hemolysin, 5 ml of this cell suspension were added to 5 ml of culture supernatant fluid. The supernatant fluid was used without dilution and also after dilution with normal saline, so that the final dilutions of culture fluid were 1:2, 1:4, 1:8, and 1:16. The tubes were incubated at 37 C for 4 hr, and the optical density was determined with a Leitz photometer at a wavelength setting of 625 mμ.

Miscellaneous. Pediococcus sp., strain SG1, was incubated at 37 C unless otherwise indicated. The strains of *P. homari* were incubated at 30 C and the strains of *P. cerevisiae* at 37 C. Growth was estimated by optical-density determinations with a Bausch & Lomb Spectronic-20 colorimeter at a wavelength setting of 600 mμ. Tween 40 and Tween 80 were purchased from Hill Top Laboratories, Cincinnati, Ohio.

RESULTS

Morphology and physiology. Characteristically, growth of *Pediococcus* sp., strain SG1, in broth cultures occurs only at the bottom of the tube. If the culture is shaken, the cells readily disperse, giving a uniform turbidity. Upon primary isolation, the individual cocci were exceedingly small. Subsequent transfers in laboratory media resulted in a cell size comparable to that of *P. homari*. In stained preparations from broth cultures, a distinct tendency to form gram-positive tetrads was noted.

Growth of the organism was indifferent to oxygen when tested in agar-shake cultures. Unlike most strains of *P. homari*, strain SG1 was never observed to accumulate hydrogen peroxide when grown aerobically. Both the catalase and benzidine tests (Deibel and Evans, 1960), however, were repeatedly negative. Peculiarly, the organism does not tolerate 0.02% sodium azide in agar or broth media.

The fermentation of glucose did not produce significant amounts of CO_2. Unlike *P. cerevisiae* and similar to *P. homari*, strain SG1 produced a rather high final pH in the glucose fermentation (Table 1). Only 7 (glucose, mannose, fructose, sucrose, maltose, sorbitol, and mannitol) of 25 carbohydrates tested were fermented.

Cultures of the organism in Triple Sugar Iron Agar evidenced a weak, but distinctly positive, production of H_2S. Additional experiments were conducted in which the following semisolid medium was employed: Tryptone (Difco), 10 g; yeast extract (Difco), 5 g; K_2HPO_4, 4 g; Tween 80, 0.5 g; sodium thiosulfate, 0.2 g; ferrous ammonium sulfate, 0.2 g; agar, 1.5 g; and distilled water, 1 liter (pH 7.2). Production of H_2S was not observed in cultures in the basal medium after 24 hr of incubation. The addition of either cysteine (0.1%) or cystine (0.04%) resulted in a

TABLE 1. *Physiological characteristics of Pediococcus sp., strain SG1*

Characteristic	Result
Coagulase	−
CO_2 production from glucose	−
Slime from sucrose	−
Final pH (1% glucose)	4.8
Acetoin production	−
Nitrate reduction	−
Hydrolysis:	
Hippurate	−
Esculin	−
Arginine	−
Gelatin	−
Starch	Weak +
Energy utilization:	
Pyruvate	−
Gluconate	−
Malate	−
Growth:	
10 C	−
45 C	+
4.0% NaCl	+
6.0% NaCl	−

blackening of the culture within 18 hr after inoculation. Fourteen other strains of *Pediococcus* failed to produce H_2S in the cysteine-supplemented medium.

The gram-positive nature of the organism, coupled with the absence of (i) catalase activity, (ii) iron-porphyrin compounds, and (iii) production of CO_2 from glucose, tend to place this organism in the genus *Pediococcus*. The high final pH noted in the glucose fermentation as well as the cell size and distinct tendency to form tetrads are characteristics which parallel those of *P. homari*.

Growth requirement for oleate in complex media. Primary isolation of the organism from the blood-agar medium was performed in APT broth. In this medium, a maximal growth response was noted within 24 hr. Subsequently, growth of the organism was observed to be comparatively poor when tested in a variety of complex media. A deletion-type experiment was conducted with various components of the APT medium, and it was observed that omission of the Tween 80 resulted in a radically decreased growth response. Omission of the supplemental inorganic salts was followed also by a diminished growth response which indicated the desirability of including these salts in the medium.

In a series of experiments, the Tween 80 requirement for growth in the complex medium was verified. The concentration required for maximal growth was high, and varied from 0.05 to 0.08% in identical trials, each performed over a period of 3 months.

TABLE 2. *Growth response of Pediococcus sp., strain SG1, to oleate in a complex medium**

Oleate per 10 ml of medium†	Growth response‡	
	No Tween 40	Tween 40 (0.01%)
μg		
0	8	7
5	13	28
10	21	44
20	23	56
30	7	68
40	0	75
50	0	75
100	0	54

* The complex medium is described in Materials and Methods.

† Oleic acid neutralized with NaOH and added to medium prior to sterilization.

‡ Optical density times 100. Results determined after 20 hr of incubation at 37 C.

To define oleate as the active principle in the Tween 80 preparation, varying concentrations of oleate (Calbiochem) were tested in the absence and presence of Tween 40 (1.0 mg per 10 ml of medium). Without Tween 40 to detoxify the oleate (Williams, Broquist, and Snell, 1947), growth was inhibited (Table 2). In the presence of Tween 40, approximately 40 μg of oleate per 10 ml of medium gave a maximal growth response.

Saturated, aliphatic, monocarboxylic fatty acids from C-2 to C-18 did not promote growth. Linoleic acid was about one-half as active as oleate; however, erratic results were obtained with this compound. Unlike the "minute" streptococci, whose oleate requirement can be replaced by culturing the organism in a 10% atmosphere of CO_2 (Deibel and Niven, 1955), no growth-promoting activity was observed with the strain of *Pediococcus* when tested under increased CO_2 tensions.

Blood-agar studies. In the initial isolation of the organism, a peculiar hemolytic reaction was noted in the human blood-agar medium. Wide zones of hemolysis were observed; however, immediately around the colony a small area was noted in which no hemolysis occurred. In subsequent experiments, the organism was cultured in a large number of blood-agar media prepared with human blood obtained from a variety of donors. Extremely erratic results were noted, in that some blood samples afforded a hemolytic reaction whereas others did not. Blood obtained from the same individuals on different days gave variable results. The incubation period required for hemolysis (when hemolysis occurred) varied from 24 to 72 hr. Characteristically, the organism grew well in all media; and, whether or not hemolysis occurred, the colony sizes were approximately equal. In this study, a correlation was noted between time of obtaining the blood sample from the donor, and the time of the donor's last partaking of food. Blood from donors who had partaken of food 1 to 3 hr prior to bleeding showed a higher incidence of hemolysis. This observation, coupled with the oleate requirement for growth, suggested a possible relationship between fatty acids and hemolysis.

Blood samples were obtained from three donors who had fasted for 10 to 12 hr. The following media were prepared: Blood Agar Base (basal

medium), basal medium plus 0.01% Tween 40, basal medium plus 2×10^{-4}% oleate, and basal medium plus 0.01% Tween 40 plus 2×10^{-4}% oleate. Each blood sample was tested with the four media. The culture for inoculum was centrifuged, washed once with sterile-distilled water, and diluted appropriately to avoid any carry-over of Tween 80 from the growth medium. Pour-plate cultures were prepared. After 24 hr of incubation, hemolysis was observed with each of the three blood samples only in the medium containing both Tween 40 and oleate. After 72 hr of incubation, extremely weak hemolytic activity was noted in the medium supplemented with oleate alone. No hemolysis was noted either in cultures containing Tween 40 or in cultures of the basal medium. In addition, no variation in colony size was noted in the media regardless of supplementation. The basal-blood-agar medium, therefore, apparently contains a sufficient quantity of unsaturated fatty acids for growth purposes; however, an additional amount of detoxified oleate is required for hemolysis.

The above experiment was repeated with 14 additional human blood samples from presumably fasting subjects. Nine of the blood samples yielded results identical to those described above in the initial experiment. In contrast, three of the blood samples were not hemolyzed under any condition of test, and two samples evidenced hemolysis in all four test media.

The same procedures, as outlined above, were used to test the hemolysis of pooled, defibrinated, sheep's blood (Markham Laboratories, Chicago, Ill.). Seven different attempts over a 1-year period of time, each with a recently purchased sample, failed to demonstrate hemolysis under any condition of test.

In Blood Agar Base supplemented with Tween 40 and oleate (as above), 14 other strains of *Pediococcus* were tested with a fasted, human-blood sample. The six strains of *P. cerevisiae* were typically nonhemolytic; the eight strains of *P. homari* strains all produced an α-hemolytic reaction; and *Pediococcus* sp. (SG1) reacted as shown (Fig. 1).

Determination of soluble hemolysin. Repeated trials with cultures grown in Veal Infusion Broth (see Materials and Methods) showed no evidence of soluble hemolysin when tested with the procedure described.

Animal studies. Six mice and six guinea pigs were inoculated subcutaneously and intraperitoneally with 3.2×10^5 viable cells of strain SG1. After 2 weeks, no untoward effects were observed in any of the animals. Upon necropsy, none of the animals appeared abnormal in any respect.

Attempts to isolate similar strains. On human blood-agar, without and with supplementation (0.01% Tween 40 plus 2×10^{-4}% oleate), numerous specimens of sputum and bronchioscopic aspirates as well as nose and throat swabs were cultured in an effort to isolate additional strains. Each batch of medium was tested with the original isolate to insure proper performance. Over 300 specimens were cultured in this manner; however, an organism similar to that described was never encountered.

The patient from which *Pediococcus* sp., strain SG1, was isolated had chronic chest pains for a 7-month period. Upon hospital admission, roentgenographic studies revealed calcified foci around the hilar area of the lung, with a moderate degree of fibrosis. Repeated sputum cultures failed to yield acid-fast bacilli. Stained preparations of the bronchioscopic aspirate, from which the *Pediococcus* was isolated, also failed to reveal acid-fast bacilli. After 3 weeks of incubation, however, cultures of the aspirated material gave rise to 15 to 20 colonies of acid-fast bacilli. A guinea pig was also inoculated with a preparation of the aspirated material; and, after 3 weeks, the animal was sacrificed. Smears of the liver showed large numbers of acid-fast bacilli.

In the ensuing 4 years, an attempt was made to isolate an organism similar to strain SG1 from recently diagnosed, tuberculous patients. In cultures from approximately ten of these patients, no organism similar to strain SG1 was detected.

DISCUSSION

Although both *G. tetragena* and *Pediococcus* sp., strain SG1, are hemolytic and morphologically similar, the strain of *Pediococcus* differs in that it lacks catalase and other iron-porphyrin enzymes. The absence of these respiratory enzymes, the homofermentation of glucose, and the tendency to form tetrads are key characteristics of the pediococci; consequently, the organism described has been classified tentatively as a *Pediococcus*.

Garvie, Gregory, and Mabbitt (1961) described an unusual strain (*Pediococcus* sp., strain NCDC 1250) that required Tween 80 for growth in a semidefined, casein-hydrolysate medium. Although Tween 80 was added to the various com-

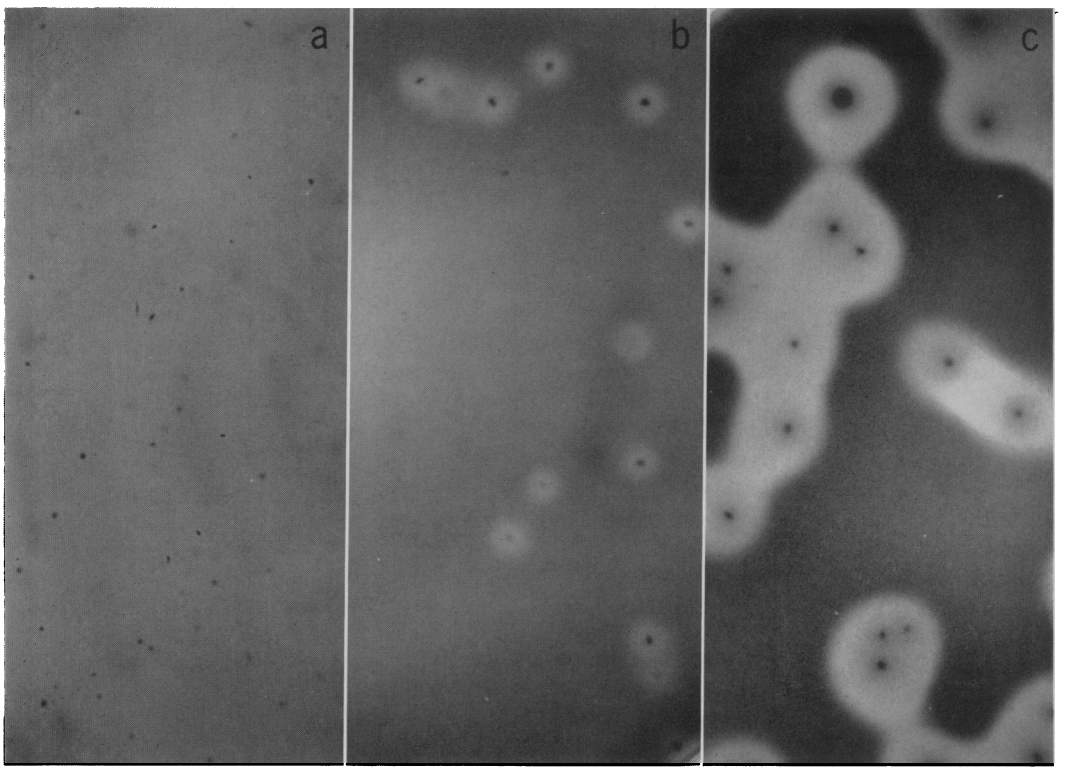

FIG. 1. *Comparison of colony characteristics on blood-agar of three strains of Pediococcus. See text for composition of medium. Incubation period, 48 hr. (a) P. cerevisiae, no hemolysis; (b) P. homari, α-hemolysis; (c) Pediococcus sp., strain SG1, β-like hemolysis. P. homari incubated at 30 C; other strains incubated at 37 C. Magnification, 2×.*

plex media employed to culture this organism, definitive experiments to determine its requirement in these media were not reported. This gram-positive, tetrad-forming coccus lacked catalase, and these characteristics prompted its inclusion in the genus *Pediococcus* even though the organism was an anaerobe.

The requirement by strain SG1 for oleate in complex media is deemed extremely peculiar, and, to the authors' knowledge, no other lactic acid bacterium has been reported to possess a similar requirement under this condition of culture. The organism's specific need for oleate, and the exclusion of possible indirect stimulation by use of a complex medium, may form the basis of a superior microbiological assay for this growth factor.

The hemolytic reaction caused by this organism merits further study. As seen in Fig. 1, there is a marked tendency of red blood cells immediately surrounding the individual colonies to withstand the lytic process. In areas more distant from the colony, however, complete lysis occurs. Generally, the lytic areas (proximal to the colony) are extremely clear and sharply defined. Although the term hemolysis has been employed in this study, the mechanism of this lytic process is unknown, and, conceivably, it may not involve a true hemolysin. Indeed, this possibility is suggested by our failure to demonstrate a cell-free hemolytic principle in broth cultures, even in those in which oleate had been incorporated.

The relationship of the concentration of oleate to growth and hemolysis in the blood-agar medium is not clear. No gross difference in colony size was detected in various experiments regardless of the hemolytic reaction. As noted in Table 2, approximately 4.0 μg of oleate per ml of medium afforded maximal growth, and 1.0 μg of oleate per ml was stimulatory. In the blood-agar medium, 1.0 μg of oleate per ml of medium gave rise to a strong hemolytic reaction, whereas 0.5

μg of oleate per ml of medium was ineffective even after 48 hr of incubation. The meager evidence obtained indicates that the blood medium contains enough oleate to afford maximal colony development but an insufficient amount to enhance hemolysis.

Our failure to isolate organisms similar to strain SG1 indicates that this organism is not a frequent contaminant of the human respiratory tract. The organism's inability to infect laboratory animals, and its most probable role as a secondary invader in the tuberculous patient from which it was isolated, cast doubt on its pathogenicity.

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