A Pigment-producing Spoilage Bacterium Responsible for Violet Discoloration of Refrigerated Market Milk and Cream

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Received for publication September 30, 1960

ABSTRACT

SEITZ, EUGENE W. (Oregon Agricultural Experiment Station, Corvallis), P. R. ELLIKER, AND W. E. SANDINE. A pigment-producing spoilage bacterium responsible for violet discoloration of refrigerated market milk and cream. Appl. Microbiol. 9:287–290. 1961.—A psychrophilic strain of bacteria identified as Chromobacterium lividum was established as the causative agent of an outbreak of violet discoloration in refrigerated, pasteurized retail milk and cream.

The organism was rod-shaped, gram-negative, and produced viscid colonies with abundant violet pigment on Tryptone glucose yeast extract agar. Growth was abundant at 4°C but none occurred at 37°C. Growth in milk was characterized by a dark violet ring at the surface after a few days, and the deep violet color gradually extended through the product in older cultures. Some proteolysis occurred. The pigment appeared to be similar to that of other known species of Chromobacterium and assisted in identification of the genus of the causative organism.

The isolated strain of C. lividum was destroyed by exposure to 56°C for 5 min which suggested postpasteurization contamination as the source of the spoilage organism in commercial milk and cream.

An investigation was conducted to determine the cause of an outbreak of violet discoloration in market cream from two different dairy plants in Oregon. Both plants were located in the same city. The defect occurred chiefly in cream, but occasionally also in market milk. The discoloration varied in intensity from a barely discernable shade in 2-day-old samples to a deep, intense violet in older milk and cream. In most instances, the defect occurred in the refrigerated product at temperatures ranging from 2 to 10°C. A slightly viscous consistency and glistening appearance often accompanied discoloration of cream. The violet color usually appeared first at the surface and when samples were stored for periods of several days to a few weeks, the entire container of cream became discolored. This report deals with the isolation and characterization of the causative organism of this defect which to our knowledge has not previously been reported in dairy or other food products.

MATERIALS AND METHODS

Isolation. Considerable difficulty was encountered in isolating the bacterium. First attempts to grow the organism on conventional agar or broth media inoculated with defective cream were unsuccessful. However, when 12% sterile cream was used as an enrichment medium, the organism grew upon incubation for 4 weeks at 4°C and subsequently was isolated in pure culture. The culture, after original isolation, was carried without difficulty on TGY (Tryptone, glucose, yeast extract) agar or in TGY broth media incubated in the laboratory at refrigeration (4°C) or room (25°C) temperatures.

Characterization. Preliminary observations suggested that the violet, pigment-producing spoilage organism was a species of Chromobacterium. Consequently, five similar species of Chromobacterium were included in the investigation as an aid to identification and characterization. It was possible to compare morphological, cultural, physiological, and pigment characteristics for all six organisms. Three of the stock cultures were from the American Type Culture Collection, one from the Hopkins Marine Station, California, and one from the Oregon State University culture collection.

The morphology of the organism was studied using a variety of conventional staining techniques (Society of American Bacteriologists, 1957). The electron microscope RCA EMU model D2 was used to study flagellation and general morphology.

A wide variety of nutrient media was used under varying conditions to establish properties of the violet bacterium and to enable comparison with the other species of Chromobacterium used in the study. A number of catabolic activities of the new isolate and stock cultures was studied using conventional methods to test for end products of degradation (Society of American Bacteriologists, 1957). The tests for lecithinase activity and for esculin hydrolysis were described previously by Sneath (1956). Heat resistance studies were carried out as described by Sneath (1956).

Nitrate and nitrite reduction were studied using five

1 Technical paper no. 1347, Oregon Agricultural Experiment Station. Contribution of the Department of Microbiology.
different media. These were Difeo's nitrate broth (Difeo Laboratories, 1953), ZoBell's (1932) nitrate and nitrite broths, Trypticase nitrate broth (Baltimore Biological Laboratory, 1956), and a nitrate medium containing 1.0 g Bacto-peptone, 0.5 g potassium nitrate, and 0.5 g sodium chloride per liter.

Pigment studies were conducted as a further means of characterizing the organism. Crude violacein was extracted from cell harvests of Chromobacterium violaceum strain OSU and the newly isolated species using 95% ethanol. The extract solution was centrifuged and Seitz filtered. Absorption spectra were determined in Beckman model B and DU spectrophotometers. The crude extracts of each organism were fractionated using one-dimensional ascending paper chromatographs at room temperature (25 C). The developing solvent used was isopropanol and water (6:4).

Four well-defined and parallel pigment fronts could be seen after development. Each front was eluted from the paper with 95% ethanol and then was centrifuged and Seitz filtered. The four different fractions thus obtained from both pigment extracts then were subjected to complete spectrophotometric analysis between 280 and 1,000 μ.

Results
Morphology. Pure culture studies showed the organism to be a gram-negative rod. Cells were motile, single, rod-shaped, with rounded ends, and were approximately 3 μ long and 0.6 μ wide. They exhibited little pleomorphism. The organisms appeared to be without fat bodies, capsules, or endospores. Single polar flagella were observed after staining by Leifson's (1956) method. Several electron micrographs of the new organisms revealed cells possessing polar, subpolar, and short, lateral flagella. The cells under this type of magnification were cylindrical with rounded ends. Some cells were slightly curved. Figure 1 shows enlarged electron micrographs of the organism.

Cultural characteristics. Nutrient agar slants incubated at 4 C showed violet, pigmented growth after
4 days. The pigment became much darker after several more days of incubation.

Similar results were observed using slopes of TGY agar which were more satisfactory for growth and pigmentation than nutrient agar, especially at temperatures above refrigeration. The growth on TGY slant cultures was abundant, echinulate, viscid, and deeply pigmented.

Colonies on nutrient agar after 4 days at 4°C were dark violet, smooth, convex, circular, raised, entire, and gelatinous. Discrete colonies on TGY agar appeared after incubation for 48 hr at 21.5°C. A typical plate bearing colonies is illustrated in Fig. 2. Old colonies (10 days) were 10 mm in diameter and the margins were lobate in most cases. Colonies easily could be removed intact from the agar surface, and they did not fluoresce under ultraviolet light in the dark. Odors given off by organisms growing on TGY agar plates were somewhat putrefactive. Subsurface colonies were very small and remained unpigmented.

Growth in the form of a violet surface ring occurred in TGY broth incubated at 21.5°C but not at 30°C. Cells formed a thick and viscid mass that adhered to the glass and remained at the surface. The underlying medium remained clear. Sedimentation occurred and exhibited a gelatinous consistency but no violet pigment. Generally, better growth occurred on the surface of TGY agar than in broth.

Litmus milk tubes incubated at 21.5°C for 5 days contained heavy, gelatinous, violet pellicles, and indication of an alkaline reaction was present (see Fig. 3). Considerable catalase activity was present in the litmus milk. The organism did not grow on potato slopes at 30°C, but grew very well at 4 or 21.5°C and produced dark-violet pigment. There was no starch hydrolysis at 4, 21.5, or 30°C after 5 days. Convex, smooth-surfaced, shiny, dark-violet colonies were observed on nutrient butter fat agar, but no hydrolysis of fat occurred after incubation for 5 days at 4, 21.5, or 30°C. Growth on moistened sterile rice grains was excellent and the pigment increased progressively from 24 to 62 hr.

Growth of the newly isolated organism on Difco blood agar base resulted in colonies having a butyrous, dark violet pigment. The addition of 0.5% human blood caused a change in the colonies from a smooth to a very rough form.

The organism rapidly liquefied nutrient gelatin at 21.5°C and plates of this medium were extensively proteolyzed within 40 to 60 hr. No liquefaction of gelatin occurred at 4°C after 7 days, but some liquefaction was evident after 21 days.

Growth and pigment production were excellent on Loeffler's serum slopes but no liquefaction occurred.

**Physiological characteristics.** An examination of the growth rates was conducted at various temperatures of incubation in an effort to determine the proper temperature for physiological studies. The most suitable of the temperatures used was 21.5°C. This temperature, therefore, was employed in all subsequent studies. For convenience, the organisms were designated either as psychrophiles or mesophiles on the basis of growth at 4°C and 37°C; those organisms growing at 4°C but not at 37°C were designated as psychrophiles and those growing at 37°C but not at 4°C were designated as mesophiles. The newly isolated *Chromobacterium* was placed in the psychrophilic group.

A comparison of characteristics between psychrophilic and mesophilic strains of *Chromobacterium* may be seen in Table 1. The newly isolated organism

<table>
<thead>
<tr>
<th>Test</th>
<th>Psychrophilic</th>
<th>Mesophilic</th>
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<tbody>
<tr>
<td></td>
<td>Newly isolated</td>
<td>C. amethystinum</td>
</tr>
<tr>
<td>Growth at 37°C</td>
<td>-</td>
<td>2+</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>Acid from trehalose</td>
<td>1+</td>
<td>-</td>
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<tr>
<td>Hydrolisys of esculin:</td>
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<tr>
<td>(a) Broth</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>(b) Agar</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>-</td>
<td>1+</td>
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<tr>
<td>Utilization of citrate</td>
<td>4+</td>
<td>3+</td>
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<tr>
<td>Proteolysis of milk</td>
<td>2+</td>
<td>-</td>
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<tr>
<td>Egg yolk agar lecinhasin activity</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>H2S production</td>
<td>2+</td>
<td>4+</td>
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<tr>
<td>Indole from tryptophan</td>
<td>3+</td>
<td>2+</td>
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<tr>
<td>Urease activity</td>
<td>1+</td>
<td>2+</td>
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<tr>
<td>Gelatinous growth</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Amount of fat in cells</td>
<td>-</td>
<td>2+</td>
</tr>
<tr>
<td>Pigmented sediment in broth</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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- = Negative reaction; ± = barely discernible; 1+ = scant; 2+ = fair; 3+ = good; 4+ = very good.
apparently was unable to produce acid from any carbon source unless incubated at 4 C. At this temperature, acid was produced from dextrose in 7 days.

The new isolate failed to reduce nitrate in all of the nitrate media employed. Considerable growth occurred in broth containing 7.5% sodium chloride. Greater tolerance of alkalinity than acidity was exhibited as the organism grew at approximately pH 9.5, but failed to grow at pH 5.0.

In heat resistance studies, 48-hr broth cultures of the newly isolated *Chromobacterium* were subcultured after heating at 56 C for 5 min. All subcultures were negative. The 48-hr broth cultures also were negative for growth after heating the culture tubes at 56 C for 30 min followed by incubation at 21.5 C for 48 hr.

**Pigment characteristics.** The results suggested that the pigment from the OSU strain of *C. violaceum* was closely related to the pigment of the new isolate of *Chromobacterium*. The absorption curves from the pigment extracts for both organisms showed peaks at approximately 580 m,u. Butanone and water (8:2) caused separation of a yellow fluorescent pigment from the violet pigment using the ascending chromatographic method described. When isopropyl alcohol and water (3:2) were used, the crude pigment appeared to arrange itself into four separate bands parallel to the front of the solvent. The most mobile fraction consisted of a yellow band which was fluorescent under ultraviolet light. The other three fractions were not fluorescent. The band following the yellow band was tinted yellow. It was followed by a third band which was light violet in color. This band in turn was followed by the least mobile band which was dark violet in color. The spectral analyses of the respective fractions obtained from the crude pigments were similar for the newly isolated *Chromobacterium* and *C. violaceum* strain OSU.

**DISCUSSION**

The genus *Chromobacterium* includes those organisms that produce the violet pigment, violacein. In fact, both Gilman (1953) and Sneath (1956) have excluded from the genus all organisms not producing violacein. Results of spectral analyses on the violet pigment in this investigation agree closely with those of Gilman (1953).

In contrast to the clarity of the generic designation, the classification at the species level in this genus is in a state of confusion. Species names in some instances do not clearly identify the organism.

The results of this investigation indicate that at the present time *C. violaceum* is the only species of this group that might be classed as a mesophile. Of the organisms studied by the authors, it appears that *C. violaceum* strain OSU, *C. violaceum* strain 553, and *C. violaceum* strain 6357 are all mesophiles. Furthermore, the cultural and physiological similarities shown in this study between the newly isolated *Chromobacterium, Chromobacterium violaceum* var. *amethystinum*, and *Chromobacterium amethystinum* strain 6915 indicate that these organisms are all the same species. According to Sneath (1956) the first *Chromobacterium* recognized as a psychrophile was *Chromobacterium lividum*. Each of these three psychrophiles, therefore, may be considered strains of this organism. For purpose of convenience, the newly isolated *Chromobacterium* has been designated *C. lividum* strain OSU.

The gelatinous property of *C. lividum* strain OSU, suggested that this organism originally came from soil or water. Corpe (1951) noted that gelatinous *Chromobacterium* species originated from the soil and that nongelatinous came from other sources. The fact that the organism appeared in two different dairy plants in the same city suggested the possibility of its originating on the farm, possibly from soil. From this source it could have established itself on utensils and gained entrance to the milk supply of the two plants.

Another possible source may have been the city water supply used by both plants. The *Chromobacterium* might have entered the plants by this route and gained entrance to the pasteurized product from equipment contaminated by the water supply. Heat resistance studies demonstrated that it was easily destroyed by pasteurization and thus contamination of the product must have been postpasteurization.

**ACKNOWLEDGMENTS**

The helpful suggestions of Dr. W. B. Bollen, Dr. A. W. Anderson, and Mrs. H. A. Hays are gratefully acknowledged. The courtesy of Dr. C. B. van Niel in making *C. violaceum* var. *amethystinum* available is greatly appreciated by the authors.

**LITERATURE CITED**


Difco Laboratories, Inc. 1953. Difco manual of dehydrated culture media and reagents. 9th ed. Detroit.


