similar, the ropy isolate differs from the experimental
and recorded characterizations for A. viscosus on the
following points: 1) It does not hydrolyze fat, 2) it
consistently ferments arabinose, dextrose, galactose,
and xylose with pH readings between 4.6 and 5.6, and
3) it is capable of good growth at 4.5 C and does not
grow at temperatures greater than 35 C.

The ability of the capsulated isolate to produce ropiness in milk at 5 C, the inability to do so at 36 C, and the ease of capsule formation on simple nutrient
agar at 5 C indicate the psychrophilic nature of the ropy
isolate.

Until a chemical analysis of the capsular material of
A. viscosus and a serological study comparing the ropy
psychrophile with A. viscosus can be made, the ropy
psychrophile might be regarded as a variant of A.
viscosus.

Studies on a Psychrophilic Bacterium Causing Ropiness in Milk.
II. Chemical Nature of the Capsular Polysaccharide

D. E. Wegemer and C. Gainor

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania

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Buchanan and Hammer (1915) have described ropiness in milk as a change from its normal consistency to
a condition in which long threads of viscous masses are
observed. They attributed this condition to the production
of carbohydrate (polysaccharide) derivatives. The organisms which usually produce ropiness in milk are
those possessing a large capsule, such as Alcaligenes
viscosus and members of the Escherichia-Aerobacter
group (Buchanan and Hammer, 1915; Sarles and Ham-
mer, 1933). The capsular material produced by many
bacteria consists of complex polysaccharides identified
as polymers of simple monosaccharides (Pignamm
and Goeppe, 1948; Evans and Hibbert, 1947).

It is the purpose of this paper to report on the nature
of the capsular material produced by a gram negative
organism, resembling Alcaligenes viscosus, causing ropiness in a sample of homogenized-pasteurized milk
(Gainor and Wegemer, 1954).

Experimental Methods

The isolation and characterization of the ropy milk
organism have been previously described (Gainor and

Five ml volumes of 2 per cent glucose (c.p.) solution,
sterilized by Seitz filtration, were aseptically added to
50 ml of cooled, sterile nutrient agar (8.0 grams of Difco
nutrient broth, 17.7 grams of Difco agar-agar, and 500
ml of distilled water) in Kolle flasks. Although the or-
ganism developed colonies of stringy type on nutrient
agar (pH 6.9), it was found that higher cell counts of
the organism were obtained when glucose was incor-
porated into the medium. The glucose-nutrient agar
medium (pH 6.8) was shaken to insure homogeneity of
the component parts. The flasks were incubated for 48
hours at 37 C for a sterility check, and then each was
incubated with 1.25 ml of a saline suspension of the
stringy isolate, previously incubated at 25 C, containing
9 X 10^8 cells per ml. The inoculated 1 per cent carbohy-
drate agar was incubated at 25 C for 48 hours. The
flasks showed the presence of typical stringy colonies.

The growth was removed from the surface of the agar
and suspended in 25 ml of sterile distilled water in a
50-ml centrifuge tube. The water suspension of the or-
ganism was spun in an angle centrifuge at 3,000 rpm
at room temperature for one-half hour to remove the

1 This work was submitted in partial fulfillment of the re-

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cells. To the viscid supernatant (cell free) were added two volumes of absolute ethyl alcohol followed by storage at 5°C overnight to precipitate the polysaccharide. The crude polysaccharide was spun at 3,000 rpm for 15 minutes and dissolved in 20 ml of distilled water containing 2 grams of sodium acetate and 0.5 ml of glacial acetic acid. The polysaccharide was repeatedly precipitated from this solution by the addition of absolute alcohol. After a final precipitation, the polysaccharide was dissolved in the sodium acetate-acetic acid mixture. A Biuret test made on a sample of the polysaccharide indicated a small amount of protein material. The protein material was removed from the acetate-acetic acid solution of polysaccharide by repeated mixing with an equal volume of chloroform and n-amyl alcohol (4:1 ratio). A slight zone of precipitated material formed at the interface of the two liquids. The precipitate was separated by centrifugation and discarded. The polysaccharide was again precipitated by the addition of two volumes of absolute alcohol, centrifuged, and dried in a desiccator containing CaCl₂.

The polysaccharide was hydrolyzed in 1.0 N H₂SO₄ for 2½ hours in a water bath at 100°C. One-half ml of the acid was used for 20 mg of the polysaccharide. The hydrolysate was neutralized by adding 1.0 N Ba(OH)₂. The hydrolysate was centrifuged at 3,000 rpm to remove the insoluble BaSO₄ precipitate. The supernatant was concentrated for analysis by evaporation in a desiccator containing CaCl₂ within an incubator at 50°C.

Chromatographic techniques based on the work of previous investigators (Consden et al., 1944; Partridge, 1948; Forsyth, 1948; Hawthorne, 1947; Fisher et al., 1948) were employed. Sample amounts of 0.02, 0.04, and 0.06 ml were placed on the base pencil line of a single dimensional chromatogram. The solvent used was a mixture of n-butanol, acetic acid and water (4:1:5 ratio). The water layer of the solvent plus NH₄OH (1 per cent by volume) was placed in an open Petri dish in the bottom of the chromatocab* to maintain saturated conditions. The paper was irrigated for 14 hours by the descending method, removed from the chromatocab, and the solvent front marked with ink. The paper was dried for one-half hour at 105°C to evaporate the solvent. The chromatogram was sprayed either with ammomiacal silver nitrate (equal volumes of 0.1 N AgNO₃ and 5 N NH₄OH) or with acidified resorcinol, which consisted of a 0.2 per cent ethanolic solution of resorcinol acidified by adding first an equal volume of 0.25 N HCl and then 0.1 volume of orthophosphoric acid. The chromatogram was then dried for 10 minutes at 105°C to develop the spots. The position of the spots was marked and the Rf value and color reaction of the spots were determined.

Sugar standards having an Rf range of 0.21 to 0.28 were used as controls, since a preliminary run of the polysaccharide hydrolysate showed a spot within this range. A 0.02-ml amount of each of the standards (1 per cent arabinose, dextrose, galactose, fructose, xylose and mannose) was placed on the base pencil line of the chromatogram and chromatographed.

An attempt was made to shift the colonial phase of the stringy isolate to determine if a nonstringy variant could produce ropiness. Previous investigators (Haddley, 1927; O'Neal, 1933) have shown that highly viscous encapsulated bacteria can be shifted to nonencapsulated types by repeated transfer through an impoverished medium or in alkaline peptone water.

Three loopfuls of a 24-hour 5 per cent peptone broth culture of the stringy isolate were streaked on peptone agar of pH 7.8 containing 5, 3, 2⅔, 2, and 1 per cent Difco Bacto-peptone. The inoculated plates were incubated at 25°C for 48 hours. There was no visible growth on the 1 per cent plates, pin-point colonies developed on the 2 per cent plates, and stringy colonies were found on the 3 and 5 per cent peptone plates. After 1 week, a number of the colonies on the 2½ per cent peptone plates showed nonstringy evaginations while the rest of the colony was stringy. A portion of an evagination was streaked on fresh 2½ per cent peptone agar plates of pH 7.8, a drawn sterile glass rod being used as an inoculating needle. Three subtransfers of the original evaginated area incubated at 25°C yielded colonies that were white, flat, circular with regular margins, and butyrous. The colonies maintained this morphology for 12 consecutive subtransfers, at which time the culture was considered to be stable. Cellular studies showed the presence of gram negative rods (0.4 to 0.5 by 0.9 to 1.7 microns), single, in pairs, and in chains of short length, nonmotile and nonencapsulated. Biochemical studies gave the same reactions as were obtained with the stringy isolate (Gainor and Wegemer, 1954).

**Results**

The supernatant of the water suspension of the ropy organism was viscid. Upon the addition of absolute alcohol, a white, cottony precipitate was formed. The hydrolysate gave a positive result with the Molisch reagent, a negative test with the Benedict reagent, and a positive result with the Seliwanoff reagent. The supernatant of the nonropy variant was nonviscid, gave negative carbohydrate tests, and showed no change on addition of absolute alcohol.

The developed chromatogram indicated that the hydrolysate from the ropy organism contained only fructose when the spots of the hydrolysate were compared to the fructose standard in both Rf value and color reaction. The Rf value of fructose was 0.28 (the temperature of irrigation was 30°C), with a black spot being developed with the acidified ethanolic resorcinol spray. The other standards of arabinose (0.27 green)
dextrose (0.23 tan), galactose (0.21 yellow), xylose (0.23 bluish-black), and mannose (0.26 green) did not correspond in Rf value and/or color reaction.

No carbohydrate spots were detected when the supernatant of the nonstringy variant was chromatographed.

**Discussion**

Numerous investigators have studied the organisms responsible for producing ropiness in milk. These studies have been concerned chiefly with the identification of the causative organism. Little investigation has been devoted to the specific chemical nature of the material causing this abnormal condition. Studies concerning colonial state in relation to ropiness in milk are notably rare in the literature.

It was found in this investigation that the stringy bacterial colony isolated from a sample of pasteurized-homogenized milk possessed a large capsule. This stringy isolate produced ropiness in milk. A nonstringy variant, similar in biochemical reactions to the stringy isolate, lacked a capsule. This variant failed to produce ropiness in milk.

It is possible that the levan nature of the capsular polysaccharide of the stringy isolate is responsible for the production of ropiness in milk by this organism, and that the nonstringy variant, lacking a well-defined capsule and levan material, is unable to cause ropiness.

**Summary**

A gram negative, rod-shaped bacterium, producing ropiness in milk, was found to possess a capsular polysaccharide which was levan in nature. A nonropy variant of this organism neither produced ropiness in milk nor possessed detectable levan material.

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