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Synergistic False-Positive Coliform Reaction on M-Endo MF Medium

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The reported occurrence of synergistic false-positive coliform reactions on M-Endo MF medium was investigated. The objectives of the study were (i) to isolate populations of bacteria which produced such false-positive reactions, (ii) to determine whether false-positive sheen reactions are a result of synergistic interaction, and (iii) to determine the metabolic intermediates involved in false-positive sheen production. Samples of river water were subjected to coliform analysis by the membrane filter technique with M-Endo MF medium. Suspect sheen-forming colonies were analyzed to determine purity and identity of cultures. Mixed cultures were separated, and individual isolates were examined for sheen production. The isolates not producing a sheen were recombined and tested further for sheen production. Those mixtures reproducing the sheen were characterized biochemically and tested to determine the metabolic intermediates involved. Chromatographic analysis of the metabolites showed that individual isolates produced an assortment of neutral organic compounds including 2-butanone, 2,3-butanedione, formaldehyde, and butyraldehyde, whereas acetaldehyde or propionaldehyde was produced only by the mixed cultures. Tests showed that both propionaldehyde and acetaldehyde could react to produce a sheen on M-Endo medium. The conclusion was reached that synergistic false-positive coliform reactions do indeed occur on M-Endo MF medium.

The standard test for the detection of coliform organisms in river water has been the multiple-tube fermentation technique (MPN). It was not until 1957 that the *Standard Methods for the Examination of Water and Wastewater* (1) tentatively accepted the membrane filter technique, which, because of its ease and rapidity in providing results, is now widely used.

A positive coliform test with the membrane filter technique is defined as the production of a dark colony with a metallic sheen within 24 hr incubation at 35 C on M-Endo medium (1). The sheen produced by the coliform organisms in the membrane filter technique is the result of a complex formed by the combination of sulfite and basic fuchsin with the acetaldehyde produced by the metabolic activities of the coliform (4).

The accuracy of the method used in detecting coliforms has been questioned from both the false-negative and false-positive points of view. Melia (M.S. Thesis, Colorado State Univ., Fort Collins, 1965) showed that a high frequency of false-positive reactions occurred in the MPN technique with lauryl sulfate tryptose broth as the test medium. These false-positive reactions were found to be the result of a synergistic interaction of paracolons or *Proteus* sp. with enterococci. Fifield and Schaufus (2) observed that false-positive reactions with the membrane filter technique utilizing the M-Endo MF medium occurred as sheen-producing colonies which are enumerated with the coliform indicator organisms. It has been suggested that the majority of the false-positive reactions of M-Endo MF medium result from the interaction of coexisting bacterial populations. These mixed bacterial cultures may produce an intermediate metabolic substance not produced by the individual organisms. This intermediate could react with the sulfite and basic fuchsin in a manner similar to that of the acetaldehyde and be responsible for the sheen characteristic of the positive coliform test.

The object of this investigation was: (i) to isolate and identify populations of bacteria which produced false-positive sheen reactions on M-Endo MF medium; (ii) to determine whether the false-positive sheen was being produced by individual organisms or by two or more organisms in synergistic interaction; and (iii) to determine what metabolic intermediate(s) may be involved in such false-positive sheen production.

MATERIALS AND METHODS

Test organisms. This investigation was conducted with organisms isolated from water samples collected at various field stations along the upper Cache la Poudre River in Larimer County, Colo. The organisms were isolated with M-Endo MF broth by using the membrane filter technique for total coliforms. All sheen-producing colonies were further tested for the ability to ferment lactose by inoculation into lactose broth. Cultures producing acid and gas from lactose were then checked for purity and transferred to Plate Count Agar (Difco) slants for further study. A typical *Escherichia coli* was used as a positive control, and *Serratia marcescens* was used as a negative control in all biochemical studies.

Media and solutions. The media used throughout this investigation were products of Difco Laboratories, Inc. In addition, a chemically defined minimal medium as described by Wendt (M.S. Thesis, Colorado State Univ., Fort Collins, 1966) was used in the biochemical studies. The medium contained (grams per liter): K_2HPO_4, 7; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1; $NH_4H_2PO_4$, 1; lactose, 10. The medium was adjusted to *p*H 6.8 to 7.2 and sterilized by membrane filtration.

Growth conditions. Growth studies to determine the time to reach the late exponential growth phase were done in screw-cap tubes (16 by 125 mm) containing the chemically defined minimal medium. Incubation was at 35 C in an automatic temperature-controlled shaker incubator (model R26, New Brunswick Scientific Co.). Growth was determined by measuring the increase in turbidity with a Bausch & Lomb Spectronic-20 colorimeter at 420 nm. At the end of the incubation period, each culture was filtered through a 0.5-μm membrane (Millipore Corp., Bedford, Mass.), and the filtrate was collected for characterization of the intermediates.

Analytical procedures. Filtrates obtained from the cultures were collected, and 2,4-dinitrophenylhydrazones were formed and extracted by the procedure developed by Smith (6) and modified by Thayer and Ogg (7). Identification of the neutral carbonyl compounds was made by the method of Thayer and Ogg (7).

The paper chromatography technique of Lynn, Steele, and Staple (5) was used to identify aldehydes and ketones as their 2,4-dinitrophenylhydrazones. Descending chromatography was carried out on Whatman 3MM filter paper.

Identification of hydrazones. The neutral carbonyl compounds which were subjected to paper chromatography were compared with the movement of the hydrazones of known aldehydes and ketones. The individual chromatographic zones containing the hydrazones were then eluted with ethyl acetate, and descending chromatography was repeated on the eluates to determine the purity of the zones. Individual paper strips from the chromatograms were evaluated on the Densitometer model 525 (Photovolt Corp., New York, N.Y.) by plotting distribution curves of the fractions separated on the filter-paper strip.

RESULTS

Colonies suspected of giving false-positive coliform reactions were obtained from M-Endo MF medium and inoculated into 0.5% lactose broth. The cultures producing gas in the lactose broth were streaked on EMB and Endo agars to determine purity. The results showed that 29% (16 of 55) of the sheen-producing colonies for study were mixed colonies. Most of the organisms isolated from the mixed colonies were of the coliform type.

The mixed cultures and individual isolates of the mixtures were analyzed for their ability to reproduce a sheen on M-Endo MF medium. Individual isolates which did not produce a sheen, unless recombined to the original mixed pair, were used to study false-positive coliform reactions and to determine the metabolic intermediates responsible for the false-positive reactions. Individual isolates of the synergistic sheen-producing colonies were further studied for physiological characteristics. Results of the biochemical tests indicated that, although all isolates involved in synergistic sheen production were coliform types, there were no fecal coliforms which would grow at 44.5 C on M-FC medium.

Mixed cultures showing synergistic sheen production were inoculated into the chemically defined minimal medium with lactose as the carbon source and 0.21% sodium sulfite added as a metabolic inhibitor to prevent further metabolism of acetaldehyde. Fermentation was carried out in screw-cap and cotton-plugged tubes (16 by 125 mm) so that the increase in turbidity could be measured directly. Cultures were also grown in cotton-stoppered 125-ml Erlenmeyer flasks containing the chemically defined minimal medium. The results from the three different incubation conditions described above showed no obvious difference in the exponential growth phase. For convenience, screw-cap tubes were used throughout the fermentation studies with incubation for an 18-hr period.

Hydrazones of neutral carbonyl compounds were formed and extracted from the chemically defined minimal medium in which the mixed cultures had been grown. The 2,4-dinitrophenylhydrazones of ketones and aldehydes were identified by chromatography and are listed in Table 1. A summary of the results of the chromatographic separation of the phenylhydrazones of individual isolates and mixed cultures is shown in Table 2.

To determine whether the acetaldehyde produced by the mixed cultures was a result of true synergistic interaction, the following study was conducted. Each of the individual organisms

TABLE 1. *Mobilities of 2,4-dinitrophenylhydrazones of neutral carbonyl compounds isolated*

2,4-Dinitrophenylhydrazones	Avg R_{MEK}[a]	Avg R_M[b]
Glyceraldehyde	0.03	1.62
2,3-Butanedione	0.10	0.95
Formaldehyde	0.27	0.43
Acetaldehyde	0.44	0.07
Propionaldehyde	0.54	−0.05
Butyraldehyde	0.73	−0.40
2-Butanone	1.00	0

[a] R_{MEK} = distance travelled by solute/distance travelled by 2-butanone.
[b] $R_M = \log[(1/R_{MEK}) - 1]$.

TABLE 2. *Phenylhydrazones isolated from individual isolates and mixed cultures*

Culture no.	Carbonyl compounds[a] produced by	
	Individual isolates	Mixtures
1 and 20	A, 2-Butanone B, 2,3-butanedione	Acetaldehyde
4	A, formaldehyde B, formaldehyde	Acetaldehyde
16	A, butyraldehyde B, 2,3-butanedione	Acetaldehyde
40	A, 2,3-butanedione B, 2,3-butanedione 2-butanone	Acetaldehyde 2,3-Butanedione Butyraldehyde
44	A, 2,3-butanedione B, none	Propionaldehyde

[a] Glyceraldehyde produced by all organisms.

making up the mixed culture was grown in the chemically defined minimal medium for 18 hr. The filtrate was then collected, and the second organism making up the mixture was allowed to grow in the filtrate until maximum growth was obtained. The 2,4-dinitrophenylhydrazones formed in culture filtrates were subjected to descending paper chromatography as mentioned earlier.

Acetaldehyde was produced only when the organisms were incubated simultaneously in the medium and not with sequential incubation of the organisms in the medium. This evidence indicates that both individual organisms making up the mixed culture had to be present throughout the fermentation process before the aldehyde was produced and, therefore, the reaction could be considered truly synergistic.

DISCUSSION

The observed metallic sheen on M-Endo medium was reproduced by 38% (6 of 16) of the mixed colonies when incubated in mixed culture, whereas individuals of these mixtures were unable to produce the sheen. This reaction producing the metallic sheen was probably due to a synergistic interaction of the individual organisms making up the mixtures or possibly to a situation whereby one organism supplied metabolic intermediates for the other.

The remaining 62% of mixed colonies were classified into two different categories. One group of mixed cultures had an individual organism capable of reproducing a metallic sheen individually on the M-Endo medium and was shown to be a typical coliform; the other organism was a noncoliform and failed to produce a sheen. The second group of mixed cultures failed to produce a sheen either individually or in mixed culture. Lack of sheen production could have been due to an alteration in metabolism resulting from an environmental change or to catabolic repression. Another possible explanation is that a third organism in the original sheen-producing colony had been lost in the process of determining purity and this third part was necessary for the synergistic sheen production. The mixed cultures which failed to exhibit synergistic sheen production were excluded from further biochemical study.

The results obtained from the paper chromatographic separations suggest that different pathways were in operation in the various mixed cultures. In mixture no. 40, glyceraldehyde, 2,3-butanedione, acetaldehyde, and butyraldehyde were produced. Organism no. 40A produced glyceraldehyde and 2,3-butanedione, and organism no. 40B produced glyceraldehyde, 2,3-butanedione, and 2-butanone. One mechanism by which acetaldehyde could have been formed was by the carboligase reaction. This reaction involves the conversion of the metabolic intermediates 2,3-butanedione and 2-butanone to acetaldehyde (3). Since 2,3-butanedione was isolated from all but one of the remaining mixed cultures, the mechanism mentioned above could also be responsible for the formation of acetaldehyde.

In one of the mixed cultures, propionaldehyde was isolated instead of acetaldehyde. Propionaldehyde was tested with M-Endo medium to determine whether a sheen would be produced; the resultant sheen was less intense than that of acetaldehyde but could be read as a positive coliform colony. It is probable then that propionaldehyde was responsible for false-positive sheen production since no acetaldehyde was produced by this mixture.

The results presented indicate that the acetaldehyde and propionaldehyde isolated from mixed

cultures, but not from the individual organisms making up the mixed cultures, were responsible for false-positive sheen-producing reactions of M-Endo medium. Furthermore, from the facts that both organisms of a mixture had to grow together in the medium for the aldehyde to be produced and that sequential growth in the same medium failed to yield aldehyde, it must be concluded that acetaldehyde and propionaldehyde production which leads to false-positive coliform reaction is indeed a synergistic phenomenon.

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