BIOCHEMICAL CHARACTERISTICS OF PIGMENTED COLIFORM BACTERIA

J. RICHARD GILILLAND AND REESE H. VAUGHN

Division of Fruit Products, University of California, Berkeley, California

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I. INTRODUCTION

Chromogenesis in the coli-aerogenes group of bacteria has been known for a long time, and pigmented strains of coliform bacteria have been studied and described by a great many investigators. Fremlin (1893) in differentiating between *Escherichia coli* and the typhoid bacillus, among other characteristics noted that *Escherichia coli* grew with a yellowish tint on potato whereas the typhoid bacillus was colorless on the same medium. Later, Holliger (1902), Levy (1904), and Burri and Holliger (1909) found yellow pigmented coli-like organisms in fermenting dough. The same type of organisms was also reported by Eisenberg (1918). The studies of McConkey (1909), Wood (1919), and Perry (1929) indicate that pigmented coliform organisms were not infrequently found in their surveys. Rogers, Clark, and Evans (1916) and Rogers, Clark, and Lubs (1918) stated that there was a correlation between pigmentation and other physiological characteristics, and proposed that the chromogenic coliform organisms could be differentiated on the basis of color. However, Rogers and his associates did not continue these investigations, and the idea of classification of these organisms using chromogenesis as a differential character was abandoned.

Recently, Oesterle (1935) described a yellow pigmented organism and proposed the name *Bacterium coli flavum*. Parr (1937) isolated a pigmented coli-like bacterium from a fecal source which he named *Bacterium aurescens*. Five chromogenic strains of coliform organisms were described by Tittsler (1937 and 1939) who considered them pigmented variants of recognized *Escherichia* species.

Yellow pigmented typhoid organisms have also been reported. Dresel and Stickl (1928) reported pigmented variants in this group and named them *Bacterium typhi flavum*. These strains were reviewed by Sonnenschein (1930) who substantiated the previous workers' claim for a new species. Cruickshank (1935) criticized this work and placed *B. typhi flavum* in the genus *Chromobacterium*.

In all the above studies, differentiation was based on qualitative methods for distinguishing the different organisms. To the writers' knowledge no previous attempt has been made to use quantitative biochemical studies for differentiation of the chromogenic coliform bacteria. Results of such a study are presented here.

II. SOURCE OF CULTURES

In connection with the maintenance of a collection of coliform bacteria from olives (West, Gililland, and Vaughn, 1941) stored in the icebox at 0° to 5°C., it was noted that certain cultures displayed a yellowish color. Previously the

collection of cultures had been stored at 30° to 37°C., at which temperature no color appeared, and it was suspected that these pigmented cultures might be contaminated. However, it was established that the organisms were in pure culture and that the temperature of incubation and storage had a marked effect on the production of the pigment. A total of twelve chromogenic strains were obtained from this collection. Ten of these strains had been isolated from water, orchard soil, and sawdust. One strain had been isolated from ripe black olives. Another strain was isolated from fermenting green olives. Chromogenic cultures from other investigators were also obtained for comparison with the organisms from the Fruit Products collection. Dr. L. W. Parr kindly supplied his strain of *Bacterium aurescens*, and a chromogenic strain of *Escherichia* was received from Dr. R. P. Tittsler. A pigmented culture of *Escherichia* from the Delft collection was received from Dr. R. S. Breed.

The above fifteen pigmented cultures were studied, employing qualitative and quantitative methods. The results of these studies are presented in the following pages.

III. EXPERIMENTAL

Characterization of the cultures

All cultures were isolated or re-examined for purity according to the methods used by West, Gililland, and Vaughn (1941). The organisms in all cases were found to be facultatively aerobic, gram-negative, non-sporulating, lactose-fermenting, short rod-shaped bacteria.

A. Differential methods

Differentiation of the bacteria was carried out by the methods of Levine, Epstein and Vaughn (1934) and West, Gililland and Vaughn (1941).

The Voges-Proskauer test was carried out with 48-hour cultures grown in Difco M.R.-V.P. medium at 30°C., by the use of Barritt's (1936) reagents. The methyl-red test was made with a portion of the same cultures by adding a few drops of 0.2 per cent methyl red solution to the medium. The production of indole in tryptophane broth was determined according to Kovacs (1928). Motility was tested in hanging drop preparations and all of the organisms except one were found to be motile. The single non-motile strain (BA) was re-examined for motility by the same method using nutrient broth and nutrient broth containing 0.5 per cent glucose. One series was incubated at 30°C., and the other series at 37°C. Examinations at 12, 18, 24, and 48 hours failed to show motility by this organism. Flagella stains were prepared according to the method of Conn and Wolfe (1938). All motile cultures displayed the peritrichous type of flagellation.

The cultures all fermented glucose and salicin with the production of acid and gas. Starch was not attacked, nor was hydrogen sulfide produced from proteose peptone ferric-citrate agar. Three of the cultures decomposed glycerol with acid and gas production in Durham tubes. The ability to ferment glycerol by

these three cultures was also demonstrated under anaerobic conditions in glass-stoppered bottles.

The differential characteristics of the cultures are shown in table 1. Twelve of the cultures studied are apparently members of the genus *Escherichia*, while three of the cultures belong to the genus *Aerobacter*.

B. Carbon dioxide/hydrogen ratios and 2,3-butyleneglycol production

Harden and his associates (1901, 1905, 1905–1906, 1911, 1911–1912) first showed that fundamental physiological differences may be observed in the me-

TABLE 1

Characteristics of the pigmented strains

CULTURE	SOURCE	COLOR OF PIGMENT	MOTILITY	V.-P.	METHYL RED	CITRATE	INDOLE	GELATIN LIQUEFACTION	SUCROSE	AESCULIN	GLYCEROL	CO_2/H_2 RATIO	2,3-BUTYLENEGLYCOL	ACETYLMETHYLCARBINOL + DIACETYL
E1	orchard soil	straw yellow	+	−	+	−	+	−	−	+	−	1.2	0	0
E2	sawdust	straw yellow	+	−	+	−	+	−	−	+	−	1.2	0	0
E3	sawdust	straw yellow	+	−	+	−	+	−	−	+	−	1.1	0	0
E4	sawdust	amber yellow	+	−	+	−	−	−	+	−	−	1.2	0	0
E5	sawdust	amber yellow	+	−	+	−	−	−	+	−	−	1.1	0	0
E6	sawdust	amber yellow	+	−	+	−	+	−	−	+	−	1.0	0	0
C5	ripe black olives	mustard yellow	+	−	+	−	+	+	+	+	−	1.2	0	0
C7	orchard soil	mustard yellow	+	−	+	−	−	+	+	+	−	1.2	0	0
S2	fermenting green olives	amber yellow	+	−	+	−	+	−	−	−	−	1.1	0	0
220	R. P. Tittsler (*Escherichia*)	cadmium orange	+	−	+	−	+	−	−	+	+	1.3	0	0
Delft	R. S. Breed (*Escherichia*)	cadmium orange	+	−	+	−	+	−	−	−	+	1.6	0	0
BA	L. W. Parr (*Bact. aurescens*)	mustard yellow	−	−	+	−	+	−	−	−	+	1.7	0	0
C1	water	ivory yellow	+	+	−	+	−	+	+	−	−	2.0	+	trace
C2	soil	mustard yellow	+	+	−	+	−	+	+	−	−	2.3	+	trace
A2	soil	amber yellow	+	+	−	+	−	+	+	−	−	2.0	+	trace

tabolism of glucose by the coliform bacteria. These workers reported that *Aerobacter* decomposes glucose with the production of acetylmethylcarbinol and 2,3-butyleneglycol and the evolution of two or more volumes of carbon dioxide to one volume of hydrogen. *Escherichia coli*, on the other hand, does not form significant quantities of acetylmethylcarbinol or 2,3-butyleneglycol and produces carbon dioxide and hydrogen in approximately equal volumes.

In order to substantiate the allocation of the pigmented cultures to the proper genera, quantitative measurements were made of the carbon dioxide/hydrogen ratios and of the production of 2,3-butyleneglycol, acetylmethylcarbinol, and

diacetyl. The cultures were grown anaerobically in a M.R.-V.P. medium consisting of 0.7 per cent proteose-peptone, 0.5 per cent dipotassium phosphate and 0.5 per cent glucose in distilled water.

The carbon dioxide/hydrogen ratios and 2,3-butyleneglycol production of the cultures are shown in table 1. The 2,3-butyleneglycol and carbinol fractions were determined quantitatively on all cultures whether or not previous V.-P. tests indicated that these substances were formed.

On the basis of both gas ratios and the production of 2,3-butyleneglycol, acetylmethylcarbinol and diacetyl, it is apparent that the genera *Escherichia* and *Aerobacter* are both represented among the pigmented strains studied. It may be seen from table 1 that those organisms having a gas ratio of less than two volumes of carbon dioxide to one volume of hydrogen and producing no 2,3-butyleneglycol or acetylmethylcarbinol in the anaerobic decomposition of glucose, were correctly diagnosed as belonging to the genus *Escherichia* purely on the basis of qualitative tests. Those organisms having a gas ratio of two or more volumes of carbon dioxide to one volume of hydrogen and producing quantities of 2,3-butyleneglycol and acetylmethylcarbinol were also correctly allocated to the genus *Aerobacter*.

C. Pigment production

It has been pointed out that the chromogenesis of the cultures obtained from olives was not discovered until the organisms had been stored on agar slopes at low temperatures. This prompted the determination of an optimum temperature for maximum color production. The cultures were grown aerobically on nutrient agar slants containing 0.5 per cent precipitated calcium carbonate and incubated in the dark at 19°, 30° and 37°C. Precipitated calcium carbonate was used to present a white background and to simplify comparisons with color standards. However, pigmentation of the cultures was the same with or without the added calcium carbonate. Examinations were made after 2, 4, 7, 14, 21 and 28 days of incubation. Color of the growth was determined by comparison of the culture slopes with Ridgway's (1912) color standards. In the case of twelve of the cultures, it was found that the incubation at 19°C. resulted in maximum color after 14 days. At the higher temperatures of incubation there was little or no color, although the growth was prolific. On the other hand, strains 220 (Tittsler), BA (Parr) and "Delft" all produced color equally well at 37° and 19°C.

Some investigators have claimed that the addition of certain inorganic salts stimulates pigment production of chromogenic organisms. Kuntze (1900) and Sullivan (1905–1906) showed that magnesium sulfate increases pigment production by *Bacillus prodigiosus*. Karpov and Timakov (1933) demonstrated that 0.5 per cent magnesium chloride has a similar effect on the coloration of a yellow *Bacillus paracoli*. Oesterle (1935) also obtained increased pigment production by *B. coli flavum* with 0.5 per cent magnesium chloride. To test the effect of the addition of magnesium, iron, nickel, copper and manganese salts on color production, the organisms were next grown on slants of nutrient agar with calcium car-

bonate as described earlier, but to which had been added 0.5 per cent magnesium chloride, 0.01 per cent ferric sulfate, 0.01 per cent nickelous nitrate, 0.01 per cent copper sulfate and 0.01 per cent manganese chloride, respectively. Although slight increase in pigment production was observed on media with added magnesium chloride and manganese chloride, it did not seem to be sufficiently marked to warrant further investigation.

The following media were similarly tested for their effect on coloration.

1. 0.3 per cent beef extract + 0.5 per cent proteose-peptone
2. 0.3 per cent beef extract + 0.5 per cent tryptone
3. 0.3 per cent beef extract + 0.5 per cent peptone + 0.5 per cent glucose
4. 0.3 per cent beef extract + 0.5 per cent peptone + 0.5 per cent glycerol
5. fresh beef infusion + 0.5 per cent peptone
6. fresh beef infusion + 0.5 per cent tryptone
7. fresh liver infusion + 1.0 per cent tryptone + 0.25 per cent glucose
8. 0.5 per cent yeast extract (Difco)
9. yeast infusion (adjusted to pH 7.0)
10. potato infusion
11. 5 per cent Bacto-liver extract
12. synthetic glycerol medium (Bunting, 1940)

To all the above media 1.5 per cent agar and 0.5 per cent precipitated calcium carbonate were added. No differences in color production on these various media were noted in any of the cultures on incubation at 19°C. for two weeks. It is interesting to note moreover, that the organisms produced pigment on a purely synthetic medium (no. 12) thereby eliminating the possibility that color production might be due to the absorption of chromatic substances from biological media, or to a reaction of the metabolic products of the organisms with some complex constituents of the media.

The effect of pH on pigment production was limited to the effect of pH on the growth of the organisms. Maximum pigmentation was obtained at pH values optimal for the growth of the bacteria (about pH 7.0).

Table 1 shows the typical colors produced by the organisms when grown on nutrient agar with 0.5 per cent precipitated calcium carbonate for two weeks at 19°C. The cultures 220, BA and "Delft" produce definitely different colors from the other strains. Besides, the intensity of the colors of these strains is decidedly greater for the same amount of growth as measured by visual comparison.

D. Carbon and hydrogen balances

Carbon and hydrogen balances of glucose fermentations were made to determine whether the anaerobic metabolism of these chromogenic strains differed from the metabolism reported in the literature for typical coliform bacteria of the genera *Escherichia* and *Aerobacter*.

The usual analytical methods were used to determine the amount of substrate utilized and the products of fermentation.

Tables 2 and 3 show the carbon and hydrogen balances for fermentations by two of the pigmented organisms used in this study. The hydrogen balances were

TABLE 2

Fermentation of glucose by culture C2 (aerobacter)

Medium: 1.0 per cent glucose, 0.7 per cent proteose-peptone, 0.5 per cent K_2HPO_4, in distilled water. Incubation at 30°C.

SUBSTANCE	MILLIMOLS	PER CENT CARBON	MILLIEQUIVALENTS OF AVAILABLE HYDROGEN	PER CENT AVAILABLE HYDROGEN
Substrate				
glucose	13.7	100.0	329.0	100.0
Products				
hydrogen*	7.9		15.8	4.8
carbon dioxide*	22.4	27.3		
acetic acid	2.2	5.4	17.6	5.4
ethyl alcohol	8.7	21.2	105.0	32.0
lactic acid	4.7	16.7	55.0	16.7
succinic acid	0.2	1.0	3.0	0.9
acetylmethylcarbinol + diacetyl	trace		trace	
2,3-butyleneglycol	6.0	29.0	132.0	40.0
Total		100.6		99.8

* Ratio $\frac{CO_2}{H_2} = 2.8$.

TABLE 3

Fermentation of glucose by culture C7 (escherichia)

Medium: 0.75 per cent glucose, 0.7 per cent proteose-peptone, 0.5 per cent K_2HPO_4, in distilled water. Incubation at 30°C.

SUBSTANCE	MILLIMOLS	PER CENT CARBON	MILLIEQUIVALENTS OF AVAILABLE HYDROGEN	PER CENT AVAILABLE HYDROGEN
Substrate				
glucose	6.3	100.0	151.0	100.0
Products				
hydrogen*	2.9		5.8	3.9
carbon dioxide*	4.4	11.7		
acetic acid	3.4	18.0	27.1	17.9
ethyl alcohol	3.1	16.5	37.3	24.8
lactic acid	4.8	38.4	58.0	39.6
succinic acid	0.6	6.0	8.4	5.5
acetylmethylcarbinol + diacetyl				
2,3-butyleneglycol				
Total		90.6		91.7

* Ratio $\frac{CO_2}{H_2} = 1.5$.

calculated by the method of Barker (1936). There seem to be no fundamental differences between the carbon balances obtained with the two organisms tested and the carbon balances reported by Kay (1926) and Scheffer (1928) for the decomposition of glucose by the genera *Escherichia* and *Aerobacter*. Apparently the fermentations carried out by the two pigmented organisms reported here are in no way different from those effected by typical members of these genera.

IV. DISCUSSION

From the experimental observations on the relation of temperature to chromogenesis in members of the coliform group of bacteria, it would seem probable that these pigmented organisms are more prevalent in nature than has been reported in the literature. In laboratories where collections of coliform bacteria are not stored at low temperatures chromogenesis might easily be overlooked. However, from a taxonomic standpoint it can be seen that the production of a pigment by a coliform organism is of no value in the allocation of that organism to its correct genus. The relation of these yellow pigmented organisms to the peritrichously flagellated, yellow phytopathogenic bacteria, on the other hand, is probably of greater significance. Until these phytopathogenic organisms and other closely related gram-negative chromogenic bacteria have been subjected to quantitative physiological studies and until more information is available on chromogenic coliform bacteria, it does not seem advisable to create new species names for the bacteria under investigation. In the present studies dealing with but a few cultures, two distinct colors were observed, i.e. yellow and cadmium orange. There were several shades of intensity of the yellow pigment which occurred in both the *Escherichia* and *Aerobacter* types. If new specific names were given to these organisms on the basis of pigmentation such names would tend to complicate further the taxonomy of the coliform bacteria. On the other hand, it is conceivable that use of varietal names designating pigment production may be very useful when more is known of the pigmented coliform bacteria and related groups.

V. SUMMARY

1. A collection of fifteen chromogenic coliform organisms has been studied. On the basis of both qualitative and quantitative data, twelve of the organisms were placed in the genus *Escherichia*, and three in the genus *Aerobacter*.

2. Several factors influencing pigmentation were investigated. Incubation at relatively low temperatures was found to be necessary for pigment production by twelve of the cultures; these isolates produced pigment at 19°C. but not at 30 or 37°C. However, three of the cultures were able to produce pigment at 37°C. Pigmentation was observed with organisms grown on a mineral salts, glycerol, agar medium.

3. The anaerobic glucose metabolism of typical strains was studied and compared with fermentations by members of the genera *Escherichia* and *Aerobacter* as reported in the literature. No significant deviations from the typical dissimilations were observed as shown by the carbon-hydrogen balances.

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