Escherichia-Aerobacter Intermediates from Human Feces

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THE phrase, "*Escherichia-Aerobacter* intermediates," has been variously employed in the literature of the past decade. At present it customarily refers to coliform bacteria which differ from typical Escherichia or Aerobacter strains in one or more of the major differential reactions, such as the Voges-Proskauer, methyl-red and citrate utilization tests. Recent discussion has tended to confine the designation to those organisms which are Voges-Proskauer-negative, methyl-red-positive, and capable of multiplying in a medium containing citrate as the sole source of carbon.

Apparently the intermediates are found as ubiquitously as are any of the other coliform bacteria. The first reports of such intermediate bacteria were made by Koser [12-16] who isolated a number of strains from soil, water, and human and animal feces. Lewis and Pittman [21] also obtained intermediates from water, either polluted or unpolluted. Intermediates have been found in feces by Kline,[10] Skinner and Brudnoy,[32] Parr,[22] Shunk,[30] and Tittsler and Sandholzer.[33] The latter authors also studied strains isolated from urine, water and soil. Kline,[10] Kon,[11] and Yale [37] obtained citrate-positive, methyl-red-positive, Voges-Proskauer-negative coliform bacteria from milk and other dairy products. Levine, *et al.*[20] found similar organisms in eggs. Hajna and Perry [9] obtained intermediate strains from the intestinal contents of various cold-blooded animals, and Griffiths and Fuller [8] likewise found intermediates in commercial fish and fillets.

Intermediates have also been reported in water by Ruchhoft, *et al.*,[27] Gray,[7] Poe,[24, 25] France,[6] and Parr and Caldwell.[23] The latter authors found that 8.4 per cent of 1,407 samples of well water contained intermediate coliform bacteria. They stated that in no case where intermediates occurred could the possibility of fecal pollution be excluded. A question which naturally arises concerns the frequency with which intermediates occur in human feces. The present investigation was undertaken as an attempt to provide data upon this point.

EXPERIMENTAL

A. *Isolation of Strains*

Four hundred and sixty-six samples of human feces were plated on eosin-methylene-blue agar after a preliminary 8 hour enrichment in lactose broth. One or more colonies of both Escherichia and Aerobacter types (Levine [19]) were picked and tested for utilization of citrate, and for their reactions in the Voges-Proskauer and methyl-red tests. The latter tests were performed on cultures in Clark and Lubs's [5] synthetic medium which had been incubated for 2 days at 37° C. Voges-Proskauer

tests were made by use of creatin and Leifson's [18] reagent. The Voges-Proskauer and methyl-red tests were later repeated on glucose-phosphate-peptone cultures after 3 days of incubation at 30° C. In this case acetyl methyl carbinol was detected by the Barritt [2] modification of the Voges-Proskauer test, for which a much greater delicacy is claimed. Citrate utilization was determined by growth and formation of alkali on Simmons's [31] citrate agar, and later checked in Koser's [12] synthetic liquid medium, with incubation at 30° C. and 37° C. The reactions of strains reported in this study did not change on replating or on aging.

The distribution of coliform bacteria in the 466 samples of human feces is shown in Table I. Samples are grouped in the table according to the types of coliform bacteria occurring in them. This table cannot be considered an exact index of the occurrence of coliform bacteria in all human feces, due to the fact that not all colonies on each plate were tested: the average number of colonies picked was 5. Therefore it is possible that Escherichia was simply overlooked in the 45 samples in which this type was apparently absent. The failure of growth of coliform bacteria on plates from the 12 samples in Group 8 may have been due to overgrowth by fluorescent bacteria.

It is of interest to note the relatively widespread occurrence of citrate-positive organisms in human feces: in the present investigation 49.8 per cent of the samples contained such strains. This figure is somewhat higher than those reported by many previous workers (see Ruchhoft, *et al.*[27]) and confirms the fallacy of employing the citrate utilization test as an indication of the non-fecal origin of bacteria in water. Furthermore, the presence of intermediate strains in 13.3 per cent of the samples demonstrates that this group may be of sanitary significance.

Table I also shows that from only 1 sample was an intermediate strain isolated in the apparent absence of other coliform bacteria. In this particular case, only 2 colonies were picked, the other later failing to ferment lactose. It may therefore be said that almost without exception the intermediate coliform bacteria are accompanied in human feces by either Escherichia or Aerobacter strains or both, as in Groups 3, 5, and 6. Furthermore, the data show that less than half (47.6 per cent) of the samples studied contained only Escherichia strains.

In several instances analysis of suc-

TABLE I

Occurrence of Coliform Bacteria in Human Feces

	Escherichia	*Aerobacter*	*Intermediate*	*Number of Samples*	*Per cent of Total*
Citrate	—	+	+		
M. R.	+	—	+		
V. P.	—	+	—		
Group					
1	Present	Absent	Absent	222	47.6
2	Present	Present	Absent	152	32.6
3	Present	Present	Present	31	6.7
4	Absent	Present	Absent	18	3.9
5	Present	Absent	Present	16	3.4
6	Absent	Present	Present	14	3.0
7	Absent	Absent	Present	1	0.2
8	Absent	Absent	Absent	12	2.6
Samples containing	421	215	62	466	100.0
Per cent of Total Samples	90.3	46.1	13.3		

cessive samples from one individual indicated that intermediates were always present. In 1 case this has been true for 2 years.

B. *Characterization of Strains*

One hundred and seventeen strains of intermediates, together with 8 strains obtained through the courtesy of Dr. Werkman of Iowa State College and Dr. Tittsler of the University of Rochester, were further studied in an attempt at classification. These were tested repeatedly over a period of 8 months in litmus milk and in various carbohydrate media; hydrogen sulphide production was determined in lead acetate agar and in peptone iron agar, and motility was tested in 18 hour nutrient broth cultures, and in semisolid agar. All cultures were incubated at 37° C. Carbohydrate media were prepared by addition of 0.5 per cent or 1.0 per cent of the carbohydrate to ordinary nutrient broth; the reaction was adjusted to pH 7.0. Carbohydrate cultures were observed for production of acid and gas daily for 2 weeks. Litmus milk cultures were observed daily for 10 days, and lead acetate or peptone iron agar cultures daily for 1 week.

In addition, nutrient gelatin cultures were incubated at room temperature for 30 days and observed periodically for liquefaction by placing in the refrigerator at 5° C. for 2 hours. Indol production in 1 per cent Bacto tryptone cultures was determined after 24 and 48 hours of incubation at 37° C. by use of Kovács's [17] reagent. Reduction of nitrate in a 0.1 per cent KNO_3 nutrient broth medium was determined after 24 hours of incubation at 37° C. with sulfanilic acid and dimethyl-alpha-naphthylamine. Morphology, Gram reaction, and spore formation were studied in 18 to 24 hour nutrient broth cultures.

The characteristics common to all the intermediate strains are as follows:

Gram stain	Negative
Morphology	Rods
Spore formation	Negative
Motility	Positive (4 exceptions)
Citrate utilization	Positive
Methyl-red test	Positive
Voges-Proskauer test	Negative
Gelatin liquefaction	Negative
Indol production	Negative
Nitrate reduction	Positive
Arabinose fermentation	Acid and gas
Cellobiose fermentation	Acid or acid and gas (1 exception)
Galactose fermentation	Acid and gas
Glucose fermentation	Acid and gas
Lactose fermentation	Acid and gas

It is of interest to note that all but 1 of the 125 strains fermented cellobiose with the formation of acid or acid and gas. Tittsler and Sandholzer [34] have recently reported fermentation of this carbohydrate by 38, or 84.4 per cent, of 45 citrate-positive, methyl-red-positive, Voges-Proskauer-negative strains of coliform bacteria.

The heterogeneity of the intermediate strains is shown by Table II. On the basis of 6 reactions, the 125 strains formed 28 groups of 1 to 32 strains each. In the present experiments fermentation of raffinose paralleled closely that of sucrose. Winslow and Walker [36] observed a similar correlation in studying differential tests for the colon group of bacteria.

DISCUSSION

The problem of allocating such strains as these in a system of bacteriological classification is difficult. Bergey [3] distinguished between the Escherichia and Aerobacter genera on the basis of acetyl methyl carbinol production from glucose. However, for the past few years it has been common practice to include the methyl-red test and utilization of the citrate radical as criteria of the genus to which a given coliform bacterium belongs. Braak [4] described several strains of an organism designated *Bacterium freundii,* which

TABLE II

Heterogeneity of Intermediates from Human Feces

Group	Milk*	H_2S	Sucrose†	Salicin†	Dulcitol†	α-Me-Gluc.†	No. of Strains
1	+	+	+	+	+	+	4
2	+	+	+	+	+	—	1
3	+	+	+	+	—	+	1
4	+	+	+	+	—	—	4
5	+	+	+	—	—	—	8
6	+	+	—	+	+	+	8
7	+	+	—	+	+	—	1
8	+	+	—	+	—	+	1
9	+	+	—	+	—	—	1
10	+	—	+	+	+	+	17
11	+	—	+	+	—	+	1
12	+	—	+	+	—	—	5
13	+	—	+	—	—	—	2
14	+	—	—	+	+	+	9
15	+	—	—	+	—	+	2
16	+	—	—	+	—	—	1
17	—	+	+	+	+	+	2
18	—	+	+	+	—	—	10
19	—	+	+	—	+	+	1
20	—	+	+	—	—	+	1
21	—	+	+	—	—	—	7
22	—	+	—	+	+	+	32
23	—	+	—	+	—	+	1
24	—	+	—	+	—	—	1
25	—	—	+	+	—	+	1
26	—	—	+	+	—	—	1
27	—	—	+	—	+	+	1
28	—	—	—	+	+	+	1
							125

* A (+) sign indicates acid coagulation, (—) indicates acid reaction.
† A (+) sign indicates acid or acid and gas, (—) indicates no change.

occupied an intermediate position when classified by these tests, but made no further attempt to classify them. Werkman and Gillen [35] proposed adoption of the genus name Citrobacter, designated primarily to include those organisms which produce trimethylene glycol from glycerol under anaerobic conditions. Secondarily it was stated that all strains studied were citrate and methyl-red-positive and Voges-Proskauer-negative, or only weakly positive.

This suggestion was immediately attacked by Skinner and Brudnoy,[32] who studied 63 intermediate strains isolated from human feces, on the ground that recognition of the genus would pave the way for proposal of innumerable other genera characterized by only minor differences from the established genera of coliform bacteria. It was later stated by Tittsler and Sandholzer [33] that

> . . . the heterogeneity of the *Escherichia-Aerobacter* "intermediates" renders it extremely difficult, if not impossible, to establish a new genus which will separate them from both the Escherichia and Aerobacter genera and yet in itself be sufficiently inclusive.

The above statements are emphasized by the data shown in Table II. Werkman and Gillen, studying 15 strains of trimethylene glycol-producing organisms, described 7 species to be included in the genus Citrobacter. Only 5 of our 125 strains (groups 3 and 4 in Table I) correspond to any of the species suggested by Werkman and

Gillen. These strains resemble *Citrobacter freundii* (fermentation of alpha-methyl glucoside was not reported in the original characterization).

It is therefore evident that a complete classification of the intermediate strains would involve acceptance of very many new species, as well as new genera based on different combinations of the citrate, methyl-red and Voges-Proskauer reactions. At present, however, the major problem appears to be that of proper generic allocation of the intermediates. As mentioned previously, the proposed genus Citrobacter was defined primarily on the basis of trimethylene glycol production from glycerol under anaerobic conditions. The procedure involved in this test is laborious and time consuming, and outside the scope of the ordinary bacteriological laboratory. Furthermore, it is a question whether one such feature is sufficient to justify establishment of a new genus, since to be of practical use a genus should be defined by easily applied tests which represent distinctive physiological characteristics. The genus Citrobacter could therefore be redefined to include all Gram-negative, aerobic, non-sporeforming bacteria which produce acid and gas from dextrose and lactose, and are methyl-red-positive, Voges-Proskauer-negative, and utilize citrate as a source of carbon.

The alternative solution is to include the intermediates in already existing genera. This suggestion is supported by several considerations. In the first place, the genera Escherichia and Aerobacter are distinguished by fundamental differences in their physiology, as shown by the schemes for fermentation of glucose devised by Scheffer,[29] Reynolds,[26] and others. Simple tests (methyl-red and Voges-Proskauer) are available by which these differences can be demonstrated. It has not yet been shown that a fundamental difference exists between the citrate positive and negative Escherichia strains. Production of trimethylene glycol may or may not be evidence of such a difference. However, Schaeffer[28] has shown that 12.9 per cent of 70 citrate-positive, methyl-red-positive, Voges-Proskauer-negative intermediate strains failed to ferment glycerol in a peptone-water medium, so it appears possible that strains may be found which do not produce trimethylene glycol from glycerol. Utilization of citrate may be a fundamental physiological characteristic, but this has not yet been shown. Its correlation with production of acetyl methyl carbinol in the Aerobacter genus may be purely fortuitous.

The intermediates considered in this paper could be classified as Escherichia strains which possess the ability to utilize citrate as a source of carbon. However, there exist strains of intermediate coliform bacteria which are not included in this group. Ruchhoft, *et al.*,[27] Bardsley,[1] and Barritt[2] have described intermediates which utilize citrate, and are methyl-red- and Voges-Proskauer-positive. The first named authors have also described organisms which are citrate positive but methyl-red- and Voges-Proskauer-negative. Therefore it seems logical to include all intermediate coliform strains in the genera Escherichia or Aerobacter as at present defined, at least until such time as they have been shown to possess distinct physiological differences which justify establishment of new genera. Intermediates could then be distinguished from the recognized members of these genera by sub-dividing on the basis of citrate utilization and the methyl-red test.

CONCLUSIONS AND SUMMARY

1. Citrate utilizing coliform bacteria have been found in 49.8 per cent of 466 samples of human feces. Thirteen and three-tenths per cent of the samples contained organisms of the citrate-positive, methyl-red-positive, Voges-Proskauer-negative intermediate group,

thereby demonstrating that the latter are of sanitary significance. Such intermediates do not usually comprise the sole coliform bacterial flora of the human intestine, although they may be found characteristically present in certain individuals.

2. The cultural and physiological characteristics of 125 intermediate strains showed a heterogeneity which rendered impossible any attempt to classify them according to present schemes.

3. It is suggested that the coliform bacteria be classified primarily on the basis of acetyl methyl carbinol production from glucose, and secondarily on utilization of citrate as a sole source of carbon and on the methyl-red reaction. Intermediate strains would then be allocated to the genera Escherichia or Aerobacter, and distinguished from organisms at present included in these genera by their citrate and methyl-red reactions.

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