Constancy of Characters Differentiating Intermediates in the Escherichia-Aerobacter Group and their Interpretation*

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THE choice of bacteriological methods by which organisms are to be classified within the *Escherichia-aerobacter* group offers numerous pitfalls, and whatever methods are chosen the differentiation into sections of the group will not always be sharply defined either in the laboratory or in the field.

The basic tests in most general use are methyl red and Voges-Proskauer tests, utilization of citrate as the sole source of carbon, detection of indol, and ability to grow at 46° C. (Eijkman Test). Three genera are commonly recognized as comprising the group: Escherichia (MR +, VP —, Cit. —, Ind. ±, Eijk. +); Citrobacter (MR +, VP —, Cit. +, Ind. ±, Eijk. ±); and Aerobacter (MR —, VP +, Cit. +, Ind. ±, Eijk. —).

While classification by means of these reactions may be challenged in the case of individual atypical cultures, and while it occasionally offers a result that is not supported by confirmatory tests, yet it seems to give a basic working differentiation of generic variations underlying the group. However, when these criteria for section differentiation are applied there always occurs a certain percentage of organisms that apparently are bona fide members of the group, but yet are intermediate so far as differential characters are concerned.[1, 2]

The constant occurrence of these intermediate forms can be explained in various ways. It may be that—

1. The group is so heterogeneous as not to permit strict classification into sections.
2. The strains in the group readily fluctuate in certain characters so that they vary in different environments.
3. As the bacteria in the group grow in natural conditions they are constantly dissociating into strains showing new reactions.
4. The various cultures showing intermediate reactions may not be pure strains but mixtures of two or more different strains.

It is the purpose of this paper to present certain experimental evidence bearing on these considerations tending to show that the above mentioned characters do not readily fluctuate with either changes in environment or colony morphology (dissociation), and that many of the intermediates are not mixed cultures.

The 6 experiments below, selected from a wide variety of similar experiences, illustrate the results obtained in respect to variation of differential characters.

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1. *Growth in Milk*—Eight various strains of the *Escherichia-aerobacter* group were cultured in milk with daily transfers for 53 consecutive days. Every week they were tested by the various differential tests. Three cultures were MR +, VP —, Cit. —, Ind. +; 1 was the same with the exception of being Ind. —; and 4 were MR —, VP +, Cit. +, Ind. —. No variations in these characters occurred throughout the experiment although 1 culture lost its power to produce gas from lactose between the time of the 14th and 21st transfer and failed to regain this power to the end of the experiment.

2. *Growth in the Presence of Inhibitory Dyes*—In growing these organisms in lactose-peptone brilliant green broth, delayed lag phases frequently occurred.[3] In one such experiment a typical *Aerobacter-aerogenes* (MR —, VP +, Cit. +, Ind. —, Eijk. —) was inoculated in a test series and delayed growth appeared in 2 tubes at 72 hours, in 2 tubes at 96 hours, and in 1 tube at 120 hours. All of these cultures were tested by the differential tests and showed no change.

3. *Growth in Cold Blooded Animals*—While studying effects of salamanders on spring water supplies,[4] several experiments were carried out to ascertain if habitation in the gastro-intestinal tract of the salamander would change the type of *Escherichia-aerobacter* organisms. When salamanders are placed in sterile water and starved they become free from such organisms. Salamanders were placed in large mouthed, one liter, Ehrlenmeyer flasks containing about 100 c.c. of sterile water, and every 24 hours were transferred to similar flasks. After each transfer the water was examined for the presence of the group and the organisms isolated were typed.

In one such experiment a large purple salamander (*Gyrinophilus porphyriticus*) was starved and then fed 1/10 c.c. of a 24 hour broth culture of *Escherichia coli* (MR +, VP—, Cit. —, Ind. —, Eijk. +). Organisms were obtained from the animal for 14 days thereafter and typed. The results were constant with the exception of the indol tests. Indol positive cultures were encountered in 20 of the 36 tubes showing fermentation.

An exactly similar experiment used a frog. This frog after being fed a culture of the same strain of *Escherichia coli* showed organisms for 30 days. These were typed and their reactions were again constant with the exception of indol. Indol producers were encountered in 64 of the 111 cultures.

4. *Growth on Stones Immersed in Water*—As control of the above animal experiments, small stones were soaked in similar cultures and transferred from flask to flask of sterile water in the same manner as the animals. These experiments gave peculiar results in that in some cases the organisms died out very soon, as early as the 8th day, and in others they persisted for very long periods; in 1 case 544 days, when the experiment was discontinued.

In those experiments where the organisms died out rather promptly there was no change in type. In the experiment continued the longest the organism placed in the flask was constant in type for the first 185 days after which several atypical forms appeared.

5. *Growth in Soil*—Lengths of 3 in. stove pipe were filled with soil and carefully sterilized by repeated autoclaving. These were then joined with sterile precautions to form a pipe 20 ft. in length. All joints were taped and shellacked. Copper teats had been previously soldered to the pipe at intervals and rubber tubes and clamps were placed on these to serve as sampling points. Sterile water was introduced at the head of the pipe and permitted to seep slowly through the soil. Samples of this water drawn from all taps over a test period proved to be sterile. An emulsion (approximately a billion organisms in a liter of water) of a carefully purified culture of *Escherichia coli,* recently isolated from human feces was then run into the soil column. Thereafter, sterile water was run through the column at the rate of about 1 gal. each 24 hrs. The organism was recovered from the first tap (about 8 in. of soil) in 3 hrs., after 6 hrs. it was recovered 7 ft. from the inlet, and after 36 hrs. it was uniformly recovered from the entire length of the pipe. Thereafter it was recovered intermittently from all levels of the pipe for a period of 410 days, when the experiment was discontinued. On the 410th day organisms were isolated from the 1st and 4th taps which were exactly the same in all the above characters as the original culture. In all 726 cultures were isolated and typed from this pipe (Chart I).

6. *Effect of Dissociation*—An unstable culture of *Escherichia coli mutabile* (MR +, VP —, Cit. —, Ind. +, Eijk. +) giving rough and smooth colonies was transferred daily, roughs being picked from roughs and smooths from smooths. After 21 days the cultures gave the same reactions showing that no change in these differential characters accompanied a change in colony morphology.

PURIFICATION STUDIES

Three methods are available for the purification of cultures: (a) Repeated

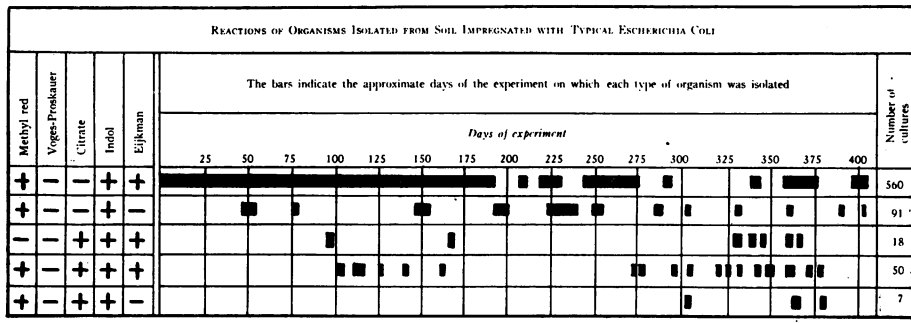
TABLE I
REACTIONS OF CULTURES OBTAINED AFTER PURIFICATION

Types		Methyl Red	Voges-Proskauer	Citrate	Indol	Eijkman	Number Obtained by	
							Selective Enrichment	Single Cell Isolation
Escherichia	Typical	+	—	—	+	+	14	11
	Atypical	+	—	—	—	+	1	..
		+	—	—	+	—	..	2
		+	—	—	—	—	11	..
Citrobacter	Typical	+	—	+	—	—	13	11
	Atypical	+	—	+	—	+	1	6
		+	—	+	+	—	2	6
		+	—	+	+	+	..	6
Aerobacter	Typical	—	+	+	—	—	7	16
	Atypical	—	+	+	+	—	..	1
		—	+	+	—	+	..	4
		—	+	+	+	+	..	1
		Non-members					4	2
		Totals					53	66

TABLE II
TYPES OF ESCHERICHIA-AEROBACTER ORGANISMS ISOLATED FROM NATURAL HABITATS

Source	Escherichia	Citrobacter and Intermediate	Aerobacter	*Source*	Escherichia	Citrobacter and Intermediate	Aerobacter
Human feces	273	29	2	Prepasteurized milk	54	14	89
Bovine feces	47	6	0	Grade "A" raw milk	31	10	45
Sewage	4	0	1	Certified milk	9	2	12
Streams	65	120	71	Pasteurized milk	29	8	13
Wells	34	37	38	Hay and grain	3	4	68
Springs	66	90	52	Pasture soil	23	5	2
Salamanders	37	54	8	Totals	675	379	401

CHART I

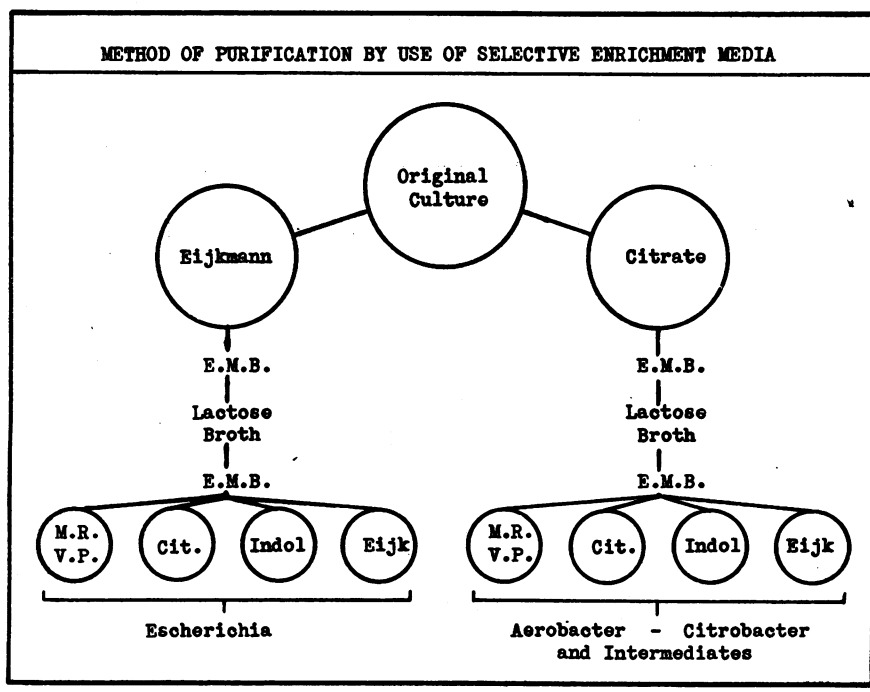


plating of thin emulsions or very early cultures. (b) Plating after growth in selective enrichment media (Citrate media or Eijkman tests). (c) Single cell isolation. In our laboratory all three methods have uniformly failed to isolate only "typical" cultures of *Escherichia-aerobacter*. Intermediates of various kinds were always found. The method used for purification by enrichment is shown in Diagram I. Single cell isolations were performed with the aid of a micromanipulator, picking single cells from minute droplets of emulsions or from thin agar smears of dilute emulsions. The types of organisms recovered after these painstaking purifications are shown in Table I.

OCCURRENCE OF VARIOUS TYPES IN DIFFERENT ENVIRONMENTS

There are many references in literature to show that all types of cultures can be obtained from any natural source if proper enrichment methods

DIAGRAM I



are used.[2] Our experience confirms these general findings and shows that no special type is solely present in any given environment. The various series of cultures isolated from different habitats during our several years experience are divided among the three main divisions of the group, as shown in Table II.

DISCUSSION

The *Escherichia-aerobacter* group of bacteria consists of a few simple generic types and many intermediate forms. These intermediate forms are frequently mixed cultures but even the most painstaking methods of purification fail to reduce the group to a few standard types. All of these are comparatively stable in pure culture and do not fluctuate in regard to basic differential characters in short periods even though their environment is altered, nor do they dissociate to new types although they may show marked changes in colony morphology.

No natural environment is exclusively the abode of any single type of organism and no single type of organism is found exclusively in any natural environment. Some environmental conditions favor the development, and consequent overgrowth, of special types, and some types grow very poorly or not at all under certain environmental conditions. Therefore, mixed cultures will fluctuate with environmental changes through the overgrowth of one or the other component of the mixture.

Tests such as the utilization of citrate and the Eijkman test which have been suggested as differentiating fecal and non-fecal strains of the group do not indicate the *immediate* source of the organisms, since laboratory experiments show that any variations in these reactions were matters of long periods in altered environments.

For practical purposes a very general grouping of the various strains into generic groups will yield all the information that can be utilized in practical field work and exact classification into smaller groups may be left to the research laboratory.

APPLICATION TO PROBLEMS OF WATER ANALYSIS

Since the *immediate* source of the various strains is not indicated by their cultural characters, interpretation of the presence of certain members of the group through such tests or groups of tests on either pure or mixed cultures is apt to lead to erroneous conclusions. However, the proportionate numbers of the various generic groups present in an inoculum may, in the future, justify drawing conclusions concerning the immediate environment through a knowledge of what environmental conditions favor the overgrowth of certain groups.

Interpretation of this knowledge will have to be based on experience, and this experience can only come after present enrichment methods are discarded and new methods developed for the recognition of sections of the group without preliminary enrichment. Future efforts to clarify the meaning of the presence of the group, both regarding laboratory methods and interpretive values, should be in this direction.

SUMMARY

Intermediate cultures of the *Escherichia-aerobacter* group isolated from natural habitats are frequently mixed cultures but careful purification studies including single cell isolations have failed materially to reduce the number of kinds of intermediates.

Strains of the group have been tested for constancy of the methyl red and Voges-Proskauer tests, utilization of citrate as the sole carbon source, detection of indol and ability to grow at 46° C. (Eijkman test). Pure cultures were grown in milk and brilliant green broth, fed to cold blooded animals,

inoculated into soil, and grown on stones immersed in water.

All the above characters were constant during weekly tests when 8 strains were grown in milk and transferred daily for 53 consecutive days. When a salamander and a frog were starved and then fed broth cultures of *Escherichia coli,* cultures recovered daily for 14 and 30 day periods respectively yielded constant characters with the exception of indol formation. Twenty of the 36 cultures (55.5 per cent) recovered from the salamander, and 64 of the 111 cultures (57.6 per cent) recovered from the frog were indol positive.

Sterilized soil which was held in a 3 in. stove pipe 20 ft. in length was inoculated with a carefully purified culture of *Escherichia coli.* Sterile water was permitted to seep slowly through the soil for 410 days. Of 726 cultures isolated at various sampling points during this period, 560 cultures (77.1 per cent) retained the same characters as the original culture (MR +, VP —, Citrate —, Indol +, Eijkman +). The remaining 166 cultures (22.9 per cent) varied in respect to the methyl red, citrate or Eijkman tests, but no variation occurred before the 47th day. The fact that 75 cultures (10.3 per cent) isolated after the 95th day were able to utilize citrate as the sole source of carbon suggests that citrate fermenting forms may be strains of species in the genus Escherichia and that there may be no justification for classifying them in a separate genus (Citrobacter).

While the results of these studies indicate that different environmental conditions result in changes in the characters of members of the Escherichia group, particularly with respect to indol production, the utilization of citrate and the Eijkman test, the complete persistence of the characters which differentiate Escherichia from other types of colon organisms in changed environments in a large percentage of the cultures for long periods of time shows that as an index of fecal pollution, reliance should be placed on the identification of this species rather than to expect these variable tests to indicate the *immediate* environment from which the organism is isolated.

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