ATYPICAL (SLOW) LACTOSE FERMENTING B. COLI

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During studies on infectious diarrhea (Jones and Little (1931)) in cows, motile Gram-negative rods which failed to attack lactose were frequently encountered among the colonies developing on the surfaces of lactose agar plate cultures streaked with fecal suspension. Their numbers varied greatly; at times they made up 90 per cent of the organisms, at others they were present in relatively small numbers and in many cases no such colonies were found. In one instance they were found throughout the small intestine of a cow slaughtered during an attack of diarrhea. At first it seemed to us that we had to deal with organisms of the paratyphoid group but sharp differences in agglutination affinities between these organisms and both types of animal paratyphoid indicated little immunological relationship. In addition, cultural differences were readily manifested.

Many have reported the presence of atypical non-lactose-fermenting *B. coli* in the intestinal and urinary tract of man and in a few instances they have been noted in the feces of horses and cattle. Gilbert and Lion (1893), appear to have been the first to describe Paracolon bacilli. Morgan and Ledingham (1909), encountered such types in human feces and divided them into three groups. On the basis of cultural characters they reported them as numerous in cases of diarrhea but regarded them as of little pathogenic significance. On the other hand, György (1920) suggested that they played a considerable part in diarrhea in both man and calves. Stewart (1926), who studied the Mendelian variation in organisms of this group, recognized that two distinct types existed: (1) paracolon bacilli, which fail to attack lactose

after long periods, and (2) mutable colon bacilli which possess a routine Mendelian variation with respect to the fermentation of such substances as lactose, sucrose and dulcitol. In this respect Stewart broadened the original definition of *B. coli-mutable* which Neisser (1906) and Massini (1907) applied to the organisms whose action on lactose only was variable.

THE SOURCE OF ATYPICAL COLON BACILLI

In our earlier studies on infectious diarrhea of cows we encountered in the feces actively motile organisms which, on the surface of lactose agar plates, failed to attack the carbohydrate. In certain outbreaks all specimens of feces contained them while in others their appearance was irregular and in later epidemics during 1930 and 1931 we failed to find them. In addition to our own isolations, Dr. W. A. Hagan (1925), Professor of Bacteriology at the New York State Veterinary College, Cornell University, supplied us with several strains which he had obtained from intestinal material, pond water, etc., during his study of Braken poisoning of cattle. Furthermore, we examined the feces from 50 normal cows drawn from 5 herds in this vicinity and cultivated atypical colon bacilli once. Another culture was isolated from the urinary tract of a healthy calf. From this it would appear that such organisms are not present in large numbers in the intestinal tract of normal cows although they may be the predominating types during certain intestinal disorders; but, as we have shown, there is little evidence to incriminate them as the etiologic agent in such maladies.

THE CULTURAL CHARACTERS OF THE GROUP

When the surfaces of agar plates, containing brom-cresol-purple, in addition to 1 per cent lactose, are streaked with feces or culture, smooth round translucent colonies of actively motile bacilli develop. The surrounding medium retains its purple color even after incubation or storage in the room for two weeks or more. In our hands, buds or protrusions which ferment lactose have not occurred, although strains may, as will be shown later, under certain conditions, develop both rapid lactose and

slow lactose fermenters. When digest broth containing carbohydrate and indicator (brom-cresol-purple) is inoculated with culture, many of the carbohydrates (table 1) are promptly fermented with acid and gas production. Lactose broth, however, remains alkaline for varying periods. When the cultures were freshly isolated, there was no appreciable change in reaction during the first three or four days of incubation and, in some cases, during the first two or three weeks. One strain isolated from the urine failed to attack lactose, as judged by acid production and

TABLE 1

The biochemical characters of atypical colon bacilli

NUMBER OF STRAINS	GLUCOSE	LACTOSE	SUCROSE	MALTOSE	MANNITOL	XYLOSE	RAFFINOSE	SALICIN	DULCITOL	VOGES-PROSKAUER	INDOL	METHYL RED	LIQUEFACTION OF GELATIN	PRODUCTION OF H_2S
7	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	−	+	+	−	−
1	A.G.	Slow fermentation	A	A.G.	A.G.	A.G.	0	A.G.	A.G.	−	+	+	−	−
1	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	0	−	+	+	−	−
2	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	0	A.G.	−	+	+	−	−
12	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	A.G.	−	+	+	−	−
15	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	0	−	+	+	−	−

gas formation, during thirty days. However, after repeated transfer it now begins to ferment this substance after four or five days. As is brought out in table 1, all strains readily attacked glucose, mannitol, maltose, and xylose, with the production of acid and gas, and failed to ferment raffinose, but fermented lactose slowly. In addition all gave a negative Voges-Proskauer reaction, produced indol, gave a positive methyl-red reaction, and failed to liquify gelatin or produce H_2S. In the fermentation of such substances as sucrose, dulcitol, and salicin, differences were noted as indicated in table 1.

From the data in table 1, it appears that of the 38 strains the majority (27) fell into one group when judged by inability to attack sucrose; this large group could again be subdivided on the basis of dulcitol fermentation since 12 fermented this carbohydrate and 15 failed to do so. Eight of the 11 strains which attacked sucrose fermented both dulcitol and salicin. Of the other 3 sucrose fermenters, 2 fermented dulcitol but not salicin and the other the reverse.

Our cultures, then, could be divided into two groups on the basis of sucrose fermentation. The larger, which failed to attach sucrose, could be subdivided into two subgroups based on the property of attacking dulcitol. The smaller group, all strains of which fermented sucrose, was more complex in that it contained individuals which differed in their behavior in broth containing dulcitol and salicin.

The apparent failure of the organisms to utilize lactose when grown on the surface of lactose agar plates aroused our interest. Several facts were observed in this connection. The cultures grew well on the lactose plates. When true *B. coli* was seeded on the same plate with atypical strains, it readily attacked the sugar. The results then could not be attributed to poor growth or alteration of the carbohydrate. Further, the odor of the culture was different. *B. coli* was characterized by a sour odor, while the atypical type produced a strongly disagreeable ammonical one. When Durham tubes containing 1 per cent lactose are seeded with culture even after a year or more of cultivation, growth begins promptly but the indicator remains unchanged for two or more days. Acidity is first noted at the bottom of the tube and the reaction gradually changes, accompanied by a moderate gas production. Ten days or more may be required to change the color of the indicator completely. When lactose agar plates are seeded and incubated anaerobically the result is the same as when the plates are incubated in the air; the reaction of the medium is unchanged. Furthermore when certain strains of true *B. coli* of bovine origin are grown on the surface of lactose agar plates the medium is promptly acidulated. If, however, growth is artificially checked by a sharp reduction of temperature

the medium becomes alkaline after a few hours, but the onset of optimum growth conditions will again inaugurate acid production. There exist then among the true colon bacilli races which under optimum conditions readily attack lactose with the formation of acid, but during retardation of growth produce sufficient alkali to shift the pH toward the alkaline side.

That perhaps there existed in our cultures of atypical colon bacilli some such balance between carbohydrate attack and alkali production seemed plausible and the following observation was designed to test this possibility.

Sugar free digest broth, pH 7.2, was distributed in amounts of 50 cc. into 100 cc. flasks. Sufficient 20 per cent lactose was added to make a concentration of 2 per cent. The flasks were inoculated in pairs with 0.2 cc. of an eighteen-hour broth culture of the organism. Half of the flasks were incubated at 38°C., and half at 22°C. The series included in table 2 contained 5 cultures of the slow lactose fermenting organisms, 1 *B. coli* which, when once it had acidulated the medium of a lactose agar plate culture, never became alkaline, and another which readily produced acid under optimum growth conditions but during retardation produced alkali. In addition the sterile medium was held for control purposes. After two-and four-day intervals the amount of lactose was quantitatively determined by means of Benedict's solution. The hydrogen ion concentration was determined and the effect of the addition of formol on pH was recorded. The results are recorded in table 2.

These observations were repeated on 3 occasions with similar results. From the data in table 2, several points are readily recognized. First, that the atypical colon bacilli utilize lactose from the start. It is true that at 38°C. the utilization is not so marked as that of typical colon types. On the other hand the amount of lactose utilized at 22°C. is comparable with that consumed by *B. coli* under the same condition. Another significant point is the effect of lactose consumption on pH. This is best illustrated in the titrations after four days of growth at 22°C. In the case of the atypical cultures, the utilization of lactose is comparable to that of colon bacilli. Nevertheless, the acidity is

relatively low, varying between pH 6.8 and 7.4, whereas under the same conditions *B. coli*, while consuming no more sugar, has shifted the pH to 5.2 in one instance and 4.4 in the other. The addition of the same quantity of neutral formol to all cultures likewise reveals sharp differences. When added to the *B. coli* cultures it either had no effect (i.e., failed to reduce the final pH of 4.4) or depressed it to 4.7. The same treatment of the atypical culture revealed about the same buffer content as that possessed by the original medium, as indicated by a shift to pH 5.9. In

TABLE 2

The utilization of lactose and acid production by various cultures

CULTURE	2 DAYS						4 DAYS					
	38°C.			22°C.			38°C.			22°C.		
	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol	Lactose utilized	pH	pH after addition of formol
	mgm.			*mgm.*			*mgm.*			*mgm.*		
Atypical 3497-K	6.6	6.0	5.0	7.0	7.0	6.8	11.5	6.4	5.2	8.0	7.4	5.9
Atypical A-74	9.0	7.8	6.0	8.4	7.4	5.8	9.5	5.9	5.4	9.3	6.8	6.0
Atypical 182	7.7	7.2	6.0	9.7	7.0	5.8	9.0	7.8	5.9	10.0	7.4	5.8
Atypical 894	7.4	7.0	5.6	7.0	7.0	5.8	9.0	6.8	5.4	10.9	6.8	5.9
Atypical brom 5-7	7.6	6.9	5.9	6.8	7.4	5.9	8.2	5.6	4.4	11.0	7.0	7.0
B. coli S H 2	10.5	4.4	4.4	10.0	5.0	4.4	12.8	4.4	4.4	11.0	4.4	4.4
B. coli 1736*	9.8	5.0	4.4	5.7	5.6	5.0	12.5	4.4	4.4	8.0	5.2	4.7
Uninoculated media		7.4	5.9								7.4	5.9

* Acid during rapid growth on lactose agar plate but alkali when retarded.

the instance of culture brom 5-7, it required the addition of twice the amount of formol to produce this result.

There exists, then, experimental evidence that the atypical colon bacilli possess the property of utilizing lactose but at the same time produce alkali, for a time at least, in sufficient quantities to mask the acidity of fermentation.

THE IMMUNOLOGICAL PROPERTIES OF ATYPICAL COLON BACILLI

Rabbits were immunized with several strains of typical colon bacilli and with the atypical strains. In general, little relation-

ship existed between individual strains even when drawn from the same source, as illustrated by the results recorded in table 3. The serum was prepared by immunizing a rabbit with strain 182-II and titred against other cultures identical in cultural characters and from the same plate culture.

All cultures were tested against various sera of both the typical and atypical types drawn from the same sources but no definite grouping could be established.

Stewart (1926) commented on the fact that the cultures with which he worked possessed the property of acquiring certain fermentative characteristics. This was established by observ-

TABLE 3

Illustrating the heterogenous agglutination affinities of atypical B. coli cultures obtained from the same material

CULTURE	SERUM DILUTIONS									
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	C
Homologous 182 II	C	C	C	++++	+++	+++	++	++	+	−
182 II-0	++	+	±	−	−	−	−	−	−	−
182 II-I	C	C	+++	++	+	−	−	−	−	−
182 II-02	+	++	+	+	+	+	±	±	±	−
182 III	C	C	++	+	+	+	+	±	±	−
182 IV	++	+	−	−	−	−	−	−	−	−
182 II 40	+	++	−	−	−	−	−	−	−	−

ing colonies which produced buds along their borders. The organisms of the buds frequently acquired the property of fermenting the carbohydrate, an attribute not possessed by the bacteria of the original culture. This has not been true with our cultures. When buds have been formed they behave toward the environment (lactose) as did the parent colony. Many cultures were rapidly passed through lactose broth, and of these a number developed two types of colonies. One type produced acid when growing on the surface of lactose agar plates and the other behaved in all respects like the atypical culture. Certain of our strains then possessed two elements both capable of fermenting lactose but one presumably producing sufficient alkali to mask acidity and the other apparently lacking this property.

On the other hand, once a prompt lactose fermenting type had been obtained from an atypical strain this character became fixed and rapid transfers through media containing no sugar, or rich in blood serum, never produced a reversion to the atypical form.

There were then two components in some of our strains. One, which when cultivated in a proper environment, was a true colon bacillus; the other in which the dominant factor was the atypical type.

It seemed of interest to show whether or not such a change had altered the antigenic nature of any of the forms. For this purpose 2 series of cultures were chosen, but only 1 series of experiments is reported in detail. The parent types consisted of subcultures carried on in the usual manner, the others were selected after rapid passage through broth containing lactose; those that promptly attacked lactose after several passages are referred to as Y, and those that still failed to do so under these conditions, as B. Individual rabbits were immunized with parent A, Y and B strains and the various sera tested with all the strains. Each serum was tested with both homologous and heterologous cultures. The initial titers of the various sera produced from strain 1306 are given in table 4.

The parent strain and the others possess certain antigenic properties in common as judged by agglutination. The slow lactose-fermenting B type agglutinates much better in all sera than any of the others. The character of the clumps differed sharply. B always produced the fluffy type of flocculation and the others the granular type. For a clearer understanding of these differences further experiments were necessary.

The first step consisted in the usual absorption tests. Sufficient of the heterologous strain was added to reduce greatly the agglutinin for the homologous strain. The results of this procedure are shown in table 5.

On the whole the results were unexpected. The parent strain A and the rapid lactose-fermenting type Y behave the same, each absorbing agglutinin for the other. The results with the B type, which utilized lactose slowly, appear to suggest that a new antigenic entity appeared during the course of selection, but certain

TABLE 4

The antigenic affinities of the parent (A), slow lactose fermenting (B), and prompt lactose fermenting (Y) strains

CULTURE	ANTISERUM A (PARENT)							ANTISERUM B (SLOW LACTOSE FERMENTING)							ANTISERUM Y (PROMPT LACTOSE FERMENTING)							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	C
A	C	C	++++	++	+	+	+	++++	+++	+	+	+	+	+	C	C	++++	++	+	+	+	−
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	−
Y	C	C	++++	++	+	+	±	++++	+++	+	+	+	+	+	C	C	++++	+	+	+	+	−

TABLE 5

The effect of absorption of all sera with usual amounts of culture

CULTURE	ABSORBED WITH PARENT STRAIN A							ABSORBED WITH STRAIN B (SLOW ACID PRODUCTION IN LACTOSE)							ABSORBED WITH STRAIN Y (RAPID ACID PRODUCTION IN LACTOSE)						
	Dilution of serum																				
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Serum 1306-A (parent)																					
1306-A	+	+	+	+	+	+	±	±	±	±	±	±	±	±	+	+	+	+	+	±	±
1306-B	C	C	C	C	C	C	C	++++	++++	++	+	+	+	+	C	C	C	C	++++	++++	++++
1306-Y	±	±	±	±	−	−	−	±	±	±	±	−	−	−	±	±	±	±	±	−	−
Serum 1306-B																					
1306-A	±	±	±	±	−	−	−	±	±	±	±	−	−	−	±	±	±	±	−	−	−
1306-B	C	C	C	C	++++	++++	++++	++++	+++	+	+	+	+	+	C	C	C	++++	++++	++++	++++
1306-Y	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Serum 1306-Y																					
1306-A	+	+	+	+	+	+	±	+	+	+	+	+	+	±	+	+	+	+	+	+	+
1306-B	C	C	++++	+++	+	+	+	C	C	++++	+++	+	+	+	C	C	C	C	C	C	++++
1306-Y	±	±	±	−	−	−	−	±	±	±	±	±	−	−	±	±	±	±	±	−	−

data belie this. It is recorded in table 4 that the antiserum produced by both the A (parent) and Y strain agglutinated the B culture better than the homologous strains. Therefore, both A and Y must have contained sufficient antigens similar to B to produce B antibody. That both A and Y strains possessed relatively little of such antigen can be argued from the fact that even when sufficient A and Y culture was used to reduce greatly the agglutinin for these strains the agglutinin for B remained relatively unaffected. Culture B seemed to possess the property of absorbing agglutinin for both A and Y.

TABLE 6

The effect of massive absorption of serum 1306 upon the agglutinin

SERUM	STRAIN	SERUM DILUTIONS						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Unabsorbed serum	A	C	C	++++	++	+	+	+
	B	C	C	C	C	C	C	C
	Y	C	C	++++	++	+	+	+
Absorbed with massive quantities of strain A	A	++	+	±	±	+	+	+
	B	++	+	+	+	+	+	+
	Y	++	+	+	+	+	+	+
Absorbed with massive quantities of strain Y	A	++	++	++	+	++	++	+
	B	++	++	++	+	++	++	+
	Y	++	++	++	++	++	++	+

If, then, strains A and Y possessed the dominant antigen for B in small quantities only it should be possible to remove agglutinin from these antisera provided large enough quantities of culture were used for absorption. This proved to be the case, as bought out in table 6.

Antiserum A was diluted 1:10 with NaCl and was repeatedly absorbed with massive quantities of either A or Y strains. As an instance, the A serum was absorbed on 6 occasions with all the bacteria obtained from the surface of 9 Blake bottles of culture Y. Another portion of A serum was absorbed with large quantities

of homologous culture. The effects of such absorption on the agglutinins for B are shown in table 6.

It is evident then that the parent A strain and the active lactose fermenting Y type actually possess B antigen in sufficient quantity to produce antibody. The quantity is so small as hardly to affect the agglutination titer when the serum is absorbed by moderate quantities of A and Y cultures, but when large numbers of these organisms are employed for absorption the B agglutinin is removed. The apparent antigenic difference then is quantitative and it appears that A and Y possess similar antigens to B but in B the antigens which produce the fluffy agglutination are present in greater quantities.

In another less complete experiment both the Y and B strains developed similar antigenic qualities and agglutinated better in all sera than the parent strain.

DISCUSSION

We have described certain characteristics of a type of colon bacilli encountered in many cases of infectious diarrhea in cows. In other papers we have shown that these organisms, although frequently predominating in the feces and readily obtained from various portions of the intestinal tract of cows suffering from diarrhea, cannot be considered as the cause of the disease. Our experiments indicated that when fed to calves the organisms, either as pure cultures or fecal suspensions failed to establish themselves in the intestinal tract. In three instances cases were followed from early in the course of the disease until recovery. At the height of the attack the organisms in question were present in the feces in large numbers, during convalescence their numbers diminished, and they could no longer be found when health was restored.

From their appearance on lactose agar plates these organisms may readily be mistaken for paratyphoid bacilli especially when freshly isolated and cultivated in glucose, lactose, and sucrose. The relative tardiness with which they ferment lactose is at first deceptive although they are readily differentiated from the paratyphoids by agglutination and more complete cultural study.

We prefer to designate the organisms with which we worked as atypical colon bacilli in contra-distinction to the mutable and para types of Stewart. Our organisms invariably attacked lactose in contrast to the para type of Stewart and differed from the mutable type since it was not possible to show that they acquired new fermentative properties. On the basis of our data it seems preferable not to regard them as true mutants since we were unable to show that new characters had been developed or original properties had been lost. We have called attention to certain races of colon bacilli which readily attack lactose under optimum growth conditions and as readily produce alkali when such conditions are abruptly changed. There exists naturally a race of *B. coli* which utilizes carbohydrate under certain conditions and presumably attacks nitrogenous matter under different conditions. Our strains possess both properties. Although they utilize the carbohydrate (lactose) a little more slowly than do true *B. coli*, nevertheless during this phase they form sufficient alkali, probably ammonia, to stabilize the reaction of the media, and hence the indicator retains its original color. By rapidly passing certain strains through lactose it has been possible to obtain 2 types, one of which attacks lactose with ordinary rapidity and is essentially a true colon type and the other, which is the atypical organism. The former has not reverted to the atypical form in our hands. If, then, the atypical organism is one which differs from the typical quantitively in its ability to produce more alkali and perhaps to utilize a little less lactose, its appearance in the intestine in large numbers under abnormal conditions might be explained on the grounds of a more favorable environment during illness which facilitates the growth of this type. The lack of proper conditions might explain the apparent rarity of such organisms during health and their disappearance from the feces with a return to normal.

Certain of the immunological findings bear out the contention that members of the group differ only in degree, rather than constitutionally. The experiments reported for culture 1306 strongly support this view. Here, the parent strain and one of the derivatives which readily attacked lactose possessed identical

agglutination affinities. The slowly fermenting lactose culture apparently possessed a different antigenic complex. Further experiments, however, indicated that apparent differences could be explained on quantitative grounds, so that in reality there were no appreciable antigenic differences.

The organisms are of interest to those concerned in the matters of public health since they may readily gain access to milk from the feces and at first be mistaken for paratyphoid bacilli.

SUMMARY

This paper deals with the cultural characteristics of atypical colon bacilli frequently encountered in the feces of cows suffering from intestinal disorders. In lactose agar plate cultures the organisms fail to change the reaction of the media and for this reason they may be mistaken for paratyphoid bacilli. It has been established that in broth containing lactose there is a phase in which the carbohydrate is utilized without changing the reaction of the medium, followed by a phase in which acid is slowly produced. Such organisms are not regarded as true mutants of *B. coli* since we were able to show that no new qualitative differences had developed but that apparent cultural and antigenic differences could be explained on quantitative grounds.

REFERENCES

GILBERT, A., AND LION, G. 1893 La Semaine Méd., **13,** 130.
GYÖRGY, P. 1920 Centralbl. Bakt., Abt. I, Orig., **84,** 321.
HAGAN, W. A. 1925 Cornell Vet., **15,** 326.
JONES, F. S., AND LITTLE, R. B. 1931 Jour. Exp. Med., **53,** 835.
MASSINI, R. 1907 Arch. f. Hyg., **61,** 250.
MORGAN, H. DER., AND LEDINGHAM, J. C. G. 1909 Proc. Roy. Soc. Med., **2,** Part II, Epidemiol. Sect., p. 133.
NEISSER, I. M. 1906 Centralbl. Bakt., Abt. I, Ref., **38,** Beiheft, p. 98.
STEWART, F. H. 1926 Brit. Jour. Hyg., **25,** 237.