A STUDY OF *BACILLUS ALBOLACTIS*

by

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PART I

A STUDY OF [illegible] IN A LOCAL [illegible].

STATEMENT OF THE PROBLEM

During a period of observation over several years, milk from a creamery in the vicinity of Blacksburg, Virginia, has frequently shown an abnormal fermentation in the form of rapid acid liquefaction of the curd. Previous work has indicated *Bacillus albolactis* as the cause (21,22).

The following investigation was made of the milk supply in an attempt to determine the prevalence and action of *B. albolactis*, as well as factors influencing its appearance and increase.

HISTORY AND GENERAL ASPECTS OF THE PROBLEM

Milk, the normal secretion of the mammary glands, is one of the most complete food preparations elaborated in nature. Among its more important constituents are lactose, glycerides of oleic, palmitic, myristic, and butyric acids, casein, lactalbumin and lactoglobulin, and traces of other organic and inorganic compounds, such as citrates and phosphates (19). It is amphoteric in nature and nearly neutral, with a normal p H range of 6.5 to 7.2. Thus, as milk leaves the cow's udder it is a very satisfactory medium for the growth of many bacterial species, and in consequence it will undergo many changes. The processes producing these changes are termed milk fermentations, and these may be considered as normal or abnormal depending mainly on the regularity of their occurence (11).

Normal fermentations of milk.

The commonest change occurring in milk is the decomposition of lactose to lactic acid, with a consequent coagulation of the casein. This souring of milk is regularly found under usual conditions, and is called the normal fermentation of milk (11).

Other changes such as the production of unusual flavors and odors, ropiness, sweet curdling, liquefaction of the curd, gas production, etc., constitute the abnormal fermentations (11,15,23).

Originally believed to be due to something in the casein, souring of milk was proved to be caused by microorganisms by the work of Pasteur in 1857, when he demonstrated the existence of organisms capable of producing lactic acid, and of Lister, who isolated one of these forms in 1878. (11).

The main organism involved in the normal fermentation of milk is Streptococcus lactis. The type species rapidly produces only lactic acid from lactose and, since this has no flavor or odor, gives what is called a "clean sour" milk in the dairy industry. It produces up to one percent acidity, or more, in milk, but its growth is somewhat inhibited above this and the Lactobacillus group begin to grow up, and to produce acidities of over two and one half percent. Although their growth is a normal occurrence, and desirable in many cheeses, it is considered undesirable in butter manufacture. The main organisms of this group are Lactobacillus casei, L. bulgaricus, and L. acidophilus. They produce principally lactic acid from lactose. (11,19).

Abnormal fermentations.

There are many types of abnormal fermentations, and these may be caused by a single species, or groups of microorganisms.

Escherichia coli and Aerobacter aerogenes are two of the commoner causes of gassy fermentations and off-flavors and odors.

They are almost always found in raw milk, and come from dust and feces. Certain members of the genus Clostridium, the anaerobic spore-formers, and some non-sporeforming yeasts, (Torulae), also produce this abnormal type of fermentation. (11,12).

The genus Pseudomonas, members of which produces a water-soluble blue pigment, is responsible for blue milk, while members of the genus Serratia produce "bloody milk". (11,12). Ropy or slimy milk may be caused by Alcaligenes viscosus, or highly capsulated strains of Aerobacter aerogenes, or S. lactis var. hollandicus, among others. (11,17). Sweet curdling, i.e. coagulation of milk by the production of the enzyme rennet by bacteria, rather than by acid production, is encountered mainly in warm weather and when milk must be held for considerable periods after pasteurization. (11). The causative agents are generally members of the aerobic, spore-forming group, Bacillus, e.g. B. cereus, B. subtilis, B. coagulans, etc. (15); although members of the genus Proteus (11), and various yeasts and molds may cause this trouble. (23).

Sources of contamination.

Milk, as drawn from the udder, normally contains bacteria. Counted by the plate method the average is about 500 organisms per cc., though counts have been reported of over 100,000 per cc. Staphylocci and micrococci are the commonest organisms found. (11,23).

From then on everything the milk comes into contact with serves as a further source of contamination: air, the animal coat, the milker, and probably the most important source of bacteria in milk, all the dairy utensils such as milking machines, coolers, milk cans, etc. (11).

Importance of *Streptococcus lactis*.

Streptococcus lactis, previously mentioned as one of the commonest milk organisms, is undoubtedly one of the most important for two reasons. It produces only lactic acid in milk, and so gives a "clean sour" milk that may be used in all types of dairy manufacture; and it is a very rapid growing organism, and the acid it produces, though at first insufficient to taste, will inhibit the growth of other organisms. (11,23).

It is this latter factor which is of the utmost importance in keeping down the growth of abnormal fermenters, particularly when their relative numbers are low. Organisms causing slimy milk, for example, or those such as *Alcaligenes fecalis*, and certain *Micrococci* causing bitter milk, are relatively acid-intolerant and will be held down by the acid production of *S. lactis*. (20). Members of the coli-aerogenes group, if not present in too great a proportion, will also be inhibited by lactic acid production. (11).

However, in certain abnormal fermentations, the rapid growth and acid production of *S. lactis* will not be inhibitory (5); or

certain unsound dairy practices such as overpasteurization may so lower their numbers, or increase the relative numbers of other hardier organisms that the action of S. lactis will be practically worthless. (12,20).

The Importance of Bacillus albolactis.

The type of abnormal fermentation with which this investigation has been concerned is described as acid liquefaction of the curd. In this, there is a rapid production of acid from lactose resulting in coagulation of the casein, i.e. curd formation. This is immediately followed by proteolysis or liquefaction, in which casein, or its insoluble derivative, is broken down to water soluble compounds. This is generally accompanied by bitter flavor developing in the milk, as well as bad odors, due to some breakdown products of the casein, and possibly from lactose as well. (11,23). Streptococcus liquefaciens, Bacillus laterosporus, Bacillus albolactis, and certain members of the genus Clostridium are the organisms commonly responsible for this abnormal fermentation, (11,15), as well as certain Sarcina and Torulae in associative growth. (11,17,18). Previous work (21,22) has indicated Bacillus albolactis as the cause of this particular abnormal condition in the milk supply being studied here.

In 1904, Flugge, Ford, and Pryor pointed out that milk heated to temperatures above 60° C. showed excessive development of spore-bearing bacteria, ordinarily inhibited by the lactic-acid-producing bacteria present in milk. (23).

Loeffler in 1887 first noted an organism in boiled milk which acidified, coagulated, and peptonized it, and he proposed the name _Bacillus lactis albus_ for it. This was changed by Lawrence and Ford in 1916 to _Bacillus albolactus_ Migula (15), although the following year Conn claimed that this organism was merely a lactose-fermenting variety of _Bacillus cereus_. (4). In the first four editions of "Bergey's Manual of Determinative Bacteriology" this organism was listed as a separate species, _B. albolactis_ Migula. (1). In the fifth section, however, this has been changed to _Bacillus cereus var. albolactis_ Migula. (2). Soriano also lists the organism as a variety of _B. cereus_. (24).

As synonyms for _B. albolactis_ Lawrence and Ford list _Bacillus lactis albus_ Loeffler (as does Bergey (1)), _B. teres_ Neide, _B. corragatus_ Migula, _B. lactis No. II_ Flugge, and _B. bernensis_ Lehmann and Neumann. (15).

They were the first to indicate the importance of _Bacillus albolactis_ in the liquefaction of pasteurized milk. They also mention it as a contributary factor to bitter milk occasionally obtained when pasteurization is carried on between 60° and 65° C. (15). The organism has also been widely reported in soil (4,24), which Bergey's Manual lists as its habitat. (2).

METHOD OF PROCEDURE

Fresh samples of both raw and pasteurized milk were collected from the dairy over a period of two and a half months. Milk was brought to the creamery from two different sources, and these are treated differently, and so were kept separate in the analyses made. Milk from source (A) is pasteurized and bottled, while that from source (B) is pasteurized and put into milk cans for use in a large eating establishment.

In both cases the milk is brought in from the farms in large milk cans, poured into a weighing vat and then conveyed to a holding vat. From there it is passed through a preheater into the pasteurizer, where it is heated to 144° F (62.2° C) and held for thirty to thirty five minutes, and then held for another ten minutes at 135° F (57.2° C) before being passed through the cooler. Milk (A) is then put into a bottling machine, and bottled in quart, pint, and half pint quantities, while milk (B) is passed directly from the cooler into milk cans.

As far as possible samples from either source were taken both before and after pasteurization, and on the average of once a week the samples were taken from various points along the line. Sampling was done in accordance with the methods outlined in "Standard Methods for the Examination of Dairy Products". (26). Samples were held at 10° C in no case for longer than two hours, and all dilutions were made at least in duplicate.

Milk was plated in a routine manner using standard tryptone-glucose-extract agar (26) at a pH of 6.8 to 7.0. Where dilutions greater than 1:10 were made skim milk, and not the powder, was used.

Total counts of the organisms were made, and after a study of the colonial characteristics of *Bacillus albolactis* had been made, the numbers of this organism were estimated from the colonies on the plates, percentage of the total flora calculated. One hundred cubic centimeter portions of each sample were incubated at room temperature (21° to 24° C.) and at 37° C. and examined in twenty-four and forty-eight hours, and later if necessary, to determine the type of fermentation the milk would undergo.

Representative colonies were isolated from time to time, and the action on litmus milk noted, to serve as a check on the percentage counts, and also to determine the types of organisms present.

RESULTS

Plates with less than seventy colonies were not included in these results because it was felt that in such cases the approximate count of B. albolactis would be too inaccurate. On the other hand plates with over three hundred and fifty colonies were not used because of possible inaccuracies of the count.

Tables 1 through 6 are the results of samples taken from both sources, A and B, both raw and pasteurized, and from various points in the line. Counts were made at least in duplicate, and the average recorded. Dashes in the observations on the type of fermentation indicate no change from the preceding observation. Slight liquefaction means that less than a tenth of the one hundred cc. sample was whey.

In Table 7 a comparison is made between raw and pasteurized samples of milk from source A, types of fermentations and the per cent increases of Bacillus albolactis noted, as well as the decrease in total numbers of organisms. Table 8 does the same thing with milk from source B.

Table 9 shows the relation of type of fermentation to the percentage of Bacillus albolactis in the sample. Cases showing slight liquefaction were omitted, while those samples that gave no curd (and no liquefaction) were included under "no liquefaction". The percentages of B. albolactis were rounded out to the nearest whole number.

Table 1-- Source A--Raw milk from milk cans

Average total organisms per cc.	Average *B. albolactis* per cc.	% *B. albolactis* per cc.	37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
520,000	30,000	5.7	gassy curd	----	curd	----
118,000	6,000	5.1	gassy curd	----	curd	----
215,000	32,000	15.0	gassy curd	slight liquefaction	curd	----
201,500	35,000	17.4	gassy curd, slight liquefaction	----	gassy curd	----
176,000	19,000	10.35	gassy curd	----	gassy curd	----
236,000	10,000	4.2	gassy curd	----	curd	----

Table 2-- Source A--Raw milk from holding vat

Average total organisms per cc.	Average *B. albolactis* per cc.	% *B. albolactis* per cc.	37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
110,500	12,500	10.5	curd	slight liquefaction	gassy curd	----
185,500	20,500	11.35	gassy curd	----	gassy curd	----
71,500	15,000	20.95	gassy curd	----	gassy curd	----
32,500	5,500	17.0	gassy curd	----	curd	----
206,500	13,500	7.0	gassy curd	slight liquefaction	No change	slight liquefaction
135,000	14,000	10.35	gassy curd	1/3 liquefied	gassy curd	----

Table 3— Source A—Pasteurized milk from the cooler

Average total organisms per cc.	Average *B. albolactis* per cc.	% *B. albolactis*	37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
65,500	1,140	17.3	gassy curd, slight liquefaction	complete liquefaction	No change	curd and liquefaction
72,000	1,050	13.3	gassy curd, 1/3 liquefied	----	curd	----
53,000	11,000	20.5	curd, 3/4 liquefied	complete liquefaction	No change	curd
700	100	14.6	liquefied	----	No change	No change
4,500	3,750	81.4	1/2 liquefied, curd	----	curd, 1/4 liquefied	----
15,800	12,450	78.75	curd, 2/3 liquefied	complete liquefaction	curd, slight liquefaction	1/2 liquefied
25,000	3,200	12.8	curd	1/4 liquefied	curd	----
12,400	1,950	16.4	curd, 1/2 liquefied	complete liquefaction	curd	1/4 liquefied
13,400	2,150	16.05	curd, 1/2 liquefied	complete liquefaction	curd	1/4 liquefied

Table 4— Source A--Pasteurized milk from bottles

Average total organisms per cc.	Average B. albolactis per cc.	% B. albolactis	Sample 37° C. 24 hours	Sample 37° C. 48 hours	Sample Room 24 hours	Sample Room 48 hours
27,700	900	3.2	gassy curd	----	gassy curd	----
16,600	1,800	10.9	malty odor	gassy curd, slight liquefaction	no change	malty odor
22,400	2,500	11.2	malty odor	gassy curd, 1/2 liquefied	no change	malty odor curd
9,500	500	5.3	malty odor	gassy curd	no change	malty odor curd
13,000	2,100	16.2	gassy curd, 1/2 liquefied	complete liquefaction	curd	slight liquefaction
89,000	2,000	2.3	curd	----	curd	----
97,000	2,000	2.0	curd	----	curd	----
5,300	650	12.25	curd, slight liquefaction	1/3 liquefied	curd	----
67,000	3,000	4.5	gassy curd, slight liquefaction	----	curd	----

Table 4— Source A—Pasteurized milk from bottles.—Continued

Average total organisms per cc.	Average *B. albolactis* per cc.	% *B. albolactis*	Sample			
			37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
82,500	11,000	12.95	curd	----	curd	----
34,000	16,500	19.65	gassy curd	1/4 liquefied	no change	curd
850	200	23.75	gassy curd	----	curd	----
7,350	6,350	85.7	complete liquefaction	----	curd, slight liquefaction	----
36,400	4,600	12.65	curd, slight liquefaction	1/2 liquefied	curd	slight liquefaction
14,800	2,300	15.5	curd, 1/3 liquefied	3/4 liquefied	curd, slight liquefaction	1/4 liquefied
13,400	2,250	16.8	curd, 1/4 liquefaction	1/2 liquefied	curd	slight liquefaction
13,500	7,100	53.3	gassy curd, 1/2 liquefied	2/3 liquefied	no change	no change

Table 5— Raw milk from Source B

Average total organisms per cc.	Average *B. albolactis* per cc.	% *B. albolactis*	37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
(1) 273,000	18,500	6.85	gassy curd	----	gassy curd	----
(2) 1,610,000	30,000	1.8	gassy curd	----	gassy curd	----
(3) 3,400,000	110,000	3.2	gassy curd	----	gassy curd	----
(4) 350,000	30,000	8.6	gassy curd	----	gassy curd	----
(5) 225,000	10,000	4.4	curd	----	gassy curd	----
(6) 3,640,000	10,000	0.3	curd	----	gassy curd	----
(7) 359,000	20,500	5.7	curd	----	gassy curd	----
(8) 340,000	20,000	5.9	gassy curd	----	gassy curd	----
(9) 3,790,000	110,000	2.9	gassy curd	----	gassy curd	----

(1) Milk cans
(2) - (7) Holding vat
(8) - (9) Preheater

Table 6— **Pasteurized milk from Source B**

Average total organisms per cc.	*B. albolactis* per cc.	% *B. albolactis*	37° C.		Room	
			24 hours	48 hours	24 hours	48 hours
(1) 13,000	800	6.2	No change	No change	curd	----
(2) 14,400	800	5.6	curd	1/4 whey	No change	No change
(3) 4,330	95	2.2	curd	----	gassy curd	----
(4) 8,500	900	10.6	curd, 1/4 liquefied	----	curd, slight liquefaction	----
(5) 9,600	650	6.75	No change	No change	No change	No change
(6) 49,500	5,500	11.2	curd, slight liquefaction	1/3 liquefied	curd	----
(7) 25,750	1,450	5.75	No change	complete liquefaction	curd	----
(8) 6,750	450	6.7	curd, slight liquefaction	----	curd	----

(1) and (2) from the pasteurizer
(3) and (8) from the cooler

Table 7 **Comparison of milk in the raw and pasteurized state, from Source A.**

Total organisms per cc.	**% organisms killed**	**% _B. albolactis_**	**% increase of _B. albolactis_**	**37° C.**		**Room**	
				24 hours	**48 hours**	**24 hours**	**48 hours**
206,500 66,250	68.5	7.0 17.3	147	— ≠	≠ ≠	— —	≠ ≠
227,000 77,250	66.4	7.9 13.1	66	— —	≠ ≠	— —	— —
215,000 68,500	66.2	15.0 20.1	34	— ≠	≠ ≠	— —	— —
32,500 775	97.6	17.0 20.0	18	— ≠	— ≠	— —	— —
71,500 5,950	91.8	20.95 83.55	298	— ≠	— ≠	— ≠	— ≠
201,500 14,650	92.7	17.4 66.0	279	≠ ≠	≠ ≠	— ≠	— ≠

The first results in each block are the raw milk.
The second results refer to the samples pasteurized from these.

— normal curd   ≠ slight liquefaction
— liquefaction   N.C. No change

Table 7— Comparison of milk in the raw and pasteurized state, from Source A. Continued

Total organisms per cc.	% organisms killed	% *B. albolactis*	% increase of *B. albolactis*	37° C.		Room	
				24 hours	48 hours	24 hours	48 hours
180,700 30,700	83.0	10.85 12.7	17	— ≠	— -/-	— —	— ≠
118,500 13,600	88.5	10.5 15.95	59	— -/-	≠ ≠	— ≠	— -/-
236,500 13,400	94.3	4.2 16.4	290	— -/-	— -/-	— —	— -/-

The first results in each block are the raw milk.
The second results refer to the samples pasteurized from these.

— normal curd
-/- liquefaction
≠ slight liquefaction
N.C. No change

Table 8— **Comparison of milk in the raw and pasteurized state, from Source B.**

Total organisms per cc.	% organisms killed	% *B. albolactis*	% increase of *B. albolactis*	37° C.		Room	
				24 hours	48 hours	24 hours	48 hours
3,660,000 7,680	99.8	3.0 3.9	30	— —	— +	— —	— —
345,000 10,750	96.9	7.25 8.4	16	— +	— +	— N.C.	— ±
225,000 9,600	95.7	4.5 6.75	50	— N.C.	— N.C.	— N.C.	— N.C.
3,640,000 25,750	99.3	0.3 5.75	1,820	— N.C.	— +	— —	— —
1,610,000 6,750	99.6	1.8 6.7	272	— ±	— ±	— —	— —
316,000 49,500	84.3	6.3 11.2	78	— ±	— +	— —	— —

For explanation of symbols see preceding table VII.

Table 9--Distribution of liquefaction in relation to the percent of B. albolactis of the total count.

% B. albolactis	37° C. 24 hours No Liquefaction	37° C. 24 hours Liquefaction	37° C. 48 hours No liquefaction	37° C. 48 hours Liquefaction	Room Temperature 24 hours No liquefaction	Room Temperature 24 hours Liquefaction	Room Temperature 48 hours No liquefaction	Room Temperature 48 hours Liquefaction
86		1		1				
81		1		1		1		1
79		1		1				1
53		1		1	1		1	
24	1		1		1		1	
23								
22								
21	x	1	x	1	1x		1x	
20	1			1	1		1	
19								
18								
17	x	11	x	1	11xx		x	1x
16		1111		1111	111			111
15	x	1		1	1x		1x	
14								
13	11	1	1	1	1111		111	
12		1		1	1		1	
11	11xx	1	x	111	111xx		111xx	
10	x			x	x		x	
9	x		x		x		x	
8								
7	1xx		1x		11xx		11x	
6	111xxx		1xxx		111xx		111xx	
5	1x		1x		11x		11x	
4	xx		xx		xx		xx	
3	1xx		1xx		1xx		1xx	
2	111x		111x		111x		111x	
1								
0	x		x		x		x	

1 = Pasteurized milk
x = Raw milk

DISCUSSION OF RESULTS

The samples of milk fell into four main classes, those that coagulated (normally or with gas formation), those that rapidly liquefied, those that showed a slight amount of whey, and those in which there was no visible change.

There were very few cases of really normal curds, most cases of coagulation being attended by gas production. This was particularly true of the raw milk samples. The fact that this was more common in raw than in pasteurized milk, and from several trial runs on violet red bile agar, eosin methylene blue agar, and Endo's agar, that gave a rather high coli-aerogenes counts (over 1,500 per cc. in some cases), this group is probably the cause of this gas production.

When less than one-tenth of the sample was whey it was reported as slight liquefaction. There are several possible explanations of this phenomenon. It might be due to partial inhibition of *Bacillus albolactis* so that maximum growth, and maximum activity, was not obtained; or organisms that coagulate casein by production of the enzyme rennin give a tight curd that squeezed out whey in some cases (e.g. *B. cereus* was found in several cases); or frequently gas-producing organisms may grow vigorously enough to break the curd and result in the expression of some whey.

Samples of milk showing no change, two even after forty-eight hours of incubation at 37° C., were found, although only rarely.

The percentages of B. albolactis in these cases ranged from 5.3%, in which case there was no change in twenty-four hours at either room temperature, or 37° C, though both showed a normal curd in forty-eight hours, to 53.3%, which was liquefied in twenty-four hours at 37° C, but showed no change after forty-eight hours at room temperature. In three cases of this type a predominance of Streptococcus lactis variety maltigenes was found, but outside of these samples no unusual types or numbers of microorganisms could be found.

The comparison of raw and pasteurized milk shows that the numbers of organisms killed varied from 66.2% to 99.8%. Although there was no correlation between this and the percent increase of B. albolactis, there was a significant increase in the relative numbers of this organism after pasteurization. The organism in the vegetative stage is normally more resistant than many microorganisms (11), and in the spore form is able to withstand particularly high temperatures. Thus, although their actual number decreases, since Streptococcus lactis, the main organism in milk is not a spore former, the relative numbers of B. albolactis increase. In the cases listed there were increases ranging from 16% to 1,820%. This increase is important when the milk is to be used in any sort of dairy manufacture, for, although a few samples of raw milk showed a slight liquefaction, for the most part they gave a normal curd, while pasteurized samples showed a significantly high number of cases of liquefaction.

Although there were several samples that showed no liquefaction in twenty-four hours at 37° C. when the percentage of B. albolactis ranged from 11% to 24%, all the samples that did liquefy in this period had 11% or more B. albolactis present. Below this percentage undoubtedly Streptococcus lactis, or S. lactis variety tardus, both of which have been isolated from these samples, produce enough acid to be somewhat bacteriostatic. It is also interesting to note that liquefaction occurred more rarely when samples were incubated at room temperature, and then only when the percentage of B. albolactis was at least 16%.

SUMMARY AND CONCLUSIONS

1. The type of fermentation at room temperature and 37° C, total bacterial count using the agar plate method, and relative numbers of *Bacillus albolactis* were determined in a number of samples obtained from a local milk supply.

2. In almost all samples of both raw and pasteurized milk, from the time it entered the creamery until it left, *B. albolactis* was found to be present. Its percentage of the total count generally ranged from 2% to 21%.

3. The raw milk formed a gassy curd in most cases, a normal curd in several cases, a slight amount of whey in a few, no change at all in a few, and liquefaction of the curd in two cases.

4. The pasteurized milk gave fermentations similar to those of the raw milk, although about one third of the samples liquefied.

5. A 16% to a 1,820% increase of the percentage of *B. albolactis* of the total count occurred in pasteurization.

6. Liquefaction of the milk can be correlated with the numbers of *B. albolactis* in relation to the total count, proteolysis occurring when its percentage of all the organisms present was over 11%.

PART II

A STUDY OF THE CHARACTERISTICS AND CULTURAL VARIATIONS OF *BACILLUS ALBOLACTIS*.

STATEMENT OF THE PROBLEM

In the first part of this thesis a study was made that entailed numerous observations of colonies of *Bacillus albo-lactis*. It was noted that when this organism was isolated from milk, and also when plated out in pure culture, two main types of colonies formed.

The purpose of this investigation was to make a detailed study of these two varieties of *B. albolactis* to determine any differences between them from the standpoint of morphology and biochemical activities, and to attempt to find a possible causal factor, or factors, for this variation.

HISTORY AND GENERAL ASPECTS OF THE PROBLEM

History

The question of the constancy or variability of bacteria has played an important part in the study of these microorganisms. At different times and in different countries, one or the other doctrine has held sway and been of importance in observations made, interpretations drawn, and procedures used. (30).

Early notions of variability, and transmutability, of bacteria were crystallized in their extreme possibilities by Nageli in 1877, and again in 1882, in his theory of pleomorphism. He maintained that as far as bacteria were concerned, there was a single cell type of extreme variability that was able to change its morphology, biochemical reactions, fermentative abilities, and so forth. Thus he claimed that a classification of bacterial genera and species on the basis of morphology and biochemical characteristics was unfounded. (9). Hueppe, Kruse, Lankester, Bilbroth, and others, agreed with Naegeli's work on variability, although they didn't carry the idea of pleomorphism to the lengths that he had gone. (23).

About this time Cohn (1872,1876) presented a classification of bacteria, based on stability of the morphology and physiology of bacterial species. (23). Koch in 1878, and many times after

that, insisted on the dogma of monomorphism--a constancy of form and uniformity of action. His followers in Germany, England, and this country added further support to his theory. (30). This concept led to a saner view of bacterial constancy, and rapid progress ensued in the entire field, particularly in classification. Migula, in 1897, brought out a rather comprehensive classification, and this work was followed by Orla-Jensen (1909), Buchanan (1917, 1918), Castellani and Chalmers (1919), and Bergey (1920,1923), and others. The tendency has been towards a more complicated classification, with the differences between species being split more and more finely. (9,10).

All this time, however, evidence contrary to the theory of monomorphism had been accumulating. Lehmann and Neumann offered a classification of bacteria in their "Atlas and Grundriss der Bakteriologie" (1896), but at the time they pointed out that many so-called species were quite often only varieties of one species. Neisser (1906), Massini (1907), Kowalenko (1910), Baerthlain and Eisenberg (1912), and others, worked on cultural variations of B. coli. (9). DeKruif (1921), Webster (1925), and Nutt (1927), reported on cultural and morphologic variations of the Pasteurella group, (28); Griffith (1923), and Reimann (1925) on pneumococci; Eagles (1928), Todd (1928) and others on the hemolytic streptococci; and many workers on other species, particularly on the colon-typhoid-dysentery group. (27). Topley and Wilson (27), Hadley (10), Lewis (16), and others maintain that cultural variations may probably be

seen in all bacterial strains if they are submitted to a prolonged examination under suitable conditions.

Thus, today, in spite of various classifications in general use based on the idea of constancy of bacterial species, the question, are the bacteria classifiable, is still a debatable one. Hadley (10), and Zinsser and Bayne-Jones (30) claim that they are not, nor will be until bacteriologists know what comprises a bacterial species. The trend of the species concept of bacteria is away from a restricted use of characteristics towards a concept of statistical distribution about a mean. And in the meantime, it is evident that the variability of bacteria is still unestimated in its extent and significance. (30).

Types of variation.

Salle (23), in a list of bacterial variations taken from Thompson (1935), offers sixteen bacterial characters and the variations of these that have been observed. These characters include cellular morphology--size, form, staining properties, spore formation, flagellation, and capsulation; growth on solid and liquid media; virulence, antigen and toxin production; proteolytic and fermentative powers, etc., with variations in all of them.

Cultural variation.

Cultural variations may take several forms. The progeny of a single cell, or a few cells, taken from one source may give

widely different colony forms. One of the most common of these is the change from smooth to rough colonies. (30). The smooth, or S-type, colony is usually smooth, glistening, moist, soft, homogeneous, round, and entire. The R-type, or rough colony is generally somewhat larger, wrinkled, irregular, granular, broken or fimbriate at the edge, flattened, translucent. Two or more of these differences may show up, indicating a transition from one form to the other, though the most striking of these are size, surface, and density. (9).

The commonest change is from smooth to rough, and this may be a gradual change over a period as long as several months, or a sharp change with no intermediates. Intermediate forms are designated as "I" by Koser and Styron (13,14), as "O" by Hadley (28), or, varying from true smooth, S, to true rough, R, they are Sr, SR, and sR according to Hadley (9) and Dienst. (7). The change from the smooth colony to the rough from through intermediates has been reported by Koser and Styron (13,14), Dawson (6), Dienst (7), Edwards (8), and many others. Cases of direct passage from S to R with no intermediates have also been reported by Lewis (16), and Van Roekel (29), for example. Hadley (9), however, mentions the fact that intermediate forms are all highly unstable, and suggests the possibility, because of this fact, and the great number of organisms s hown to have intermediates, that all S to R changes pass through transitional forms.

Until the beginning of this century it was believed that the change from S to R was permanent. Firtsch, Dyar, Corbett, Phillips, Zupnik, and others, in the last century, and White, Schutze, Crowell, and others in this century, working on many different species maintained that the conversion from the rough form back to the smooth was impossible. However, the work of Bordet and Sleeswyck in 1910, Baerthlein in 1918, Wreschoner in 1921, Lewis in 1934, Koser and Styron in 1930, Hadley in 1932, and many others, indicated that with sufficiently long periods of observation rough forms could be found going to smooth (in many, just as easily as the reverse) (10,23,28). Hadley indicates that this reversion does not seem to pass through intermediate forms, and raises the question as to whether this is really a reversion or further "progression". (10).

Mucoid colonies, generally formed from wedges, or sectors, of smooth colonies, are a third form that seem to be more common than previously thought. (10). Certain workers (8) have attempted to show that there was a direct correlation between M and R colonies, though most evidence seems to point to this as a distinct colonial type. Transition from R to M is particularly rare, the change generally being from smooth to mucoid, or mucoid to smooth.

The fourth main colonial type is the gonidial, or G type. This type is claimed to have been separated from parent cultures by filtration, and are at first not visible in the subcultures. In continued cultivation tiny "dew-drop" colonies form, with the cells

as minute coccus forms, usually gram-negative regardless of the original source. These colonies revert, on further subculture, back to the original S form (23,10). Not very much is known of these, however, and reports on them are rare. (30).

There are several other types of colonial variations possible. The production of daughter colonies very different from the original type in an old or dying culture, or within or on the mother colony is a rather common phenomenon. This has been reported on as far back as 1895 by Gunther. And, of course, the appearance, sometimes delayed, of secondary colonies following partial or complete lysis of a mother colony is not at all unusual.(9).

Relation of Cultural to other types of variation.

The question of correlation of cultural variations with variations in other characteristics of microorganisms has long been a point of contention among bacteriologists, and is still unanswered. Hadley (10) in the same discussion of microbic dissociation claims that each culture phase, when existing in a pure state, is associated with a definite group of characters, and that when one phase becomes transformed completely to another, some of these characters are lost and others gained; and again, that each attribute of a species may be transmitted to subsequent generations, independently.

Characters most frequently correlating with colonial type include cell morphology, motility, possession of certain carbohydrates, antigenic structure, and virulence. Such things as chromogenesis, hemolysis, fermentations, and other physiological reac-

tions seem to be unrelated to colony phase. (23).

Virulence has been correlated with colony phase at least as far back as 1911 when Preiss demonstrated that what is now called the rough form of Bacillus anthracis was virulent, and the smooth phase is avirulent. DeKruif in 1921 correlated virulence of B. lepisepticum with smoothness, and also a diffused growth in broth, while the avirulent form was rough, and granular in broth. Smoothness has often been correlated with virulence; Griffith in 1923, and Amoss in 1925, and others, with the pneumococci, and Topley and Ayrton in 1924 with Bacterium aertrycke, for example. Although here again, Todd in 1928 showed that hemolytic streptococci were virulent in the rough phase, and not the smooth. (28).

Edwards (8) on the other hand, studied rough and smooth variants of Shigella equirulus and found no serological or bilchemical differences, and equal virulense among the rough mucoid, smooth mucoid, and smooth non-mucoid phases. With Bacterium dysenteriae Sonne, in 1930 Koser and Styron reported no difference, other than colony form, with the S, I, and R strains. (14).

Thus, although many correlations can be made of other bacterial characteristics with cultural variation, it seems that each characteristic of a species is subject to independent transmissibility. (23).

Factors inciting variation.

An "inciting" substance, or condition, is one that sets up some physiological mechanism by whose activity ultimate changes

result. (9). Topley and Wilson (27), Edwards (8), Hadley (9), and others maintain that when the "normal" form is the smooth phase, the change to the rough phase is induced by unfavorable conditions. Dawson puts this in a somewhat different light when he claims that the chemical constitution of bacteria can be changed by changing the media and, with this, such things as enzyme production or agglutination may be changed. (16). Lewis (6) in his work on the coliform group, states that in some cases at least, variation can occur spontaneously and without regard to environment.

However, many things will bring about variation in bacteria. Growth at a temperature above the optimum; special food such as isodulcite, urea, lithium chloride, various sugars, and even a lack of food, i.e. use of a minimum amount of nutrients; antiseptics such as phenol, potassium dichromate, dyes, e.g. methyl violet, malachite green, gentian violet, etc.; animal passage, growth in normal serum, ascitic fluid, blood, etc.; and use of the products of growth, are some of the more important means of producing variations among bacteria. (9). Salle, however, raises the question as to whether these changes in environment merely lead to temporary changes in the organism, or whether they do lead to permanent inheritable differences. (23).

Various explanations of the phenomenon of variation have been proposed, and they include such things as fortuitous variation,

i.e. Darwinism; variation in response to environmental conditions, or Lamarckianism; mutation, in the sense of DeVries; orthogenetic variation; or cyclogeny, progressive ontogenetic changes in the form of a life cycle. (13).

Great confusion has resulted in both systematic and applied bacteriology from trying to compare cultures in different phases. The idea of a "normal" bacterial type, and variations from this seem to be untenable since the form of the organism may vary not only from one medium to the next but even on the same medium. (9,10). Although much work has been done, there is still a great deal to be done before enough is known to really explain variation, appreciate its extent and significance, and determine exactly what comprises a bacterial species. (6,9,10,16,30).

EXPERIMENTAL

Comparison of S and R forms

Smooth and rough forms of Bacillus albolactis were isolated from stock cultures of the Virginia Polytechnic Institute and from plates of the milk from a local creamery. These were transferred to tubes of litmus milk, plated from this on standard tryptone-glucose-extract agar (26), and isolations from these plates were replated and reisolated four more times. Isolations from the fifth plate were grown on ordinary nutrient agar slants and all the physiological tests mentioned in Bergey's Manual (1,2), and several others were run. Cellular morphology, including size, form, spores, capsulation, and motility was also studied.

A great number of workers in the field of microbic dissociation, including DeKruif (1921), Mellon (1919), Webster (1925), Amoss (1925), and Jordan (1926), used single cell technique and indicated that their results were the same as those obtained using colony isolations. (9). In view of this, and because colony isolations is very much simpler, it was used in this work. In all this work colonies were used only after a minimum of five isolations to insure purity of the strain.

Neither physiological or morphological differences could be found between these two forms. The following description, therefore, serves for both the smooth and rough strains.

Morphology

Size. 0.5 to 0.75 to 2.4 microns.

Spores. Central to subterminal. Ovoid.

Ends rounded; occur singly, in pairs, short chains, and occasionally long chains.

Capsulated.

Actively motile at 30° C. and non-motile at 37° C. in twenty-four hours.

Physiology

Starch not hydrolyzed.

Litmus milk acid, reduced, and peptonized.

H_2S not produced.

Indol not formed.

Nitrited not produced from nitrates.

Gelatin. Crateriform liquefaction.

No gas produced from any carbohydrates.

Acid from dextrin, dextrose, galactose, glycerol, lactose, levulose, maltose, sucrose, trehalose.

No acid from adonitol, aesculin, arabinose, dulcitol, inositol, inulin, mannitol, mannose, melezitose, raffinose, rhamnose, sorbitol, xylose.

Optimum temperature 30° C.

Cultural characteristics

The cultural characteristics of these two forms are different,

of course, and it is on this basis that they are separated. In nutrient broth both forms gave similar growth, a general turbidity, a ring at the top, and a slight amount of sediment.

The smooth type in a nutrient agar stab gives filiform growth. On nutrient agar the colony is circular, white, entire, raised to convex, smooth, shiny, with the center generally sunken, and occasionally nucleated. (Figure 1, Plate I). Microscopically the appearance is from coarsely granular in the center to finely granular throughout the rest of the colony. (Figure 2, Plate I). The edge is sometimes clear, generally entire, although occasionally notched. On continued incubation the internal appearance may become curled. On a nutrient agar slant the culture is white, raised, shiny, smooth, lobate, with a very slight depression down the center.

The rough type in a nutrient agar stab grows in a villous to a rhizoid fashion. On nutrient agar the colony is slightly spreading, flat, grey-white, ameboid to rhizoid, rough and shiny. (Figure 1, Plate II). Microscopically, particularly when young, the colony is finely filamentous. (Figure 2, Plate II). Spreading is in the form of rhizoid-like growth straight out, or in a clockwise direction. Of several thousand colonies observed only two were ever seen growing in a counterclockwise direction. Replating of these failed to give this type of growth again. The growth on a nutrient agar slant is grey-white, spreading, rough, rhizoid, and shiny.

Side View Surface View

Fig. 1. Natural Size, 96 hours old

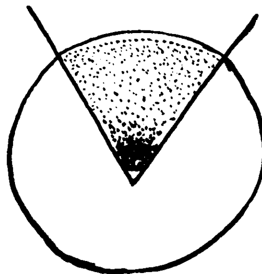


Fig. 2. 100 x, 10 hours

Plate I - Smooth Colonies on Nutrient Agar

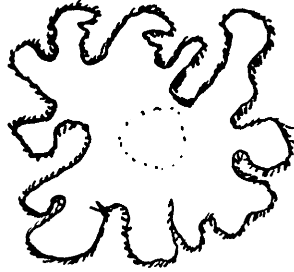


Fig. 1. Natural Size, 96 hours old

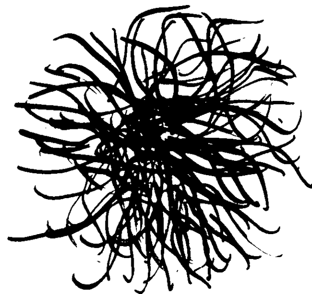


Fig. 2. 100x, 10 hours

Plate II - Rough Colonies on Nutrient Agar

Ratio of S to R, and R to S colonies

Plates made from either type of colony almost always showed several colonies of the other form. Continued replating and re-isolation of typical colonies failed to yield a strain that would result in colonies of only one type on replating; in one case a rough strain was replated seventeen times, but smooth colonies still showed up.

To determine whether there was any constancy in the ratio of smooth to rough, or rough to smooth, strains, several plates were made on tryptone-glucose-extract agar, nutrient agar, and nutrient agar with starch. The results obtained in twenty-four hours are listed in Tables 1, 2, 3. Combining these results with those of the same media listed in Table 4, gives an average of 3.2% smooth colonies from the rough strains in a range from 0% to 6.5%. Rough colonies averaged 7.2% of the smooth strain in a range from 1.6% to 12.9%. This fairly narrow range seems to indicate a constancy in these ratios.

The effect of the medium.

Using twelve different media counts of the colonies were made to see if the ratio of the smooth and rough colonies was the same as that previously observed.

The following is a list of the media used, and their composition.

<u>Nutrient agar</u>

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water--1 liter	

<u>Tryptone glucose extract agar</u>

Beef extract	3.0 g
Tryptone	5.0 g
Glucose	1.0 g
Agar	15.0 g
Distilled water--1 liter	

<u>Beef Lactose Agar</u>

Infusion from 450 g beef heart.	
Proteose-peptone	5 g
Bacto (B.) lactose	10 g
B. agar	15 g
45 gms. per liter of distilled water.	

<u>Blood Agar Base</u>

Infusion from 500 g fresh beef heart.	
B. peptone	10 g
Na Cl	5 g
B. agar	15 g
45 gms. per liter of distilled water.	

Brain Veal Agar

Infusion from 250 g calf brain		
Infusion from 500 g veal		
$Na\ H_2\ PO_4$	1.25	g
Na Cl	3.75	g
B. peptone	10	g
B. agar	15	g

55 gms. per liter of distilled water.

Corn Meal Agar

Infusion from 50 g corn meal		
B. dextrose	2	g
B. agar	15	g

19 gms. per liter of distilled water.

Endo's Agar

B. peptone	10	g
B. lactose	10	g
$K_2\ HPO_4$	3.5	g
Basic fushsin	0.5	g
Sodium sulphite	2.5	g
B. agar	15	g

Distilled water--1 liter

Levine's Eosin Methylene Blue Agar

B. peptone	10	g
B. lactose	10	g

K_2HPO_4	2	g
B. eosin Y (DE-2)	0.4	g
B. methylene blue (DA-2)	0.065	g
B. agar	15	g
Distilled water--1 liter		

<u>Galactose whey agar</u>

Whey from 1 liter of milk		
B. peptone	5	g
d galactose, Difco	10	g
B. agar	10	g

65 gms. per liter of distilled water.

<u>Lead Acetate Agar</u>

B. tryptone	20	g
B. dextrose	1	g
Lead Acetate	0.2	g
B. agar	15	g
Distilled water--1 liter		

<u>Malt Extract Agar</u>

Technical maltose	12.75	g
Dextrin, Difco	2.75	g
Glycerol	2.35	g
B. peptone	0.78	g
B. agar	15	g
Distilled water--1 liter		

<u>Nitrate Agar</u>.

B. Beef Extract	3	g
B. Peptone	5	g
KNO_3 (C.P.)	1	g
B. agar	12	g

Distilled water—1 liter

The results obtained on eight of these media are listed in Table 4, and a comparison of the twelve media is made in Table 7. The colonies obtained were typical on some cases, in comparison to those obtained on nutrient agar, but in many cases they were not. Descriptions of the variations follow.

<u>Nutrient agar</u>. For description of colonies see above. (Also Plates I and II)

<u>Beef lactose agar</u>. (Plate IV.) Smooth colonies were never larger than 5 or 6 mms. in diamter. The edges were undulate to auriculate.

Rough colonies were large, grey-white, spreading, with "hazy" edges. The colonies were irregularly opaque to translucent.

<u>Blood agar base</u>. (Plate V) Colonies were the same as on nutrient agar, though the color of the medium made them stand out even more clearly. The edges of the smooth colonies were dark. The rough colonies typically showed growth in a clockwise direction. After incubation for 48 to 72 hours most of the smooth colonies changed over to rough.

Table I--Comparison of S and R ratios on tryptone-glucose-extract agar

Plates of R strain			Plates of S strain		
Total number of colonies (R and S)	S colonies	% S type	Total number of colonies (S and R)	R colonies	% R type
120	2	1.6	148	6	4.1
241	11	4.6	79	8	10.1
250	10	4.3	86	9	10.5
340	7	2.1	420	30	7.1
111	7	6.3	254	16	6.3
63	1	1.6	213	12	5.6
220	6	2.7	187	21	11.2
210	11	5.2	256	33	12.9
321	6	1.9	69	2	2.9
46	0	0	117	7	6.0
Average 190.2	6.1	3.2	182.9	14.4	7.9

Table 2—Comparison of S and R ratios on nutrient agar

Plates of R strain			Plates of S strain		
Total number of colonies (R and S)	S colonies	% S type	Total number of colonies (S and R)	R colonies	% R type
254	15	6.0	245	4	1.6
92	6	6.5	189	16	8.5
367	7	1.9	232	17	7.3
51	1	2.0	169	4	2.4
268	14	5.2	110	5	4.6
172	4	2.3	74	3	4.1
158	0	0	279	20	7.2
193	4	2.1	78	3	3.8
Average 194.4	6.4	3.3	172.4	9.0	5.2

Table 3--Comparison of S and R ratios on starch nutrient agar

Plates of R strain			Plates of S strain		
Total number of colonies (R and S)	S colonies	% S type	Total number of colonies (S and R)	R colonies	% R type
250	6	2.4	180	20	11.1
310	10	3.2	400	32	8.0
130	3	2.3	460	30	6.5
46	2	4.3	38	4	10.5
Average 184.0	5.3	2.9	269.4	21.5	8.0

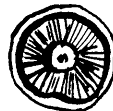
Table 4—The effect of various media on the S and R ratios. Observations in 24 hours.

Nutrient Agar			Beef lactose agar			Blood agar base			Brain veal agar		
Total count	No. S	% S	Total count	No. S	% S	Total count	No. S	% S	Total count	No. S	% S
38	2	5.3	125	0	0	340	2	0.6	30	0	0
34	1	2.9	48	0	0	45	2	4.4	360	0	0
216	1	0.5	38	0	0	67	3	4.5	48	0	0
Average		2.9			0			3.2			0
Total count	No. R	% R	Total count	No. R	% R	Total count	No. R	% R	Total count	No. R	% R
53	2	3.8	400	3	0.75	135	6	4.4	33	1	3.3
140	12	8.6	60	0	0	58	0	0	160	3	1.9
160	16	10.0	290	1	0.3	210	3	1.4	150	4	2.7
Average		7.5			60.3			1.9			2.6

Table 4—Continued

Galactose whey agar			Lead acetate agar			Nitrate agar			Tryptone-glucose-agar		
Total count	No. S	% S	Total count	No. S	% S	Total count	No. S	% S	Total count	No. S	% S
100	10	10	51	0	0	39	0	0	78	3	3.8
40	18	45	380	0	0	280	0	0	96	3	3.1
—	—	—	315	0	0	320	0	0	82	4	4.9
Average		27.5			0			0			3.9
Total count	No. R	% R	Total count	No. R	% R	Total count	No. R	% R	Total count	No. R	% R
80	0	0	35	3	8.6	78	9	11.5	80	6	7.5
54	0	0	38	2	5.4	230	53	23.0	72	5	6.9
60	0	0	—	—	—	83	12	14.5	—	—	—
Average		0			7.0			16.3			7.2

Fig. 1. Rough Form, Natural Size



Side View Surface View

Fig. 2. Smooth Form, Natural Size

Plate IV - 144 Hour Colonies on Beef Lactose Agar

Fig. 1. Rough Form, Natural Size

Fig. 2. Smooth Forms, Natural Size

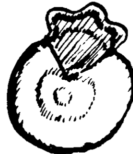


Fig. 3. Smooth Form, Showing Sectoring, Natural Size

Plate V - 144 Hour Colonies on Blood Agar Base

<u>Brain veal agar</u>. (Plate III) Distinction between the two types wasn't clearcut until after incubation for forty-eight hours.

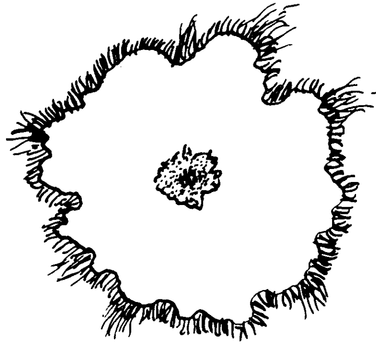
Smooth colonies were up to seven or eight mms. in diameter. The edge was raised, and there were frequently two or three raised, concentric rings.

Rough colonies were quite large, up to forty-five and fifty mms. in diameter in many cases. The edge was either lacerate, and finely filamentous for about three millimeters around the colony, or was undulate with a clear zone of from one to three millimeters around the colony. Microscopically the centers of the colonies were coarsely granular, and the rest amorphous.

<u>Corn meal agar</u>. In 72 hours no colonies were over four millimeters in diameter. All the colonies were myceloid, and though this isn't typically rough, there certainly is no resemblance to the smooth type.

<u>Endo's agar</u>. In 72 hours the colonies were still pinpoint in size, and the rough form indistinguishable from the smooth. After a week most of the colonies seemed to be either rough or intermediates.

<u>Levine's eosin methylene blue agar</u>. (Figure 1, Plate VI) Rough and smooth couldn't be distinguished on this medium, i.e. the colonies all look rough. Colonies on plates



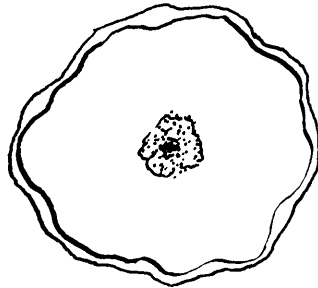


Fig. 1. Two rough forms, Natural Size

Fig. 2. Smooth Colony, Natural Size

Plate III - 144 Hour Colonies on Brain Veal Agar

Fig. 1. 144 Hour Colonies on Eosin Methylene Blue Agar. 5x.

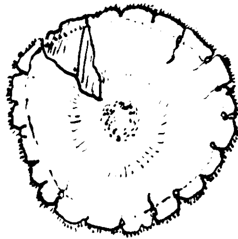


Fig. 2. 144 Hour Colony of Smooth Strain on Galactose Whey Agar, Showing Sectoring. Natural Size.

Fig. 3. 144 Hour Colony of Smooth Strain Subjected to $97.5^{\circ}C$. for 10 Minutes Showing Partial Lysis. On Nutrient Agar. Natural Size.

Plate VI - Unusual Colonial Types of *B. albolactis*

made from the smooth strain, however, were not as finely mycoolid, and were more deeply colored than colonies of the rough strains.

Galactose whey agar. Smooth colonies grew exceedingly well on this medium; some colonies were as large as forty millimeters in diameter at the end of a week. They were concentrically raised, lobate, with very fine, short mycelial-like projections surrounding the colony. Sectoring was noted on several of these colonies. (Figure 2, Plate VI).

Lead Acetate Agar. Smooth colonies were the same as on nutrient agar, though they were somewhat coarsely granular around the edges.

Rough colonies had a large wrinkled nucleus, finely granular around this, to irregular coarse granulation at the edge (auriculate). They were flat colonies and up to eighteen millimeters. Some regular rough forms were also present.

Malt extract agar (ph 4.7). No growth.

Nitrate agar. Both forms show up clearly, colonies appearing the same as on nutrient agar.

Tryptone glucose extract agar. Colonies were typical and the differences between the two forms show up very clearly.

As shown in Tables 4 and 7, the ratio of smooth to rough colonies, from either strain, will vary depending on the medium used. Beef lactose agar comes the closest of any medium to

yielding both strains in a pure form. Certain media, such as lead acetate agar, and nitrate agar, favor the growth of the R strain, plates of the R strain giving no smooth colonies, while plates of the S strain gave about twelve percent rough forms. Galactose whey agar was the only one favoring growth of the smooth type.

Colonies varying from the typical smooth and rough forms were observed in several instances. Myceloid forms were one type; smooth colonies with notched edges, or cleared, translucent edges another. And many forms evidently intermediate between the typical smooth and rough colonies were noted.

The effect of heat treatment.

A smooth and rough strain from the V. P. I. stock cultures, and a smooth and rough strain isolated from milk, all replated five times and then grown on nutrient agar slants for three days were used in this portion of the experiment. Dilutions of these four cultures were made in sterile water and these subjected to 60°, 70°, 80°, 90°, and 97°, C. for ten minutes, and then plated out on blood agar base. Counts were made in twenty-four hours, and the results are listed in table 5.

These results indicate that the smooth form is better able to withstand heat than the rough form.

Table 5--The effect of heat treatment on the S and R ratios.

	Temperatures applied for ten minutes to water suspensions of the organisms.					
	24 hours			42 hours		
	Rough Strains					
	Total count	No. S-type	% S-type	Total count	No. S type	% S-type
60°	51	20	39.4	51	2	4.0
70°	220 400	20 98	9.1 24.5	220 403	0 42	0 10.4
80°	362 45	34 2	9.4 4.4	370 45	1 0	0.03 0
90°	120 51	53 23	44.2 45.1	126 51	0 0	0 0
97.5°	178 35	170 35	95.5 100.0	179 35	92 0	51.4 0

Table 5--Continued

	24 hours			72 hours		
		Smooth strains				
	Total count	No. R-type	% R-type	Total count	No. R-type	% R-type
60°	272	6	2.2	256	32	12.5
70°	324	0	0	324	163	50.3
80°	50 380	2 36	4.0 9.5	51 376	29 34	56.9 9.0
90°	220 210	5 20	2.3 9.5	235 207	196 193	83.4 93.2

<u>The effect of lengthened incubation</u>.

Lengthened incubation at 30° C. seems to favor conversion of the S form to the R form. Table 5 indicates the result of lengthened incubation. Plates made of the heat-treated strains of <u>B</u>. <u>alboloctis</u> were counted in twenty-four and seventy-two hours, and a marked decrease in the numbers of the smooth colonies was noted.

Several partially lysed colonies were observed on plates of the smooth strain that had been heated at 90° C. and 97.5° C. (Figure 3, Plate VI).

<u>The effect of growth in milk</u>.

Two smooth and two rough forms of <u>B</u>. <u>alboloctis</u> isolated from milk, and two strains of each type isolated from the V. P. I. stock cultures, after five replatings were transferred to sterile milk, and retransferred on milk three times. The cultures were incubated for one week and then plated out using typtone glucose extract agar.

Normal percentages of smooth and rough colonies from the opposite strain were obtained; i.e. about 3% smooth colonies on plates made from the rough strains, and a little over 4% rough colonies from the smooth strains. The results are recorded in Table 6.

Growth in milk evidently had no effect on conversion of either the smooth to the rough form, or vice versa.

Table 6--Effect of growth in milk on the S and R ratios.. Observations in 24 hours.

Rough strains			Smooth strains		
Total count	No. of S colonies	% S-type	Total count	No. of R colonies	% R-type
87	2	2.3	238	11	4.6
60	2	3.3	69	0	0
250	0	0	180	12	6.7
178	12	6.7	295	12	4.1
249	6	2.4	130	7	5.4
Average percentage		2.9	Average percentage		4.2

The effect of p H

The various media used in the first part of this experiment on the ratio of smooth to rough colonies, had a range in p H values from 4.7 to 7.55. Table 7, indicating the p H values of these media, as well as the carbohydrates present, shows that there seems to be no correlation between either of these factors, and the favoring of the growth of either the rough or smooth form.

Table 7—Effect of pH and carbohydrates on S and R growth.

pH	Medium	Type favored. 0—neither favored	Carbohydrate in medium
4.7	Malt extract	No growth	Maltose, dextrin
6.0	Corn meal	R	Corn meal infusion, dextrose
6.4	Galactose whey	S	milk whey, galactose
6.6	Lead acetate	R	Dextrose
6.6	Nitrate	R	----
6.7	Nutrient agar	0	----
6.8	Beef lactose	0	Lactose
6.8	Blood agar base	0	----
7.0	Tryptone-glucose-extract	0	Glucose
7.1	Eosin methylene blue	R	Lactose
7.5	Endo's	R	Lactose
7.55	Brain veal	0	----

DISCUSSION AND CONCLUSIONS

Work on the isolation of Bacillus albolactis from milk, and studies of it in pure culture, indicated the presence of two main colonial varieties.

The colony of the smooth type is round, white, entire, raised to convex, smooth, glistening, generally with a sunken center, and occasionally nucleated, and averages five to seven millimeters in diameter (Plate I). The rough colonial form is flat, slightly spreading, ameboid to rhizoid in shape, with lacerated or ciliated edges, grey-white, rough, glistening, and larger than the smooth colonies (Plate II).

No morphological or physiological differences could be found between these two forms, although rather extensive studies were made.

Pure cultures of either form, when plated out on nutrient agar, or tryptone glucose extract agar, always yield a certain small percentage of the opposite type. These percentages are fairly constant on particular media, though they vary from one medium to another.

Certain media will favor one form over the other, but there seems to be no indication of why this should be so. The composition and the pH seem to have no effect on this.

The medium will also have an effect on the type of colonies formed. Mycoloid colonies (Figure 1, Plate VI) form when there are bacteriostatic substances present, such as methylene blue in eosin methylene blue agar, for example; or possibly pH values at the

lower limits of the organism's tolerance, as in corn meal agar at a pH of 6.0.

Reported quite commonly with many organisms (23,30), and generally resulting in variant forms, sectoring was noted on several occasions (Figure 3, Plate V; and Figure 2, Plate VI). Isolations, however, were never made of these forms. Partial lysis of colonies, due to weak strains of bacteriophage, has also been commonly reported (9, 10, 30). This was observed only on plates of the smooth strain that had been heated at 90° and 97.5° C. for ten minutes(Figure 3, Plate VI).

Although intermediate forms were observed, and a varying percentage of smooth forms will change over to the rough type of colony on continued incubation, it does not seem to be necessary for passage to be through an intermediate form in changing from smooth to rough, or rough to smooth. Nor is there any change of physiological or morphological characteristics going from smooth to rough and back again to smooth.

Both forms were capsulated, and their colonies glistening, which would make them mucoid, as described with other organisms by Edwards (8), Soule (25), and others. In no case were colonies seen which might be described as non-mucoid. Phantom colonies, as noted by Soule (25) with closely related forms (e.g. B. cereus) were not seen; nor were gonidial colonies ever observed.

The question as to which of these two main forms might be considered "normal", and which the variant, seems to be unanswerable.

The normal form may be defined as that one growing best on artificial media, but this varies not only from one medium to another, but even on the same medium. The normal form might be defined as the most easily isolated form, but this varies from one milk sample to the next. The smooth type, however, seems to be somewhat more common in milk. On the other hand, from the standpoint of stability, the rough form converts less easily than does the smooth form.

Thus neither form may be considered more normal than the other, and any system of classification, or description, must take into account the wide variation possible within this single species.

SUMMARY

1. *Bacillus albolactis* exists in two main cultural forms on solid media, one described as a smooth form, the other as a rough type.

2. Smooth, rough, and intermediate forms are morphologically and physiologically identical.

3. The rough form is somewhat more stable than the smooth, but plating out pure strains of either the rough or smooth type will yield some colonies of the other type. On nutrient agar and standard tryptone glucose extract agar the rough type yields about 3% smooth type colonies, while the smooth form yields about 7% of the rough form.

4. Various media, although yielding both colonial types, may favor the growth of one or the other form. There seems to be no correlation between this and either the composition of the medium, or its pH.

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