COLONY AND ANTIGENIC VARIATION IN KLEBSIELLA PNEUMONIAE TYPES A, B AND C

WILLIAM A. RANDALL

Department of Bacteriology and Immunology, Georgetown University School of Medicine

INTRODUCTION

It is commonly recognized that *Klebsiella pneumoniae is* closely related to the *Escherichia-Citrobacter-Aerobacter* group of gram-negative bacteria, the main differential factor being the capsule which is formed by *Klebsiella pneumoniae.* However, *Klebsiella pneumoniae* is usually placed in a separate category because its cultural reactions are not typically those of either the *Escherichia* group or the *Aerobacter* group, and also because it is often associated with pathological conditions in man and animals. The precise relationship of this organism to the *Escherichia, Citrobacter,* and *Aerobacter* groups remains to be established.

The confusion concerning this relationship is emphasized by the fact that Edwards (1929) showed that many cultures ascribed to the genus *Aerogenes* are serologically identical with certain strains of *Klebsiella pneumoniae.* This relationship was studied further by Julianelle (1937), who found that some strains of encapsulated *Aerogenes* cross-agglutinate with type B of Friedlander's bacillus, and with type II pneumococcus in serological tests. However, absorption tests revealed differences between the capsular material of the type B Friedlander's bacillus and *Aerobacter aerogenes.*

Obviously, the presence or absence of a capsule does not constitute a reliable criterion for the separation of *Klebsiella pneumoniae* from the members of the Colon-Intermediate-Aerogenes group.

All members of the coli form group, may, under certain conditions assume a mucoid form. Revis (1910) caused a colon

organism to develop a mucoid form of growth by cultivating it in sterilized soil. Gratia (1922) reported the development of mucoid colonies of colon bacilli when non-mucoid types were subjected to the action of bacteriophage. Smith (1925) found a mucoid colon organism connected with calf scours, and Parr (1933) has repeatedly isolated mucoid colon organisms from normal feces.

Furthermore, experience shows that Friedlander's bacilli, when decapsulated, exhibit a smooth, translucent colony form characteristic of the colon bacillus. This is a form which Julianelle in his studies on dissociation of *Klebsiella pneumoniae* did not describe.

In an investigation of the colony variants of *Klebsiella pneumoniae*, Julianelle (1928) reported direct transition from the smooth, mucoid colony form to the rough colony form when cultures of the mucoid bacilli were grown in homologous immune serum. The transformation of *Klebsiella pneumoniae* from the mucoid colony type to the rough colony type was also reported by Hadley (1925) and O'Neal (1933).

Dawson (1934) pointed out that there exist three main colonial forms within the species *Diplococcus pneumoniae*. These are mucoid, smooth and rough. The mucoid colony consists of encapsulated, virulent organisms. The smooth colony consists of organisms which have lost their virulence and their capsules but which form stable suspensions in broth. The rough colony consists of organisms which are non-encapsulated, avirulent, and which form a sediment in broth.

This paper presents evidence that an analogous situation more accurately describes the variants of Friedlander's bacillus, and that capsules and colonial form cannot be relied upon to separate Friedlander's bacilli from other coliform bacteria.

DISSOCIATION OF KLEBSIELLA PNEUMONIAE[1]

The three serological types, A, B, and C of *Klebsiella pneumoniae*, in mucoid form were seeded into 0.5 per cent lithium

[1] The strains used in this work were obtained from Dr. John Hanks of George Washington University and from Dr. L. A. Julianelle of Washington University. They were Type A Strain Sc., Type B Strain E., and Type C Strain F10.

chloride peptone water and incubated at 37°C. for one week. At this time plates of meat infusion agar were streaked and the resulting colonies examined for any variation from the normal. When colonies differing from the original mucoid type were observed some were picked, reseeded into lithium chloride peptone water and again incubated for one week. At each picking several variant colonies were selected for morphological and biochemical study. This process was continued until the variants to be described later were obtained.

The dissociative process took place at a different rate for each of the types A, B, and C, but the first step in each case consisted in a change from the mucoid colony form to a translucent colonial variant which remained stable in subculture on meat infusion agar. The second step was the gradual roughening of the translucent variants until typical rough variants were obtained. Type A gave rise to translucent variants after three weekly transfers. Representative translucent colonies were again transferred to lithium chloride peptone water and produced stable rough colonies after six weekly transfers. Type B produced translucent variants after three weekly transfers. Colonies of this translucent form produced rough colonies after four weekly transfers in lithium chloride peptone water. Type C gave rise to translucent variants after two weeks. These variants gave rise to rough colonies after three more weekly transfers in lithium-chloride peptone water. .The mucoid, translucent, and rough colony forms of each strain have been kept on meat infusion agar for the past four years without further detectable variation.

When types A and C gave rise to translucent variants, there also appeared very small dew drop colonies. When they were replated these dew drop colonies gave rise to translucent forms or to a mixture of translucent and dew drop colony types. The type C translucent colonies were purified so that they no longer produced dew drop colonies but the translucent variant from type A still retains this tendency after four years.

The translucent variant derived from type A grows poorly on agar and in broth. In twenty-four-hour agar cultures, the colony size varies from very small dew drop colonies to larger forms. Sometimes, growth fails to occur unless a large inoculum

is used or else it is delayed from 36 to 72 hours. The rough variant of type B (Br) obtained from the translucent variant of type B (Bt) is also difficult to grow. Large inocula are necessary to initiate growth if the culture has been stored for several weeks in the ice box. Growth occurs more readily if blood agar is used. However, if the culture is transferred for several generations at 37°C. on meat infusion agar it will grow fairly profusely in 24 hours. The colonies produced vary in size from pin head types to forms 5 or 6 mm. in diameter.

The colonial appearance of the translucent variants resembled very strikingly the colonial form of *Escherichia coli* and other non-capsulated members of the coli-form group.

DESCRIPTION OF THE COLONY VARIANTS ON INFUSION AGAR AFTER 24 HOURS INCUBATION AT 37°C.

For ease in referring to the original colonies and variants, an abbreviated system of notation was used: As, type A, smooth and mucoid; At, translucent variant derived from type As; Ar, rough variant representing further dissociation of At. The same system was applied to types B, and C, i.e., Bs, Bt, Br; and Cs, Ct, and Cr.

Description of colonies: 1. As: Mucoid, opaque, white, about 3 mm. in diameter; round, entire edge, surface smooth, glistening and convex; viscous consistency.

2. At: Non-mucoid, translucent, greyish white; smaller than As; round, entire edge; surface smooth, glistening, and slightly convex; butyrous. There is considerable variation in the size of the colonies.

3. Ar: Rough, translucent, greyish white, about 2 mm. in diameter; round with a crenated edge, surface slightly dull, and flat with a thin, narrow periphery; butyrous.

4. Bs: Similar to As except the colonies appear more of a pasty white.

5. Bt: Similar in appearance to the larger colonies of At. The colonies of Bt are of uniform size, 2 to 3 mm. in diameter.

6. Br: Rough, opaque, dirty-white frosted glass appearance; size varies from small to quite large; round with a crenated edge;

surface dull and flat; not viscous, but tough and difficult to emulsify. On ageing, the colonies appear to enlarge by spreading a thin growth outward from the raised center.

7. Cs: Similar to Bs.

8. Ct: At first gave rise to the dew drop colony forms, but later became stabilized and now resembles Bt.

9. Cr: Rough, opaque, dirty-white ground-glass appearance; about 2 to 3 mm. in diameter; the colonies are often of an irregular oval shape; edge crenated; surface dull and flat; not viscous, but difficult to emulsify.

MORPHOLOGY AND STAINING CHARACTERISTICS OF THE ORIGINAL TYPES AND THEIR VARIANTS

The organisms in the 24-hour mucoid colonies of all three types were plump, gram-negative rods, occurring singly or in pairs. Capsules were readily demonstrated when these organisms were emulsified in serum and stained with Wright's stain.

All of the translucent variants were very similar in morphology to each other. They were short, gram-negative, non-encapsulated rods.

The rough variants did not possess capsules and differed from the mucoid and translucent varieties in appearing as long rods or filaments. Certain individual differences were noted. The rough variant of type A was a gram-negative organism occurring as a mixture of rather long rods, together with many shorter forms resembling the organisms from the translucent colonies. Br was very long and filamentous and showed some evidence of branching. The cytoplasm was granular and gram-negative with some sections of the rods being gram-positive. Cr was a long gram-negative rod, longer than Ar, but not as filamentous as Br.

CULTURAL AND BIOCHEMICAL REACTIONS

1. Growth in broth

Within 24 hours all transplants of the three mucoid cultures produced a diffuse turbidity in broth with a ring at the surface and a viscid sediment. The translucent variants grew with a uniform turbidity and produced a slight non-viscid sediment

in 24 hours. A ring at the surface appeared only after cultivation for a period of two to five days. The rough variant of type A (Ar) grew with a diffuse turbidity and a heavy pellicle which settled to the bottom on slight agitation to form a heavy granular sediment. The Br variant grew slowly in broth, producing a very faint turbidity and a fluffy sediment at the bottom of the tube. Upon prolonged incubation, the Br culture grew entirely at the bottom of the tube, leaving a clear supernatant. The Cr variant produced a faint turbidity, a slight surface film, and grew chiefly as a sediment at the bottom of the tube.

TABLE 1

ORGANISMS	2 DAYS*		4 DAYS VOGES-PROS-KAUER	21 DAYS		7 DAYS REDUC. OF NITRATES	21 DAYS	
	Indol	Methyl red		Citrate	Gelatin		Glucose†	Lactose
As.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
At.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A-alk.
Ar.......	Pos.	Pos.	Neg.	Pos.	Neg.	Pos.	AG	A
Bs.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
Bt.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	A	A
Br.......	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.
Cs.......	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	AG	AG
Ct.......	Neg.	Neg.	Neg.	Pos.	Neg.	Pos.	AG	AG
Cr.......	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.

* This refers to the incubation period at 37°C.

† The reactions of the various strains in sucrose and mannitol are the same as those recorded in glucose.

2. Biochemical reactions

During the course of this work, the different strains showed some variation in their Methyl Red and Voges-Proskauer reactions. In 1925 Small and Julianelle found that most of their strains of *Klebsiella pneumoniae* were Methyl-Red positive and Voges-Proskauer negative and that these reactions were of no value in the classification of this group of organisms. The original strains and the variants used in this study were Methyl-Red positive and Voges-Proskauer negative, with the exception of Br and Cr which fermented no sugars and, therefore, were negative in both tests.

On Koser's citrate medium, all strains, except Br and Cr, grew and utilized the citrate.

With the exception of the rough variant of type A all strains failed to produce indol.

All the cultures and variants, except Cr, reduced nitrates to nitrites.   The variant Cr also was the only strain or variant which liquefied gelatin.

The reactions on several carbohydrate substrates (see table 1) were uniformly constant during the year or more during which the original types and the variants were under frequent observation.   This has been an aid in checking the purity of the different strains during this investigation.   The translucent variants of all three mucoid types had the same biochemical reactions as the parent strains.

The rough strains showed marked cultural differences from the mucoid and translucent strains.   The Ar variant differed from the parent strain in producing gas in glucose, sucrose and mannitol and in producing indol.   The Br variant was inactive on all test substances except nitrates.   The Cr variant lost its ability to ferment sugars and to reduce nitrates, but gained the power of liquefying gelatin.

AGGLUTINATION REACTIONS

Suspensions for the agglutination test and for animal inoculation were prepared by growing the organisms on meat infusion agar, washing off the growth with saline solution, and heating the suspensions at 60°C. for one hour.   Sterility tests were then made and the suspensions stored in the ice box.   Agglutination tests were incubated for 2 hours at 52°C.

Julianelle (1926) (1937) reported that rough strains derived from different types of Friedlander's bacilli cross agglutinate with one another, producing a granular type of agglutination.   This granular type of agglutination is considered by Julianelle to be characteristic of rough organisms which lack the specific polysaccharide, while specific agglutination, which is due to the capsular material, is like a precipitin reaction and produces a cake in the bottom of the tube.   Bamforth (1928) in an investigation of the

Capsulatus-mucosus group, reported that he obtained specific agglutination of a granular type with some of the strains he investigated. Consequently, he did not believe that specific agglutination was necessarily of the "compact-disc" type.

In the present work, three kinds of agglutination have been observed. First, the compact-disc type which is seen when encapsulated bacilli are agglutinated by their specific antisera. Second, granular agglutination which was observed with all the translucent variants and their specific antisera and third, a "fluffy" type of agglutination observed when the rough variants Br and Cr were acted on by their specific antisera. In the latter

TABLE 2

Antisera

ORGANISMS	As	At	Ar	Bs	Bt	Br	Cs	Ct	Cr
As........	10*								
At........	80	640							
Ar........		160	640						
Bs........				20					
Bt........				160	1,280				
Br........						640			
Cs........							10		
Ct........							80	640	
Cr........									2,560

* This number represents the highest dilution of antisera giving an easily discernible reaction.

type of agglutination, the organisms settled rapidly to the bottom of the tube, and, when shaken resuspended without the formation of granules.

The agglutination reactions reveal (See table 2) that the dissociants do not cross react with variants from strains belonging to the other serological types.

The serological reactions of Ar are different from the reactions of the other rough variants. While the Br and Cr variants are specific and agglutinate only with the homologous antiserum, Ar is agglutinated by antisera against At, although to a lower titer than by its own (homologous) antiserum.

DISCUSSION

The results obtained in this study indicate that it is possible to obtain from mucoid encapsulated *Klebsiella pneumoniae* smooth translucent colony variants which resemble the colonial form of *Escherichia coli*.   It is from these translucent variants that one derives the rough colony type described by Julianelle (1928), which resemble rough variants of other members of the coli-form group.   Furthermore, not only do the translucent colonies look like those of *Escherichia coli* but if acid and gas are produced in carbohydrate media, as in the case of Ct, it is impossible, because of the utilization of citrate, to distinguish the organism from some of the members of the *Citrobacter* group.

The similarity between the translucent variants of *Klebsiella pneumoniae* and organisms of the *Citrobacter* group is shown by their essentially identical colony morphology and biochemical reactions.   Still another analogy can be found in the serological reactions of the translucent variants and the serological hetero-geneity of the coli-form group.

Lovell (1937) described similar translucent variants produced by ageing cultures of encapsulated colon bacilli from "white scours" of calves and from one strain of *Aerobacter aerogenes*. Some of his translucent strains were serologically specific, while others reacted with each other in agglutination and precipitation tests.   The grey (translucent) colonies described by Lovell were smooth and were composed largely of non-encapsulated or-ganisms.   It appears that the translucent variants of Lovell and the variants of *Klebsiella pneumoniae* described here correspond more closely with the smooth non-capsulated variant of the pneumococcus which Dawson (1934) described.   Dawson's sug-gestion that the "R-1" colony variant described by Julianelle (1928) corresponds to the smooth, non-encapsulated colony form of the pneumococcus is not supported by the observations re-ported in this work.

Julianelle (1937) has reported that the rough variants of several serological types of *Klebsiella pneumoniae* cross-react.   The re-sults of this investigation do not confirm the occurrence of cross-

reactions among the rough variants of encapsulated *Klebsiella pneumoniae*.   Perhaps, the use of two different means of inducing dissociation explains the different results, and, as shown by Julianelle, even rough variants derived from the same serological type of Friedlander's bacillus are not always serologically identical.

SUMMARY

1. By growing mucoid encapsulated strains of *Klebsiella pneumoniae* bacilli types A, B, and C in lithium chloride peptone water two distinct colony variants have been obtained from each type.   These are designated "translucent" and "rough" forms.

2. The translucent colony variant is smooth and composed of non-encapsulated short rods resembling those of the parent strain.

3. The translucent variant has always been the first to appear in the dissociative process.

4. The rough variant was derived from the translucent type and produces a rough appearing colony which is composed of non-capsulated long rods or filaments.

5. The biochemical reactions of the original types and the derived variants have been recorded and their relations to the *Escherichia*, *Citrobacter*, and *Aerobacter* groups discussed.

6. The serological reactions of the original types and the variants derived from them have revealed a marked type specificity and in some cases an individual specificity within the type.

REFERENCES

BAMFORTH, J. 1928 An investigation of the bacilli of the Capsulatus-mucosus group. J. Hyg., **27**, 268–278.
DAWSON, M. H. 1934 Variation in the pneumococcus. J. Path. Bact., **39**, 323–344.
EDWARDS, P. R. 1929 Relationships of the encapsulated bacilli with special reference to Bact. aerogenes. J. Bact., **17**, 339–353.
GRATIA, A. 1922 The Twort-D'Herelle phenomenon. II. Lysis and microbic variation. J. Exptl. Med., **35**, 287–302.
HADLEY, P. 1925 The action of the lytic principle on capsulated bacteria. Proc. Soc. Exptl. Biol. Med., **23**, 109–111.
JULIANELLE, L. A. 1926 Immunological relationships of encapsulated and capsule free strains of Encapsulatus pneumoniae (Friedlander's bacillus). J. Exptl. Med., **44**, 683–696.

JULIANELLE, L. A. 1928 Bacterial variation in cultures of Friedlander's bacillus. J. Exptl. Med., **47**, 889–902.

JULIANELLE, L. A. 1937 Immunological specificity of bacterium aerogenes and its antigenic relation to pneumococcus, type II and Friedlander's bacillus, Type B. J. Immunol., **32**, 21–33.

JULIANELLE, L. A. 1937 Immunological relationships of encapsulated gram negative rods. Proc. Soc. Exptl. Biol. Med., **36**, 245–248.

LOVELL, R. 1937 Classification of bacterium coli from diseased calves. J. Path. Bact., **44**, 125–139.

O'NEAL, H. E. 1933 Dissociation of encapsulated bacteria. J. Bact., **26**, 521–538.

PARR, L. W. 1933 Mucoid Bacterium coli in feces of normal subject. Proc. Soc. Exptl. Biol. Med., **31**, 226–227.

REVIS, C. 1910 The Stability of the physiological properties of coliform organisms, Zentr. Bakt. Parasitenk., Abt. II., **26**, 161–178.

SMALL, J. C., AND JULIANELLE, L. A. 1923 Biologic and serologic studies of Bacillus mucosus group. J. Infectious Diseases, **32**, 456–470.

SMITH, T., AND BRYANT, G. 1927 Studies on pathogenic B. coli from bovine sources. J. Exptl. Med., **46**, 133–140.

. PLATE I

FIG. 1. Colonies of Ar; 24 hours.
FIG. 2. Colonies of Br; 24 hours.

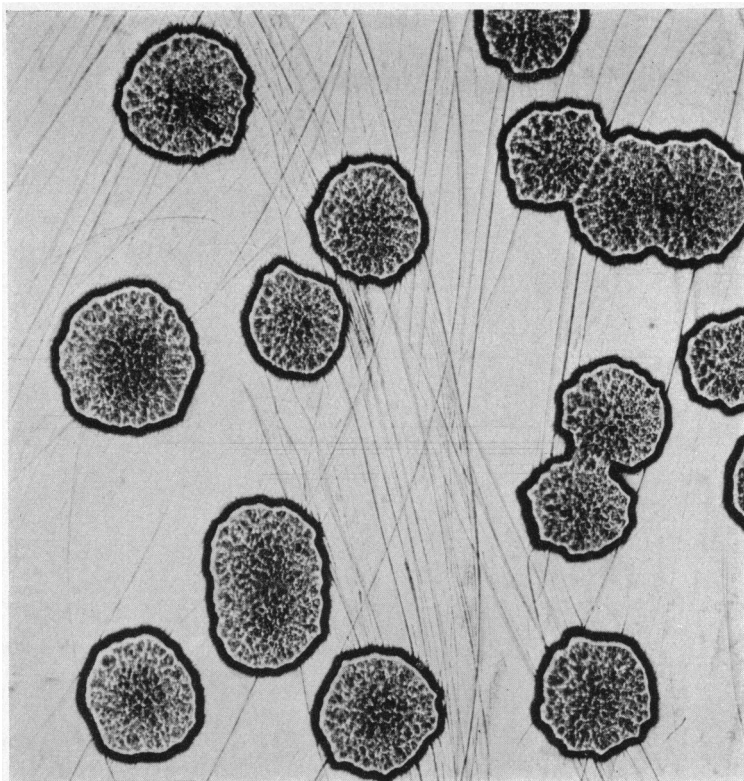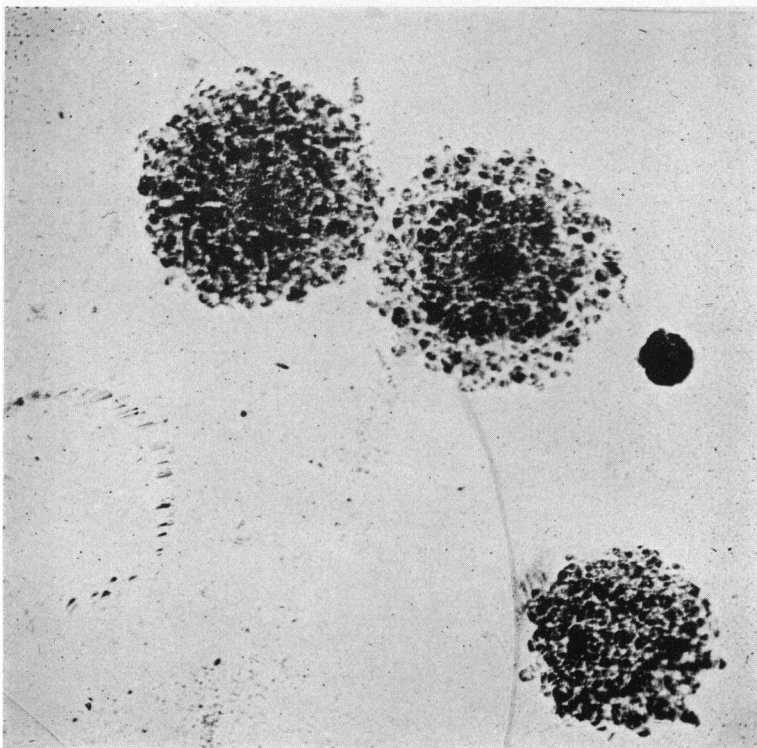FIG. 1

FIG. 2

(William A. Randall: Variation in Klebsiella Pneumoniae)

PLATE 1—*Continued*

FIG. 3. Colonies of Cr; 24 hours.
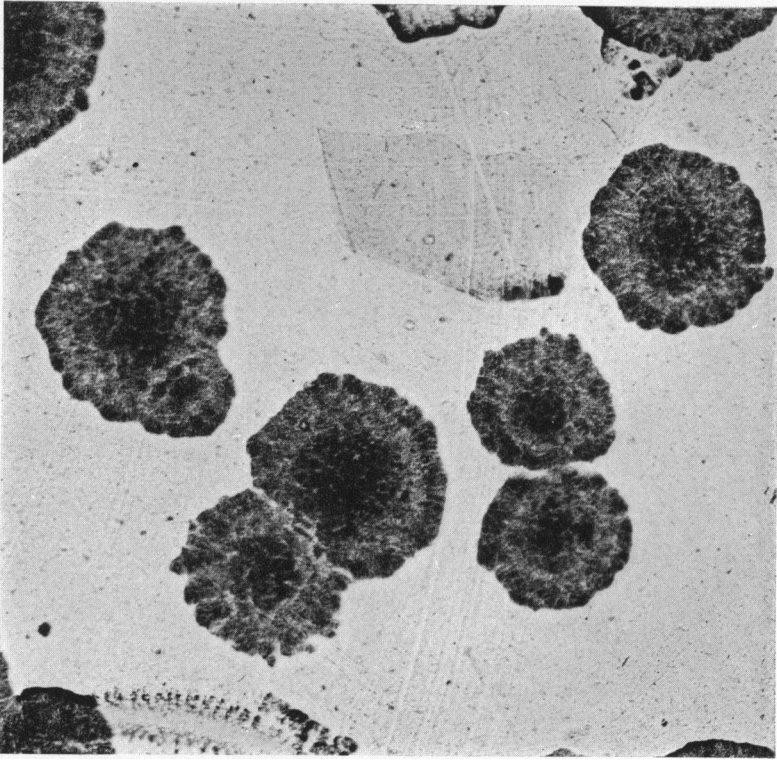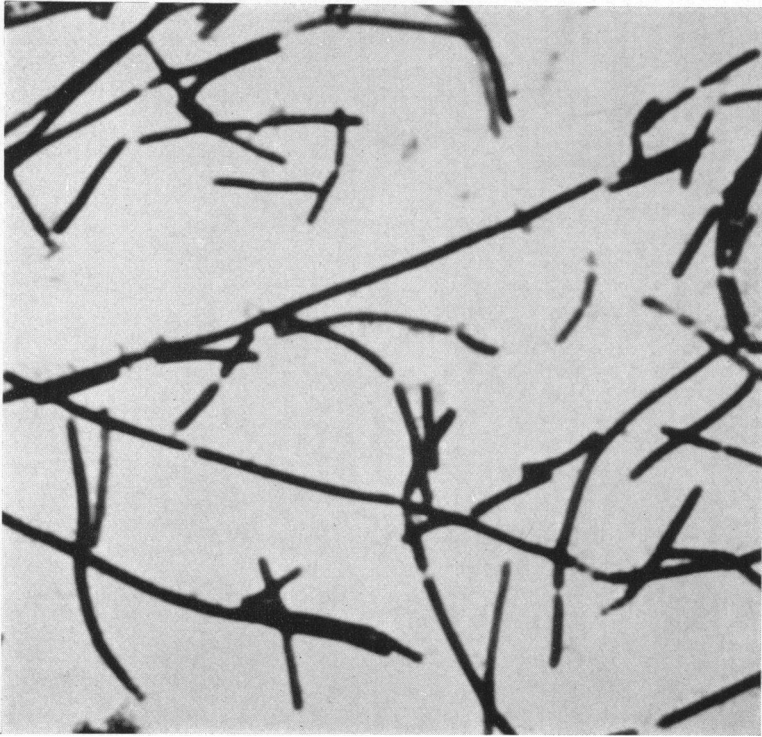FIG. 4. Gram stain of organisms in Br colony 24 hours.

FIG. 3

FIG. 4

(William A. Randall: Variation in Klebsiella Pneumoniae)

PLATE 1—*Continued*

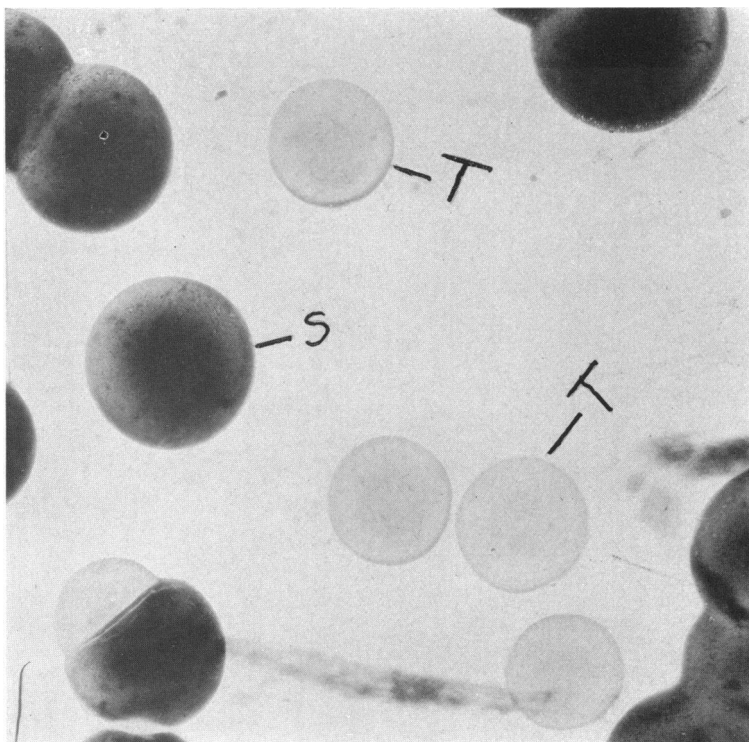FIG. 5. Colonies of B translucent and B smooth and mucoid; 24 hours.

FIG. 5

(William A. Randall: Variation in Klebsiella Pneumoniae)