BACTERIAL VARIATION

The Influence of Environment upon the Dissociation Pattern of *Klebsiella pneumoniae**

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The ability of a bacterial culture to manifest more than one set of culture characters is now recognized as a general phenomenon. Since the pioneer studies of Stryker,[21] Baerthlein,[3] Arkwright,[2] and de Kruif[6] two or more rather stable culture complexes, which, however, are capable of intertransition in a seemingly orderly manner under certain circumstances, have been described as occurring in many bacterial species. The more commonly observed of these complexes have been designated by the term "culture phase," and the transformation of one phase into another is referred to as "phasic variation" or "bacterial dissociation." The reader is referred to Hadley[9, 10] for general reviews.

The variation pattern of *Klebsiella pneumoniae* has been the subject of considerable study, as the result of which it is now considered that the phase pattern of this species is composed of at least three culture character complexes.[1, 5, 14, 16, 17, 18] Since these complexes seem analogous to the M (mucoid), S (smooth), and R (rough) culture phases of the pneumococcus,[5] these designations have been applied to the corresponding pneumobacillus variants.

The variation trend of the pneumobacillus, like that of the pneumococcus, is usually from M to S to R. Transition from the M to the S phase is indicated by a change from the opaque mucoid colony type to the translucent "coli-like" smooth colony type and by the loss of a number of culture characters including encapsulation, type specificity, the ability to stimulate the production of type-specific antibodies, specific polysaccharide production, and virulence. The S to R phase transformation is indicated by a further change in

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colony morphology—a flat, somewhat irregular colony with a finely rugose surface resulting,—by the loss of or change in an antigenic surface component of the cells, an increased tendency to agglutinate spontaneously in physiological saline and to give granular growth in broth, and by a change in cell morphology in the form of a marked tendency toward cell elongation.

Few of the variation studies that have been made on Friedländer cultures have included an attempt to correlate biochemical reactions with culture phase variability, and such attempts as have been made have resulted in seemingly contradictory findings.

Julianelle[14] reported that his colonial variant strains (R^1, R^2) gave the same reactions in dextrose, lactose, sucrose, maltose, and mannitol as did the parent culture. Also, the R colony culture of Alloway[1] had retained the power to ferment these carbohydrates.

In opposition to these findings, Randall[18] reported the rough colony cultures isolated by him from types A, B, and C pneumobacilli to show certain biochemical differences from the precursor smooth colony cultures. The type A rough culture had gained the ability to produce gas in dextrose, sucrose, and mannitol, and the property of producing indol. The type B rough culture had lost the ability to ferment any of the carbohydrates tested and could no longer utilize citrate as the sole source of carbon. The type C rough culture had lost all fermentative ability, it no longer reduced nitrates to nitrites, and it had gained the power to liquefy gelatin.

Experimental procedures and results

Cultures: Two strains of type A *Klebsiella pneumoniae** were used in the course of the work. Originally, they were isolated from two cases of pneumonia in man, the Sc strain by Julianelle, and the 108 strain by Levy-Bruhl of the Pasteur Institute. Both were gram-negative, non-motile, non-sporulating, encapsulated, facultatively anaerobic, rod-shaped bacteria that grew in the form of mucoid colonies on solid media. Gelatin was not liquefied and H_2S was not produced. Nitrates were reduced to nitrites, ammonia was produced, and H_2O_2 was broken down. The imvic reaction of Sc was —, ±, +, + and of 108 it was —, —, +, +. The two strains fermented a wide variety of substrates, including lactose and dex-

* The cultures were kindly furnished by Dr. Elizabeth Osterman.

trose; strain 108 producing acid and gas from each substrate attacked whereas strain Sc produced acid only. Antigenically, the two cultures stimulated the production of capsular agglutinins in the rabbit and gave cross-agglutination in these type-specific immune sera. Both organisms were virulent for mice as indicated by death following intraperitoneal injection.

Because the cells of both cultures gave rise to colonies of the mucoid type, were surrounded by capsules, stimulated the production of capsular antibodies, and were virulent it was concluded that these strains were in the M culture phase.

Just prior to beginning the transfer procedures pure line cultures were obtained by mechanical isolation of individual cells. Essentially the method described by Barber[4] in 1908 was used. However, for initial growth each freshly isolated cell was introduced into a droplet of sterile liquid medium on the surface of a sterile cover-slip. The cover-slip was then transferred, droplet down, to the top of a glass ring to which it was sealed, and which in turn was mounted in a shallow layer of agar in a Petri dish. Thus, the agar served to anchor the ring to the bottom of the dish and, as a source of moisture, prevented the droplet on the cover-slip from evaporating during the incubation period. With the strains used it was found necessary, in order to stimulate growth of the isolated cells, to inoculate the surface of the agar within the ring with another bacterium. For this purpose *Bacillus cereus* was used. During the period of incubation this spore-former developed as a solid film of growth on the agar surface, and as the result of this procedure approximately 60 per cent of the isolated cells developed into clones. In no instance was the spore-former found growing in the droplet. Since the only continuity between the two growths was a common atmosphere it must be concluded that the stimulation of the growth of the Klebsiella isolations was due, at least indirectly, to a change in the gaseous content of the atmosphere, and probably to an increase in the CO_2 tension, as this gas has been shown to be essential for the initiation of cell multiplication in many bacterial species.[8] After a few hours of incubation at 37° C. the droplet was observed microscopically. If growth had occurred the microculture was transferred to a tube of some suitable medium.

The several clones isolated from each of the two Klebsiella strains were morphologically, culturally, bio-chemically, and serologically like the respective parent cultures. A single clone from

each culture has been used in all of the variation experiments to be reported in this paper. These two clones have been given no special names, but are referred to by the names of the parent cultures (Sc or 108).

Transfer procedures: The physical and chemical agents used were LiCl, brilliant green, heat, and homologous antibodies. By adding the various agents to a basic medium composed of 1 per cent Bacto-peptone and 0.5 per cent NaCl in distilled water, or by subjecting this medium to different physical conditions during culture, the arbitrarily chosen environments were set up. Simultaneous serial passage in the basic medium served as a control. Since the immune bodies were added in the form of rabbit serum, which is not only a highly complex material but also an excellent source of nutriment, these added factors were controlled by passage of the strains in the basic medium to which a like volume of normal rabbit serum had been added.*

The two strains were grown serially in quadruplicate cultures in each of the different environments; one pair of each quadruplicate set being transferred daily and the other pair weekly. Because organisms in the R culture phase are often characterized by granular growth in liquid media and therefore tend to settle to the bottom of the culture container it was the procedure to agitate thoroughly the first tube of each culture pair just prior to transfer. To insure the carrying over of an average sample of the previous culture population to each fresh tube of medium in a series, a 4 mm. loop was routinely used for inoculation purposes. The volume transferred by this loop was approximately 0.01 ml.

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* The complete media, with the exception of those containing serum, were dispensed in ¾-inch tubes in volumes of approximately 10 ml. and sterilized by autoclaving at 15 lbs. for 20 minutes. In the preparation of the serum media, the serum was first sterilized by filtration through a Chamberland S-3 porcelain filter and one volume was added aseptically to 9 volumes of autoclaved basic medium. The serum media were dispensed in 3 ml. volumes into sterile ½-inch tubes, and after 24 hours of incubation at 37° C., were stored in the refrigerator pending use. It was also found necessary to store the media containing brilliant green in the refrigerator as the dye slowly lost its growth-inhibiting effect at room temperature. The media were made up weekly in order to avoid excessive concentration of the ingredients due to evaporation.

All media were adjusted (colorimetrically) to pH 7.5 during the first 11 weeks of the experiment, and to pH 7.0 thereafter.

The environmental conditions in which the two clones were grown serially were as follows:

I. Fixed environments.
  (1) Incubation temperature of 37° C. (± 1° C.).
    (a) The basic medium.
    (b) The basic medium + brilliant green (1:5,000,000).
    (c) The basic medium + LiCl (1 per cent).
    (d) The basic medium + immune serum (10 per cent).
    (e) The basic medium + normal serum (10 per cent).
  (2) Incubation temperature of 40° C. (± 1° C.).
    (a) The basic medium.

II. Changing environments. (These series were begun as offshoots of the corresponding cultures of the fixed environments.)
  (1) Incubation temperature of 37° C. (± 1° C.).
    (a) The basic medium + brilliant green (increasing concentration).
    (b) The basic medium + LiCl (increasing concentration).

Serial transfer in each of the above environments was carried on for a period of 20 weeks. In the varying environmental set-ups, the concentration of the agents was increased in a step-wise and leisurely manner, greater concentrations being substituted for the current ones only when the cultures subjected to daily transfer showed, in the presence of the new concentration, sufficient growth from the standard inoculum during 24 hours of incubation to allow the continuation of this transfer interval. With each increase in agent concentration in a daily transfer series, a like increase was instituted in the parallel weekly transfer series. In order to obtain growth in these latter cases, it was usually necessary to use a larger inoculum and to allow an incubation period longer than 24 hours for the appearance of growth. In general, 5 drops of the old culture sufficed to inaugurate growth within 48 hours. This result seems to indicate that the ability of a strain to grow from a standard inoculum in the presence of increasing concentrations of these agents is not only a function of time but also of generation.

At the conclusion of these series, Sc was being grown in a 1:1,000,000 concentration of brilliant green and in a 2.5 per cent concentration of LiCl, whereas 108 was being transferred in a 1:500,000 brilliant green concentration and in a 3.5 per cent LiCl concentration.

The ability of the strains to grow in the various environments

was judged by the occurrence of and degree of visible clouding in the media. In every environment strain 108 grew more eugonically than did strain Sc.

The fixed environments: With both strains, the initial clouding occurred earlier and the final turbidity was greater in the normal serum media than in the basic medium. The appearance of growth was also accelerated in the media containing anti-serum, but due to the granular or floccular nature of this growth turbidity comparisons were not possible.

In the concentrations used, brilliant green and LiCl had little effect either on the rate of multiplication or on the total amount of growth produced by these strains.

Growth in the basic medium at 40° C. had an accelerating effect on the multiplication rate of strain 108, whereas the same conditions caused a retardation of the growth of the Sc strain. However, the total turbidities produced by these cultures when grown in the basic medium at 40° C. seemed to be about the same as those resulting in this medium at 37° C.

The changing environments: In the culture series containing brilliant green the concentration of the dye seemed to have little effect on the total turbidity produced. If the strain was able to initiate growth in the presence of a particular concentration the final turbidity of the culture was approximately the same as that produced in media containing more dilute solutions of this agent. Although the growth of both strains was retarded in rate during the first transfers in an increased concentration of the dye, the organisms tended to overcome this effect and, indeed, 108 seemed to become able to do so completely.

In the changing environments containing LiCl, both the multiplication rate and the total amount of growth were decreased in the presence of higher concentrations of this agent. Over a period of time the strains tended to overcome the growth-retarding effect. However, above agent concentrations of about 3 per cent in the case of 108 and of about 2.5 per cent for Sc the medium seemed no longer suitable for the support of so large a population and the final turbidity was less.

Examination of the cultures for the occurrence of variants: At the completion of the impression procedures duplicate inoculations of each substrain were made into deep tubes of 0.5 per cent basic medium agar. After an initial 24 hours of incubation at 37° C.

one set was stored in the refrigerator and the second set was kept in a dark cabinet at room temperature. For further study of a substrain a heavy inoculum was transferred from the stock culture to a tube of nutrient broth, and after incubation this broth culture was used for further inoculation purposes. Thus, the substrains were always two culture generations removed from the impressing environments when the final study of their culture attributes was carried out. For comparative purposes like studies were repeated on the parent cultures.

Emphasis was placed on the study of those characters that have been reported to be involved in the phasic variation pattern of this species.

Biochemical character studies: The parent cultures and the substrains were tested for the ability to ferment dextrose and lactose, to utilize citrate as the only source of carbon, to liquefy gelatin, to grow anaerobically, to produce indol, acetoin, hydrogen sulfide, and ammonia, to break down hydrogen peroxide, to reduce nitrates to nitrites, and for their reactivity in the methyl-red test.

As the result of these procedures it was found that no qualitative changes had occurred in any of the substrains in the biochemical attributes thus determined. The biochemical activities of the substrains in every instance paralleled those of the parent strain.

The results of these biochemical tests are strong evidence that no gross contamination of the substrains had occurred during the course of the transfer procedures.

Colonial character studies: The parent cultures and the substrains were studied colonially by smearing agar plates with a loopful of a suitable dilution of each culture. Basic medium agar to which were added 0.5 per cent dextrose and 0.25 per cent Na_2HPO_4 was used; good colony differentiation resulted on this medium. Using this technic 100 to 200 well-isolated colonies were generally obtained on the plates.

The parent strains gave rise on this solid medium to circular convex, opaque colonies with a glistening surface, an entire edge, and a mucoid appearance. This colony form is referred to as the M (mucoid) colony form. Such a colonial examination of the substrains revealed that in many instances these cultures were still composed entirely of organisms giving rise to colonies of the M type. However, colonial variations had occurred in a majority of the substrains. In many of these cultures a variant type had completely

supplanted the parent organisms, hence, resulting in a pure colony culture of the variant. In others the cultures were found to be composed of a mixture of the original and the variant colony organisms. And in still others the cultures were made up of a mixture of variant types.

Two of the variants occurred very commonly and in substrains from both clones. On the basis of colony topography they have been given the designations S and R.

The *S colony* was circular, low-convex, and translucent with an entire or slightly undulate edge, a smooth dull surface, and an amorphous or finely granular internal structure.

The *R colony* was circular and flat with a somewhat irregular, often crenated appearing edge, a dull finely rugose surface, and a reticulated and granular internal structure.

No further variant types were obtained from the Sc-culture series but several other colony forms occurred on the plates from the 108 cultures.

The *MS colony*, so-called on the supposition that it might be an intermediate form, was a circular, low-convex colony with an entire edge, a smooth surface, and a finely granular structure. These colonies resembled the M type in having a mucoid appearance, but were like the S type when viewed by transmitted light, that is, they were translucent. They were observed on the plates from only one of the culture series and were the sole colony type obtained from this culture.

The *MR colony* resembled the M type in physical attributes other than topography. The surface of this variant was very rugose, giving a radially wrinkled appearance. Under the conditions of the experiment this variant occurred only in mixed culture with the M colony type, but could be obtained in pure colony culture by colony selection.

The *C colony* occurred in only one culture in which it had become the sole colony type. It was a small, convex colony with an undulate edge and an otherwise smooth appearance. The average diameter of this type was about half that of the average S colony.

The colony variants described above are mean colony forms and a certain amount of fluctuation occurred around these means. However, these fluctuations were not of such a degree that a continual gradation occurred between types and in most cases no difficulty arose in placing the colony type of the cultures.

The results of the colony type determinations are given in

TABLE 1

COLONY TYPE DISTRIBUTION IN THE SUBSTRAINS

*Substrain**	*Colony types*	
	Strain Sc	*Strain 108*
	Fixed Environments	
C-D-1	M	m & S
C-D-2	M	S
C-W-1	M	M & S
C-W-2	M	m & S
BG-D-1	S & R	S
BG-D-2	S & r†	S
BG-W-1	s & R	M & S
BG-W-2	S	M
LC-D-1	S	M & S
LC-D-2	M	M
LC-W-1	M & S	M & s
LC-W-2	M	S & R
NS-D-1	M	M & MR
NS-D-2	M	M
NS-W-1	m & S	M
NS-W-2	M	M
AS-D-1	R	S
AS-D-2	M	R
AS-W-1	R	S
AS-W-2	R	M & MR
H-D-1	S & R	S
H-D-2	S & R	S
H-W-1	R	M
H-W-2	M	M
	Changing Environments	
BG-I-D-1	R	S
BG-I-D-2	S & R	S & R
BG-I-W-1	S	S
BG-I-W-2	S	C
LC-I-D-1	S & r	MS
LC-I-D-2	m & S	M & mr
LC-I-W-1	M	M
LC-I-W-2	M	M & mr

* The various serial culture procedures have resulted, from the descriptive standpoint at least, in the production of 64 substrains from the two original clones. In order to prevent undue verbosity the following abbreviations have been adopted for describing these cultures:

BG = brilliant green
LC = LiCl
AS = antiserum
NS = normal serum
H = incubation at 40° C.
C = basic medium control
I = increasing agent concentration
D = daily transfer interval
W = weekly transfer interval
1 = 1st tube of culture pair
2 = 2nd tube of culture pair

The abbreviated substrain names were built up by suffixing these abbreviations to the clone name in the order; agent symbol, environment symbol, transfer interval symbol, and culture pair symbol. The absence of the symbol "I" denotes a fixed environment. The temperature of incubation was always 37° C. unless the symbol "H" is included in the substrain name. By translating the constituent parts of a substrain name according to the above definitions a complete description of the conditions of growth to which the substrain had been subjected can be obtained.

† In mixed colony cultures a colony type symbol in lower case indicates that a minority of the organisms in the culture were of this type.

Table 1. Since no two serial cultures of each strain were subjected to exactly the same environmental conditions during transfer, any detailed analysis of these results could only lead to fictitious findings. However, when series are grouped according to common environmental factors, variation is found to have occurred much more commonly under certain of the transfer conditions, although there were individual exceptions in almost every grouping.

Thus, the presence of brilliant green or homologous antibodies in an environment, or growth at the above-optimum temperature of 40° C. markedly enhanced the appearance of colony variant forms and in a majority of these series resulted in completely supplanting the parent colony type. On the contrary, growth in the basic medium enriched with normal rabbit serum tended to maintain these cultures in the M colony phase. Growth in the basic medium alone seemed to affect the two strains differently. Under these conditions of transfer the Sc cultures had remained in the M colony phase, whereas the S colony variant had appeared in all of the 108 cultures of these series.

The variation process did not seem to be differently affected to any marked degree by the two transfer intervals used, nor were there any consistent differences in the variation trends of tubes 1 and 2 of each culture pair as shown by analysis of these groups as wholes.

In order to make possible an attempted correlation of colony type with other culture characters pure colony cultures were used in further culture character studies. In those substrains composed of organisms of a single colony type at the conclusion of the transfer procedures obviously no further purification was necessary. However, in the case of those substrains composed of organisms giving rise to more than one colony type when serial transfer was concluded, pure colony cultures were produced by subculture from single, well-isolated, typical colonies of each type. Cultures breeding true to colony type were readily obtained by this method.

Morphological studies: For the purpose of morphological study cells from well-isolated typical colonies of the parent strains and of the substrains were emulsified in small drops of water on slides, fixed by heat, stained by the Gram technic, and examined under the oil-immersion objective.

Such preparations from 20- to 24-hour colonies of the parent strains showed these cultures to be composed predominantly of coccoid and short rods arranged singly and in pairs. Long rods and filaments were observed only rarely.

Also, like examination of such preparations of the subcultures revealed that many of the Sc substrains and almost all of the 108 substrains were morphologically very similar to the parent strains.

However, several of the Sc cultures and one of the 108 cultures had deviated notably from the original morphological type in the direction of cell elongation. The variation in this 108 subculture (BG-I-W-2) had occurred as a rather constant increase in individual unit length, while the change in the Sc cultures had resulted in the appearance of longer rods of varying length and of filamentous forms. In these latter cultures the short cell forms were still present in large numbers. The parent cultures and the substrains were consistently Gram-negative.

The results of the morphological studies are summarized in Tables 2 and 3. Analysis of these data reveals a lack of complete correlation between cell morphology and colony type. Although all of the R colony cultures of strain Sc were marked by a substantial increase in the proportion of longer rods and filamentous forms, a few S colony subcultures of this strain also varied appreciably in cell morphology from the coccoid and short rod units characteristic of the parent cultures. On the other hand, the R colony substrains of 108 showed no notable increase in cell length. For these reasons it would seem likely that colony roughness in these cultures may be due to either or both of two factors: (1) an increase in cell length; (2) a change in the surface character of the cells leading to a different cell arrangement in the colonies. Colony roughness was first attributed to this latter type of change by Nutt.[15]

Type of growth in nutrient broth: Stability of suspensions in physiological saline: Tubes of nutrient broth (pH 7.0) were inoculated with each culture and the character of the growth in this medium was noted after 24 hours of incubation at 37° C.

Stability in saline was tested by suspending growth from 24-hour tryptose agar slants in 0.5 per cent phenolized physiological saline. Results were read after 24 hours of incubation at 40° C.

The results of these tests on the parent cultures and on the substrains are included in Tables 2 and 3. These data show that several of the substrains have acquired the characters of growing non-homogeneously in broth and of agglutinating spontaneously in physiological saline. In most cases the results of these two tests correlated. This was expected, since it is probable that both tests are a measure of increased salt sensitivity.[22]

When an attempt is made to correlate these characters with

TABLE 2

VARIATION PATTERNS

I. The Sc substrains

(arranged according to colony type)

Substrain	Colony type	Cell morphology	Auto-agglutination		Agglutination in anti-M serum			Agglutination in anti-S serum		
			Broth	Saline	Result	Type	Titer	Result	Type	Titer
Parent culture	M	CB	—	—	+	C	80	—		
C-D-1	M	CB	—	—	+	C	40	—		
C-D-2	M	CB	—	—	+	F	160	—		
C-W-1	M	CB	—	—	+	C	160	—		
C-W-2	M	CB	—	—	+	C	160	—		
LC-D-2	M	CB	—	—	+	F	160	—		
LC-W-1	M	CB	—	—	+	C	160	—		
LC-W-2	M	CB	—	—	+	C	160	—		
NS-D-1	M	CB	—	—	+	C	160	—		
NS-D-2	M	CB	—	—	+	C	160	—		
NS-W-1	M	CB	—	—	+	C	160	—		
NS-W-2	M	CB	—	—	+	C	160	—		
AS-D-2	M	CB	—	—	+	C	80	—		
H-W-2	M	CB	—	—	+	C	40	—		
LC-I-W-1	M	CB	—	—	+	C	320	—		
LC-I-W-2	M	CB	—	—	+	C	160	—		
S-S-1*	S	CB	—	—	—			+	G	5120
BG-D-1	S	CB	—	—	—			+	G	5120
BG-D-2	S	CLB	—	—	—			+	G	2560
BG-W-1	S	CB	—	—	—			—		
BG-W-2	S	CB	—	—	—			+	G	1280
LC-D-1	S	CB	±	±	—			—		
NS-W-1	S	CB	—	—	—			+	G	5120
H-D-1	S	CLB	+	+	—			—		
H-D-2	S	CB	+	+	—			—		
BG-I-D-2	S	CLB	—	—	—			+	G	2560
BG-I-W-1	S	CB	+	—	—			—		
BG-I-W-2	S	CLB	+	+	—			—		
LC-I-D-1	S	CB	—	—	—			+	G	320
LC-I-D-2	S	CB	—	—	—			—		
BG-D-1	R	CLB	—	—	—			+	G	5120
BG-D-2	R	LB	—	—	—			+	G	1280
BG-W-1	R	LB	+	+	—			—		
AS-D-1	R	LB	+	+	—			—		
AS-W-1	R	LB	+	+	—			—		
AS-W-2	R	LB	+	+	—			—		
H-D-1	R	LB	+	+	—			—		
H-D-2	R	LB	+	+	—			—		
H-W-1	R	LB	+	+	—			—		
BG-I-D-1	R	LB	+	+	—			—		
BG-I-D-2	R	LB	+	+	—			+	G	2560
LC-I-D-1	R	CLB	—	—	—			+	G	160

CB = short rods. C = compact disc-like clumps.

CLB = long rods and filaments present. F = floccular agglutination.

LB = long rods and filaments very common. G = granular agglutination.

+ = positive reaction. — = negative reaction.

* An S colony culture isolated from a translucent sector in an M colony.

TABLE 3

VARIATION PATTERNS

II. The 108 substrains

(arranged according to colony type)

Substrain	Colony type	Cell morphology	Auto-agglutination		Agglutination in anti-M serum			Agglutination in anti-S serum			Virulence	
			Broth	Saline	Result	Type	Titer	Result	Type	Titer	Test Vol. in M.L.D. equivalents	Survival rate
Parent culture	M	CB	—	—	+	F	640	+	F	1280	1	0/32
C-D-1	M	CB	—	—	+	CF	320	—			2	5/5
C-W-1	M	CB	—	—	+	CF	160	+	G	1280	1	1/4
C-W-2	M	CB	—	—	+	C	160	—			1	0/4
BG-W-1	M	CB	—	—	+	F	160	—			1	3/4
BG-W-2	M	CB	—	—	+	CF	160	+	G[f]	10240	1	3/4
LC-D-1	M	CB	—	—	+	F	320	—			0	
LC-D-2	M	CB	—	—	+	C	160	—			1	3/4
LC-W-1	M	CB	—	—	+	CF	160	—			1	1/4
NS-D-1	M	CB	—	—	+	CF	320	—			0	
NS-D-2	M	CB	—	—	+	C	160	—			1	0/4
NS-W-1	M	CB	—	—	+	C	160	—			0.1	1/5
NS-W-2	M	CB	—	—	+	C	160	—			0.1	1/4
AS-W-2	M	CB	—	—	+	F	320	—			1	2/4
H-W-1	M	CB	—	—	+	C	320	+	G	10240	1	2/4
H-W-2	M	CB	—	—	+	CF	320	+	G[f]	10240	1	0/4
LC-I-D-2	M	CB	—	—	+	CF	320	—			0	
LC-I-W-1	M	CB	—	—	+	CF	160	—			0	
LC-I-W-2	M	CB	—	—	+	CF	160	—			0	
LC-I-D-1	MS	CB	—	—	+	F	320	—			0	
NS-D-1	MR	CB	—	—	+	F	160	—			1	4/4
S-S-1*	S	CB	—	—	—			+	G	10240	20	10/10
C-D-1	S	CB	—	—	—			+	G	20480	0	
C-D-2	S	CB	—	—	—			+	G	20480	20	4/4
BG-D-1	S	CB	—	—	—			+	G	20480	20	4/4
BG-D-2	S	CB	—	—	—			+	G	10240	0	
BG-W-1	S	CB	—	—	—			+	G	10240	0	
LC-D-1	S	CB	—	—	—			+	G	10240	20	4/4
LC-W-2	S	CB	—	—	—			+	G	2560	0	
AS-D-1	S	CB	+	+	—			—			20	4/4
AS-W-1	S	CB	+	+	—			+	G	1280	20	4/4
H-D-1	S	CB	—	—	—			+	G	2560	20	4/4
H-D-2	S	CB	—	—	—			+	G	20480	20	4/4
BG-I-D-1	S	CB	—	—	—			+	G	5120	20	4/4
BG-I-D-2	S	CB	—	—	—			+	G	2560	0	
BG-I-W-1	S	CB	+	±	—			+	G	2560	0	
BG-I-W-2	C	MB	—	—	—			+	G	20480	0	
LC-W-2	R	CB	—	—	—			+	G	2560	0	
AS-D-2	R	CB	+	+	—			+	G	2560	20	4/4
BG-I-D-2	R	CB	+	+	—			—			0	

CB = short rods. — = negative reaction. F = floccular agglutination.
MB = medium rods. 0 = not done. G = granular agglutination.
+ = positive reaction. C = compact disc-like clumps. [f] = fine.
* An S colony culture isolated from a translucent sector in an M colony.

colony form, it is evident that increased agglutinative sensitivity is most often associated with the R colony variants but that the correlation is not absolute. In an occasional culture composed of R colony organisms these reactions were negative, and in a few cultures of the pure S colony type the reactions were positive. However, in no case did an M colony culture acquire these characters.

Also, no complete correlation is found to exist between increased agglutinative tendencies and cell morphology. Increased salt sensitivity was demonstrable in several cultures composed of cells of the original morphological type and failed to be manifested by a few of those cultures in which elongated cell forms had appeared.

Agglutination studies: Antibody production was stimulated in the rabbit by the intravenous injection of 5 graded doses of a formalinized saline suspension of bacteria. Trial bleedings were made 10 days after the completion of an injection series. If the titer was unsatisfactory the animal was given a second series of injections. Antisera were prepared against M phase and what were believed to be representative S phase cultures of both Sc and 108.

Antigens for the agglutination test were prepared by suspending growth from 18- to 24-hour tryptose agar cultures in 0.5 per cent phenolized saline. An antigen turbidity of 80 to 85 (Evelyn colorimeter) was used in the test. The tube agglutination test was used, and was carried out in a water-bath for 24 hours at 40° C.

Since it was the purpose of these agglutination tests to detect changes in the surface antigenicity of the various cultures, it was necessary to use sera of known antibody content. Pure anti-M sera were prepared by repeated absorption of the M sera mentioned above with heavy saline suspensions of the corresponding strain in the S culture phase. No marked reduction in the capsular titer resulted from this procedure. The type specificity of these absorbed anti-M sera was verified by their failure to agglutinate mucoid type B and type C Friedländer strains. For the purpose of disclosing S surface agglutinogens, the antisera prepared against pure S colony cultures were used.

The degree of masking of the somatic agglutinogens by encapsulation was different for the two parent cultures. When Sc-M was set up against serial dilutions of anti-S serum no agglutination occurred. In this case the capsule seemed to screen completely the somatic agglutinogens. However, 108-M agglutinated to good titer (although to a lesser degree than did the corresponding non-encapsulated culture) in the anti-S serum in spite of the capsule.

Since the S agglutinins were removed from this serum by M organism absorption, the 108-M agglutination in this serum was due to the somatic and not to the capsular antigen.

Three types of agglutination were observed: (1) the clumping occurred as a single large disc-like flake; (2) the clumping appeared as a floccular sediment; (3) the bacteria agglutinated as granular clumps. The character of the agglutination was indicative of the antigen-antibody system operative. The disc-like agglutination occurred as the result of the reaction between encapsulated bacteria and the capsular antibody. It was never encountered in the bacteria plus anti-S serum systems. This type was especially prevalent in the Sc-M set-ups, but the 108-M cultures also tended to agglutinate in this manner in the lower serum dilutions. The floccular type of agglutination occurred most commonly in the anti-M sera and rarely in the anti-S sera. In all cases this type of agglutination was associated with organisms in the mucoid colony phase. The granular type was associated with bacteria in the S or R colony phase.

The parent strains and the substrains in pure colony culture were tested against both the anti-M and the anti-S sera. The results of these tests are included in Tables 2 and 3. A complete correlation was found to exist between mucoid colony form and agglutinability in type-specific serum. In every instance those cultures composed of organisms giving rise to M colonies on solid media were agglutinated by the anti-M sera. Conversely, the strains composed of organisms of the S or R colony types were not agglutinated by these sera. In accordance with the activity of the parent culture, the Sc substrains of the mucoid colony type were not agglutinated by the non-type-specific serum. Also, despite the fact that agglutination of the 108 parent culture was not prevented in the 108-S antiserum by its encapsulated state, most of the mucoid colony substrains of this culture had become inagglutinable under these conditions. The author knows of no plausible explanation for this difference in the shielding activity on the part of the capsule.

When the ability of the anti-S sera to agglutinate the substrains of the S and R colony types was tested, it was found that many of the Sc and a few of the 108 series were not agglutinated. Furthermore, in this group there is no correlation, seemingly, between the inagglutinability of the substrains and the colonial form. Not only were some of the R colony cultures agglutinated by the S antisera, but also many pure S colony cultures failed to agglutinate in these sera. Furthermore, such changes in agglutinative activity in anti-S

sera were not found to parallel the occurrence of either morphological or saline-sensitive variations in every instance.

Pathogenicity studies: The virulence studies were carried out in a heterozygous strain of Swiss albino mice. For testing purposes 12- to 15-hour basic medium cultures were diluted with sterile tap-water and the test doses, contained in 0.5 ml. volumes, were injected intraperitoneally. Mice weighing between 17 and 22 grams were used. In most cases viable counts were determined by serial plate dilutions. Death within 7 days was taken as the criterion of virulence.

For determining the relative virulence of the substrains two arbitrary standards were used. The smallest volume of the parent culture which consistently killed the injected mice was determined. A like volume of those substrains which had remained in the M colony phase, and therefore might be expected to have retained this character, was used in the test for virulence. In the case of those strains no longer in the M colony phase a culture volume of 20 times this dose was used in the test for lack of virulence. In those cases in which more information seemed desirable doses more varied in size were injected.

The pathogenicity of Sc-M was found to be very low. One-tenth of a milliliter of undiluted culture failed to kill mice consistently. Because of the low virulence of this strain pathogenicity studies on the Sc substrains were not done.

A titration of 108-M revealed that this strain was markedly the more pathogenic of the two parent cultures. On the basis of this preliminary titration it was concluded that 1 M.L.D. of this strain was contained in about 0.005 ml. (ca. 1,000,000 bacteria) of culture volume. Subsequent use of this volume proved, without exception, that at least one lethal dose of 108-M was always contained therein.

The results of the virulence studies are included in Table 3. These findings show clearly that virulence variations of a descending order had occurred in a majority of the substrains, and indeed, that complete loss of this culture attribute, as shown by the criteria applied, had become manifest in many cultures. Furthermore, there was a qualitative correlation of virulence and colony form.

In agreement with the findings of most previous workers,[7, 12, 17] virulence was found to be associated with the mucoid colony phase only. All of those substrains tested that had remained of the M colony type had retained the ability to infect mice fatally. Contrarily, all of the variant colony cultures that were examined for this

character failed to kill the mice even when the dose volume was equivalent to 20 M.L.D. of the parent strain.

However, a consideration of the quantitative aspects of the association of virulence with mucoid colony type does not lend support to the concept that pathogenicity and mucosity are manifestations of a single mechanism of the cell. Many of the substrains had undergone a marked decrease in mouse pathogenicity without at the same time showing any notable change in their colonial characteristics or in their agglutinability in type-specific antiserum. In one instance (subculture C-D-1) virulence appeared to be completely lost, as measured by the virulence test dose only. Since a marked reduction in the virulence of some of the substrains had resulted in the absence of coincident change in mucosity (therefore, supposedly without loss of encapsulation) and without any accompanying change in agglutinability in type-specific immune serum it seems probable that the two characters are controlled, at least in part, by different hereditary mechanisms.

The incomplete loss of virulence by these cultures, coupled with the fact that the extent of the reduction in this character varied in degree in different substrains, indicate that the loss of virulence in these cultures was a gradually occurring process.

When the virulence data on the M type substrains are considered, certain relations between the degree of virulence and the environments under which serial transfer was carried out can be seen. In general, those substrains that had been transferred serially in the basic medium and in this medium containing brilliant green, LiCl, or antiserum had become less virulent for mice. On the other hand, the medium containing normal serum seemed to maintain this organism in the original state of mouse virulence so long as the subculture was composed entirely of bacteria of the M colony type. Thus, it is a function of the medium which determines whether or not a culture of strain 108, when grown for long periods on laboratory media, suffers a loss in mouse pathogenicity while it retains its mucoid character and type specificity.

Discussion and summary

The character changes that occur in the culture mass during bacterial dissociation have been perhaps most often explained as being due to changes in the hereditary mechanisms of some of the cells, either as the result of spontaneous mutations or because of impressed changes in these mechanisms by the direct action of certain

environmental stimuli, followed by a selective action of the environment favorable to the mutant forms. However, in more recent years the dissociation process has been visualized by a number of workers as a normal cyclogenic development of the "species-microphyte"; the "ontogenetic" theory of Hadley.

At the present time neither of these theories is adequately, or even satisfyingly, supported by experiment. Convincing evidence for the mutation theory is very difficult to obtain in the absence of a sexual cycle. Adequate support for the cyclogenic theory must necessarily continue to be lacking until knowledge concerning the conditions under which these changes may progress is obtained and a complete cycle is shown consistently to occur.

However, if the assumption is made that one of these two theories is the correct explanation of culture-phase variation then at least one fundamental difference that is subject to experimental approach exists in these two concepts. If culture-phase transition is the result of mutations in the several characters involved, then, in the absence of character associations and in the presence of different selective or impressing environments, the sequence of character changes might be expected to occur in a haphazard manner during the interphase period. On the contrary, if the process is a cyclogenic one then the phase transition pattern should progress in an orderly fashion in those environments which allow a continuation of the phasic variation.

In the Friedländer cultures under study, the attributes characteristic of the M culture phase appear to be intimately associated with the mucoid colony form. Those cultures which had continued to be composed of organisms of the M colony type had also retained other characters indicative of the mucoid culture phase. All were composed of organisms of the typical short-rod morphology and all were agglutinated in a specific manner in type-specific immune sera. Furthermore, with a few exceptions these cultures failed to be agglutinated by anti-S sera. And, finally, all of the M colony cultures so tested showed some degree of mouse pathogenicity.

The association of these characters was further borne out by the study of the S and R colony variant cultures. In every instance in which the mucoid colony type varied to the smooth or the rough colony form the type-specific agglutinative characters had also disappeared. Also, all S and R colony cultures tested had become completely avirulent.

With the possible exception of the results of the virulence studies

these findings support the concept that all of the attributes characterizing the M culture phase of *Klebsiella pneumoniae* are the direct result of the ability of these organisms to produce specific capsular polysaccharide. Therefore, when polysaccharide productivity is lost all other M culture characters simultaneously disappear, the pure S culture phase resulting.

Contrary to the linked nature of the characters involved in the M to S culture-phase transition, the attributes indicative of the S to R phase transformation do not appear to be intimately associated. Some of the S colony cultures either had become inagglutinable in anti-S sera, or had become salt sensitive, or had developed filamentous forms, or showed combinations of these characters supposedly indicative of the R culture phase. Furthermore, an occasional R colony culture either had not become inagglutinable in anti-S sera, or failed to agglutinate spontaneously in physiological saline, or was composed of the short cellular elements characteristic of the S culture phase. In fact, when the R culture phase is defined by the criteria of colony roughness, saline sensitivity, loss of the S surface antigen, and elongated cell form, only five of the substrains can be said to have become entirely transformed to the R culture phase. This lack of correlation between colony form and other attributes of the S and R culture phases points out the fallacy of relying on colony form alone as the indicator of culture phase placing.

Not only do these results show that the character changes indicative of the S to R culture-phase transformation are capable of independent variation, but they prove also that the S to R interphase is not characterized by an orderly development of these changes in the culture attributes, at least not when the cultures are subjected to varying environmental conditions. These character changes appeared in almost every conceivable order; change in morphology, instability in saline, inagglutinability in anti-S sera, and roughening of the colonies; each occurred in one or more instances as the first indication of the beginning of the S to R phase transition.

These experimental findings fail, therefore, to support the "ontogenetic" theory of Hadley. The haphazard sequence would seem most readily explainable by the concept of cellular mutation aided by selective environmental conditions.

A similar lack of linked character variation in the S to R phase transition has been reported in several studies on members of other bacterial groups. Schütze,[20] working with salmonella types, reported "the degree of alteration in serological character varies independently

of the amount of colonial 'roughness' and the saline stability possessed by the variant." Savage and White[19] and White,[24] working with the same group of organisms, also failed to note any consistent correlation between colony roughness, saline stability, and antigenicity. They not only found that different cultures, indistinguishable in the degree of colony roughness, showed every grade of variation in their agglutinative behavior in anti-S sera, but also that several substrains derived from a single plate culture sometimes showed a variable agglutinative activity. A similar lack of correlation between agglutinative activity and colony roughness was noted in variant dysentery strains by Waaler[23] in 1935. According to the findings of this worker, a culture in the S phase serologically "could possess smooth, smooth-rough, or rough colonies."

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