THE B. MYCOIDES N HOST-VIRUS SYSTEM

I. Differences in Appearance and "Rate of Growth" of the Lysogenic and Parent Indicator Strains*

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PLATES 3 AND 4

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A survey of the literature for all articles on bacterial viruses published in the United States from 1943 to 1950 shows that 101 of 157 research studies or 64 per cent, are concerned with investigations of the T series of phages and their host *Escherichia coli* B.

Intensive study of other host-virus systems, particularly those exhibiting lysogenicity, is obviously desirable and to some extent this has been begun. Within the past 3 years, for example, there have appeared research reports dealing with the host-virus systems of *Staphylococcus muscae* (Price, 1947, 1948), *Streptococcus lactis* (Cherry and Watson, 1949), *Clostridium madsonii* (Gold and Watson, 1950), *Actinomyces griseus* (Woodruff, Nunheimer, and Lee, 1947), and *Erwinia carotovora* (Chapman, Hillier, and Johnson, 1951). In addition, an important reexamination of lysogenic strains of *Bacillus megatherium* is being carried out by Northrop (1951) and by Lwoff and his group (Lwoff and Gutmann, 1950 *a*; Lwoff, Siminovitch, and Kjeldgaard, 1950 *b*; Lwoff and Siminovitch, 1951).

As a result, it is already evident that there may be fundamental differences as well as similarities in the multiplication of bacterial viruses. Findings on the release of virus from the cell in the *Staphylococcus muscae* system (Price, 1948), on the ability of phage-infected cells of *B. megatherium* (Lwoff *et al.*, 1950 *b*) to undergo two divisions before lysing, and on the origin of virus phosphorus in a *Shigella dysenteriae* system (Goldwasser, 1949) indicate important deviations from the sequence of events postulated for bacteriophage growth in the *E. coli* phage-bacterium system.

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The present paper is the first in a series describing a study of a new bacterium-virus system, the *Bacillus mycoides* N and homologous phage.

EXPERIMENTAL RESULTS

Experiments on phage multiplication in *Bacillus mycoides* N in nutrient broth showed that there was a sudden release of free phage into the medium, following a 1 hour period of intracellular growth (Baer and Krueger, 1952). Coinciding with the liberation of phage, a decrease in the turbidity of the suspension occurred; however, clearing was invariably incomplete. With multiply infected cells, about 5 per cent of the original population remained unlysed. Some of the intact cells multiplied so that the turbidity of the suspension increased again.

Three lines of evidence warrant the assumption that all the unlysed survivors and all their progeny were lysogenic: (1) Upon picking from isolated lysogenic colonies and transferring to broth, subcultures always released phage into the medium; (2) a direct count of a suspension of well washed lysogenic bacteria corresponded closely to its plaque count; and (3) all efforts to rid the infected cells of virus were unsuccessful, even though lysogenic bacilli were subjected to pH 4, antiphage, heating, supersonic vibrations, 0.001 M iodoacetate, and glucose plus nutrient broth.

Cultures of the lysogenic strain of *B. mycoides* N, grown in nutrient broth, could be distinguished at a glance from those of the parent indicator strain. When the lysogenic strain was cultivated in shallow layers of broth in flasks on a rotary shaker at 30°C., the cells grew in isolated clumps in an otherwise clear medium (Fig. 1 *a*). The indicator strain, on the other hand, appeared to grow uniformly suspended throughout the medium, although a few large clumps were also present (Fig. 1 *b*). In nutrient broth, the lysogenic cells also seemed to multiply at a strikingly slower rate than the indicator bacilli from which they originated.

It was difficult to find a suitable method for estimating bacterial numbers. The usual techniques—turbidometry,[1] plate counts, and direct enumeration by use of a counting chamber—have no quantitative significance when applied to agglutinated suspensions. Estimation of the rate of growth was attempted by use of micro-Kjeldahl nitrogen determinations but failure of the clumps to pack well during centrifugation led to the loss of organisms when the supernatants were decanted.

Despite the imperfect settling of lysogenic cells upon sedimentation, a rough comparison of the total amounts and rates of growth of the two strains was achieved by measuring the column lengths of packed cells centrifuged into a

[1] After these studies were completed, Fisher (1951) reported the successful photometry of non-disperse bacterial suspensions by use of the Coleman model 7 photonephelometer.

capillary tube of fine bore, as described by Krueger (1930). The experiment was conducted as follows:—

The lysogenic and indicator strains of *B. mycoides* N were grown for 21 hours at 30°C. in 100 ml. of nutrient broth on a shaker. (The number of lysogenic cells used as an inoculum was 2.5 times greater than that of the indicator inoculum.) Each culture was stirred up for 2 minutes in a Waring blendor to get a temporary even distribution of cells. A Klett reading for each culture was taken. Then 1 ml. of the lysogenic strain was pipetted into the small capillary tube; the same procedure was carried out for the indicator suspension. The length of the cell column after centrifugation was measured by means of vernier calipers. The cell crop of the indicator strain greatly exceeds that of the lysogenic strain at the end of 21 hours (Table I).

TABLE I

Difference in Total Amount of Growth of 21 Hour Nutrient Broth Cultures of Lysogenic and Indicator Strains

	Indicator strain	Lysogenic strain
Klett reading (red filter; 660 mμ)	230	47
Volumn length of packed cells,* *mm.*	17.0	7.5

* Centrifuged into the small capillary tube of Krueger (1930).

The blended lysogenic and indicator cultures were then diluted 1:7 and 1:17 respectively with broth in order to get lag phase suspensions containing equal cell volumes per milliliter. Aliquots of 2 ml. of the lysogenic strain were distributed to a number of small tubes which were agitated at 30°C. in a water bath. At intervals a 1 ml. sample from a tube of the lysogenic strain was placed in a small Krueger tube and spun down. An identical procedure was used for the indicator strain. The meniscus of the lysogenic cells was neither level nor sharp; the packing of the lysogenic sediment was less firm than that of the indicator cells. Consequently only a rough measurement of the column length of the lysogenic bacilli was achieved. Nevertheless, the cell concentration of the lysogenic strain in any particular sample taken after the start of the 23 hour experiment was far less than that of a corresponding specimen of the indicator strain (Table II).

There are two interpretations which readily suggest themselves for the apparent difference in the "rates of growth" of the two strains:

1. The lysogenic cells have a longer generation time than the indicator bacilli, because the effect of aggregation on the growth of the lysogenic cell is to decrease the availability of the nutrients of the medium to the cell.

2. The lysogenic population is subject to an active lytic process. If some cells were lysing, it would presumably explain how release of phage from the lysogenic cell occurs. Clump formation (resulting in a decrease in oxygen tension,

Eh, effective viscosity for diffusion processes, etc.; Mitchell (1951)) may favor active phage multiplication which in turn would lead to lysis of the carrier cell. There was also a difference in the way that the lysogenic and normal strains grew on solid media. The indicator strain forms the mold-like colonies on nutrient agar which characterize members of this species (Fig. 2). In Fig. 2, two more or less separate "types" of indicator colonies are present—a rhizoid spreader and an amorphous fuzzy form in which few distinct filaments are discernible. The change from one type to the other seems to be governed largely by local moisture conditions on the surfaces of the plate.

TABLE II

Comparison of "Rates of Growth" of Lysogenic Strain and Parent Indicator Strain by Measurement of Column Lengths of Packed Cells Centrifuged into a Capillary Tube

Time	Length of cell column			
	Indicator strain		Lysogenic strain	
hrs.	*mm.*		*mm.*	
0.0	0.8, 1.1*	NPT	0.9, 0.9*	NPT
2.0	3.9, 4.7*	PS	2.0, 2.0*	NPT
3.8	11.2	PS	2.4	NPT
5.0			2.2	NPT
5.5			2.8	NPT
7.5			4.4	NPT
9.2	20.0	PS	8.5	NPT
22.8	23.7	PS	6.5	NPT

NPT, cells did not pack down tightly in capillary tube; measurement therefore inaccurate. PS, satisfactory packing; level, clear meniscus.

* Duplicate samples were run.

In the lysogenic strain, the spreading propensity of *B. mycoides* is minimized. In Fig. 3, 18 hour colonies of the two strains are shown. The lysogenic colonies (Fig. 3 *b*) are much smaller than the indicator colonies (Fig. 3 *a*), most of which consist of a cloudy mass in which no individual filaments can be distinguished. After 72 hours, widely spreading outgrowths have developed from the edges of the 18 hour indicator colonies (Fig. 4 *a*), whereas the lysogenic colonies are devoid of filamentous extensions, although they do show an increase in diameter (Fig. 4 *b*).

The surfaces of another set of nutrient agar plates were carefully dried before plating. The rhizoid network comprising each individual indicator colony covers a rather large area (Fig. 5 *b*); the lysogenic colony consists of a small central spot surrounded by stubby arms (Fig. 5 *a*).

When the center of a nutrient agar Petri plate was inoculated with cell material from the lysogenic or indicator strain, the comparative rates of outward extension of the two strains could be measured (Table III). Before the

44th hour, the virus-free strain had reached the edge of the Petri dish (Fig. 6 *b*), while the growth of the lysogenic strain remained confined to the inner portion of the Petri dish (Fig. 6 *a*). Moreover, there seems to be a cleared area in the center of the lysogenic cell mass.

When the Gratia layering technique (1936) was used, a difference in the character of growth of the two strains was again observed. After 16 hours, the indicator cells enclosed within the thin layer of soft agar formed a dense uniform coating (Fig. 7 *b*); contrariwise the lysogenic culture had the appearance of an irregular patchwork of alternate areas of light and heavy growth (Fig. 7 *a*). At the end of 48 hours growth was heavier but still spotty (compare Figs. 8 *a* and 8 *b*).

TABLE III

Distance of Linear Spread of Lysogenic and Indicator Strains of B. mycoides N Inoculated at Center of Nutrient Agar Plate*

Experiment No.	Time after inoculation‡	Lysogenic strain	Indicator strain
	hrs.	*mm.*	*mm.*
1	19	11–13	30–28
	43	30–23	75–75
2	20	3–3	13–20
	45	9–7	Irregular spread over plate
3	18	2–6	20–20
	43	30–22	75–75
4	44	17–16	75–75

* The distance over which growth extended was measured from one edge to the other through the center of the plate in two directions perpendicular to each other. Plate diameter = 75 mm.

‡ Plates were incubated at 30°C. for 18 to 20 hours, then at room temperature.

Although the lysogenic and parent strains of *B. mycoides* N could be differentiated when grown in mass culture, the size and shape of the virus-infected and phage-free cells were the same when observed with the light microscope in unstained and Gram-stain preparations. In electron micrographs (Baer, Gold, Smith, and Krueger) the individual bacterial bodies are also indistinguishable; however, "poorly washed" lysogenic bacilli are surrounded by phage-like particles and the indicator bacteria are not.

Experimental Procedure

The indicator strain of *B. mycoides* N was obtained from the collection of Dr. A. P. Krueger. The phage (designated as mycoides phage N) active against the indicator strain was isolated from lake mud by applying the principle of the enrichment culture. The phage was purified by three successive plaque isolations.

Experiments were carried out in Difco nutrient broth or agar.

Maintenance of Stock Cultures and Preparation of Experimental Cultures

Indicator Strain.—Stock cultures were grown on nutrient agar slants (transferred every 10 days), incubated at 30°C. for 18 hours, then stored at 5°C. As experimental cultures, agar-grown cultures were unsatisfactory. The cell material was difficult to scrape off the surface and did not emulsify well when resuspended in broth. Consequently the broth suspension contained clumps of material. A clump-free suspension of uniform turbidity could be readily prepared by growing the cells in broth and then straining the culture through filters in which the Seitz pad was replaced by a dozen layers of gauze. Experimental cultures were grown in shallow layers of nutrient broth at 30°C. on a rotary shaker for 16 to 20 hours, filtered through gauze, and diluted with nutrient broth to the desired concentration before using.

Lysogenic Strain.—Preparing the lysogenic culture for work constituted a problem which was not entirely solved. Agar slants could not be conveniently used for making transplants. Growth was quite sparse even after 36 hours at 30°C. and adhered to the agar so tenaciously that it was not possible to remove a sufficient amount of cell material with a loop.

Transfers of the lysogenic culture were more expediently made in the form of shallow layered broth cultures grown at 30°C. on a rotary shaker in small Erlenmeyer flasks for 16 to 22 hours; cultures were stored at 5°C. Since the lysogenic cells grew in the form of large clumps which settled quickly to the bottom of the flask when it was not being shaken, some of the sediment was pipetted up and inoculated heavily into fresh broth whenever a transplant was desired.

By "homogenizing" the lysogenic culture in a Waring blendor for 1 minute and filtering through gauze, the size of the clumps could be reduced so that by macroscopic observation the culture looked uniformly suspended. Cultures were then diluted with fresh broth or sedimented down and resuspended in fresh broth. Blended-filtered cultures were suitable for making turbidity readings with a Klett-Summerson photoelectric colorimeter (660 mμ filter), since the bacteria remain dispersed throughout the medium for periods up to 4 hours.

Our thanks are due to Mr. Charles L. Walker for suggesting that differences in growth of the two strains could be shown by the method of centrally inoculating agar plates.

SUMMARY

There were differences in the way the lysogenic strain N of *B. mycoides* and the parent indicator strain grew on nutrient agar and in nutrient broth.

1. On agar, the indicator culture traveled more quickly over the agar surface than the phage-carrying strain; the total extent of spread was greater.

2. In broth, the indicator strain grew diffusely throughout the liquid, the lysogenic cells in clumps. The virus-infected strain appeared to grow more slowly. This may reflect (*a*) the effect of aggregation on the generation time of the lysogenic strain, (*b*) an active lytic process in the lysogenic population which is further enhanced by the effect of clump formation on the environment of the cell.

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EXPLANATION OF PLATES

PLATE 3

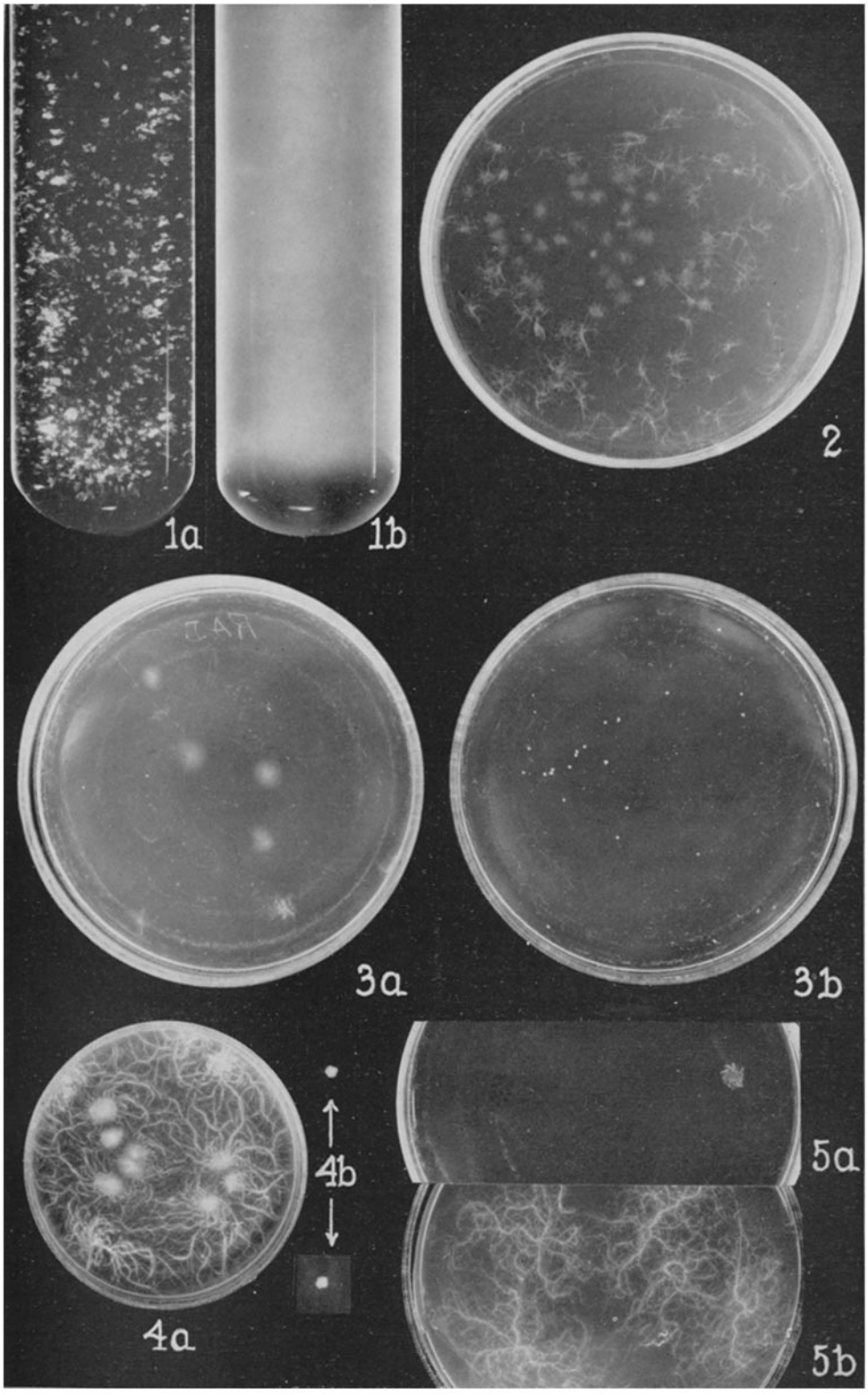
FIG. 1. Subsurface liquid growth of 20 hour old nutrient broth cultures (grown on a rotary shaker) of the (*a*) lysogenic and (*b*) indicator strains of *B. mycoides* N.

FIG. 2. Colonies of the indicator strain of *B. mycoides* N on nutrient agar after 14 hours at 30°C.

FIG. 3. 18 hour colonies of the (*a*) indicator and (*b*) lysogenic strains of *B. mycoides* N on moist nutrient agar plates.

FIG. 4. 72 hour colonies of the (*a*) indicator and (*b*) lysogenic strains of *B. mycoides* N.

FIG. 5. 18 hour colonies of (*a*) lysogenic and (*b*) indicator strains on carefully dried agar plates.



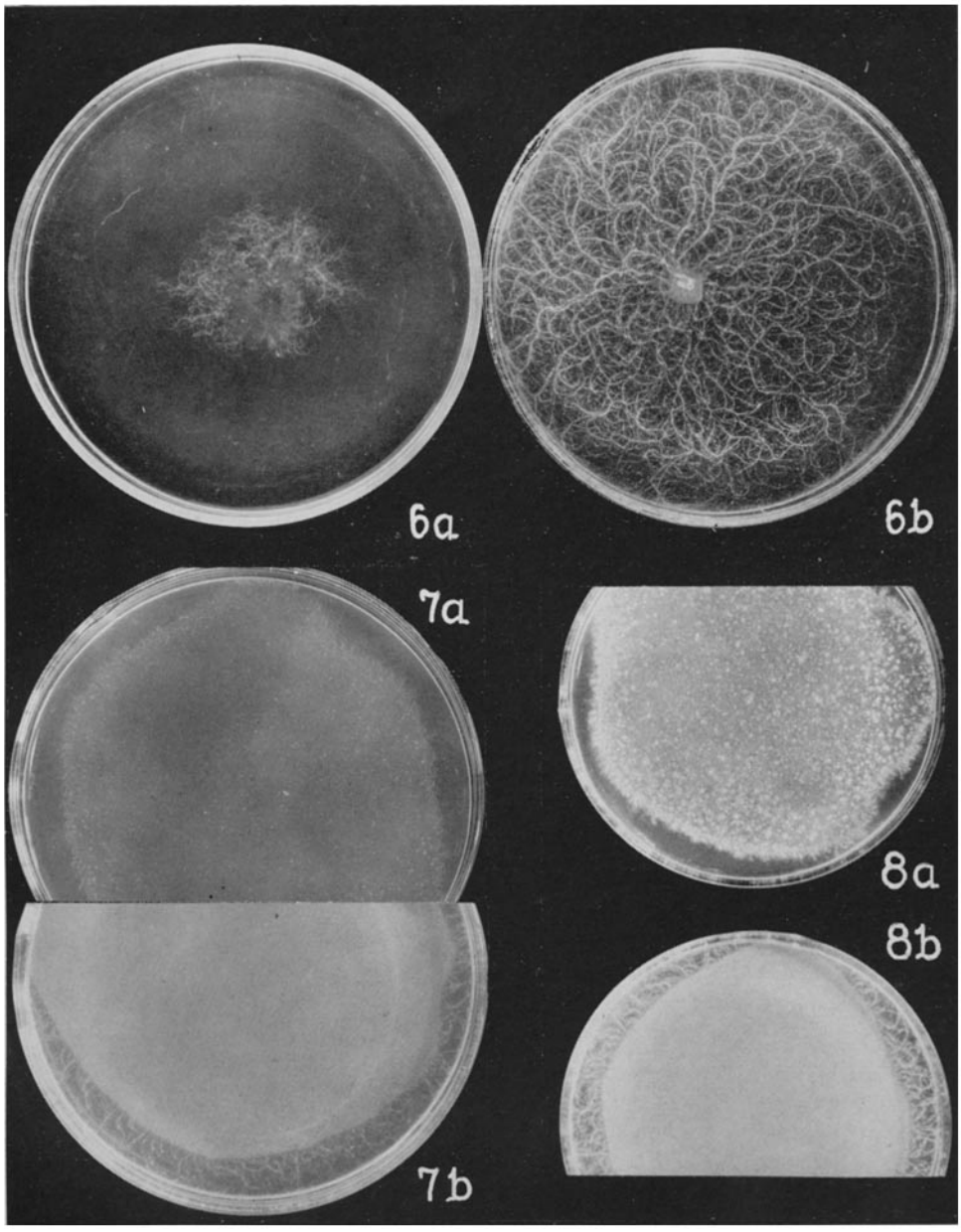
(Baer and Krueger: *B. mycoides* N host-virus system. I)

PLATE 4

FIG. 6. Difference in rate of spread and total amount of growth of (*a*) lysogenic and (*b*) indicator strains of *B. mycoides* N when cell material is inoculated in center of agar plate.

FIG. 7. Difference in character of growth when Gratia layering technique is used. After 16 hours, the indicator cells (*b*), enclosed in a layer of soft agar, form a dense uniform coating. (*a*) The lysogenic culture is an irregular patchwork of tiny islands of heavy growth surrounded by light growth.

FIG. 8. Plates shown in Fig. 7 after 48 hours. Both indicator strain (*b*) and lysogenic strain (*a*) show heavier growth.



(Baer and Krueger: *B. mycoides* N host-virus system. I)