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DISSOCIATION AND LIFE CYCLE OF

BACILLUS SUBTILIS

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By

Herbert C. Batson

A thesis submitted to the committee on Advanced Degrees, South Dakota State College of Agriculture and Mechanic Arts, in partial fulfillment of the requirements for the degree of Master of Science.

May 1934

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DISSOCIATION AND LIFE CYCLE OF BACILLUS SUBTILIS

INTRODUCTION

B. subtilis is one of the oldest known bacterial organisms, having been described by Ehrenberg as early as 1838. It was later more accurately described and placed in its present classification by Cohn in 1872. It has served as the basis of many investigations, and it is doubtful if any other aerobic sporeforming organism is now as well known as this.

The presence of dissociative forms in cultures of B. subtilis has been recorded by many investigators, the most exhaustive work on this subject being that of Soule (1928). He worked with a number of different strains of B. subtilis, isolated from various sources, and found that individuals giving rise to at least three different colony types could be isolated: the S or normal type, the R or resistant type, and the P or phantom type. He found the chief differences, among these three, to be in motility, colony formation and antigenic strength. He also found many intermediate colony formations but did not study the variation in cell morphology which may have accompanied these colony transformations. He reports but little variation in physiological characteristics and states that there is no difference at all in the Gram staining reaction. The formation of secondary colonies he considers quite common, but finds the secondary growth to be characteristically either R or S. He says nothing of variation in physiological characteristics of strains isolated from this secondary growth, nor does he report the presence of a filterable phase in any

of the strains of B. subtilis that he studied.

Löhnis and Smith (1916) report considerable evidence indicating the presence of a definite life cycle in B. subtilis. They specifically mention the presence of specialized reproductive bodies termed gonidia and they also state that the smallest of these readily pass through a Chamberland filter thus introducing a filterable phase in the life cycle.

Dr. Evans (1929) noticed the transformation of rod to streptococcus taking place repeatedly in B. subtilis, and further found considerable evidence that there was a filterable virus produced in one stage of the life cycle of B. subtilis. She found considerable evidence that this virus was either identical with or closely similar to the virus of encephalitis in rabbits. The work of Tang and Castaneda (1928) however, does not confirm the presence of the virus form.

The present investigation deals with incitants to the dissociation reaction, and with the variations in colony morphology, cell morphology, physiological characteristics, methods of reproduction, and antigenic properties of B. subtilis. The matter of a filterable phase has also been investigated, and when the various findings have been correllated they show the apparent presence of a definite life cycle.

INVESTIGATION

Source of Organisms

Three strains of B. subtilis were used in this investigation. One strain was obtained from the Department of Bacteriology and Immun

ology of the University of Minnesota, another from the Department of Botany and Bacteriology of South Dakota State College, and still another from the American Type Culture Collection (#243). Preliminary study showed all three strains to be identical in morphological and physiological characteristics. All of them produced characteristic luxuriant amoeboid colonies on agar. When stained by Gram's method they retained the stain, and appeared as long slender rods occuring as extremely long chains. Physiologically they produced acid in dextrose and sucrose, peptonized milk without producing acid, liquefied gelatin and hydrolyzed starch. All strains were moderately motile.

Factors Inducing Dissociation

For the purpose of determining the physical and chemical environmental conditions necessary to induce the dissociation reaction experiments were conducted which would reveal the influence of: (1) the consistency of the medium, (2) the volume of the medium, (3) the reaction of the medium, (4) ascitic fluid, (5) carbohydrates and glycerol, (6) aging broth cultures, and (7) surface tension depressants upon the dissociation reaction. The results obtained from these experiments were in such close agreement with those reported by Soule (1928) that it is not necessary to record them fully here. It was found, however, that it is not necessary to add traces of chemical poisons, or in any other way to modify the environment by extreme methods, in order to incite rapid dissociation. In fact, dissociation was induced by all the above methods. Not only were typical R and S forms produced in abundance, but there were also numerous intermediate

colony types. Preliminary examination of cells isolated from the different colony types revealed variations in cell morphology corresponding with the several colony types. The S colonies were characterized by extremely long chains of slender rods; the R type colonies by larger rods appearing singly, in pairs, and in short chains. Some of the intermediate colonies yielded cells of various form varying from long spirals to short cocci-bacilli which could easily have been mistaken for streptococci. In general it was found that any set of environmental conditions which tended to increase the rate of reproduction, or to prolong the period of reproduction, also brought about active dissociation. Unusual forms were produced in some instances, but as they were obvious responses to a peculiar environment, and as they could not be consistently repeated at will, they were considered of no great significance in relation to any definite life cycle.

Morphological Variation in Colonies and Cells

In an attempt to determine whether or not there existed any correlation between colony variation and cell variation, and whether or not there was a definite sequence to the appearance of certain forms, the following experiment was conducted: serial transfers were made in flasks, each containing 250cc. of Difco nutrient broth. Parallel serial transfers were made in the same manner except that 4% C. P. glycerol had been added to the broth. Loop transfers were made every 24 hrs. to the next flask in each series. After 24 hrs. incubation a loop of the broth was transferred to an agar slant. Transfers from the glycerol broth were made to 4% C. P. glycerol

At the same time that transfers were made from the broth cultures to agar, streak plates and pour plates were made, and also a giant colony bottle was inoculated. In all cases of transfer from glycerol broth the organisms were placed on glycerol agar. All subcultures were incubated at 37°C. for 24 hrs. and then left at room temperature until observations were completed.

The growth on agar slants was observed macroscopically and microscopically every six hours for sixty hours, or until the morphology of the cells had become constant. Both Gram stain and Congo red negative stain were made at each of the six hour intervals. The Congo red negative stain was found to be admirably suited to this kind of work because it revealed internal changes in the cells, and living and dead cells could be easily distinguished. At the same time that the slant cultures were examined microscopically, streak plates were made to see if any possible variation in the cells would give rise to morphologically different colonies. These plates were incubated at 37°C. for 24 hrs., observed, and then reincubated at room temperature for further successive observations at the end of 48 hrs., 72 hrs., and one week. This technique was continued until the organisms had been passed through ten flasks, each containing 250cc. of broth. Both colony and cell morphology had become constant before transferring was suspended.

Variations in Colony and Cell Morphology

Accompanying photographs show the morphological changes in both colony and in the individual cells. The changes appeared in the

order given below, but it is not wished to infer that all colonies were identical in the various stages of development. No attempt has been made to study all the various colonies noticed; attention has been paid only to that general trend of development which has been noted again and again, and which is capable of reproduction under the same given environmental conditions.

When an agar pour plate is made of the normal or S form of B. subtilis, the bacteria, which are in extremely long chains, give rise to a series of small colonies along the entire length of the chain. While the colonies are young and not fully developed the original position of the chains is quite readily traced. This is clearly shown in Fig. 1.

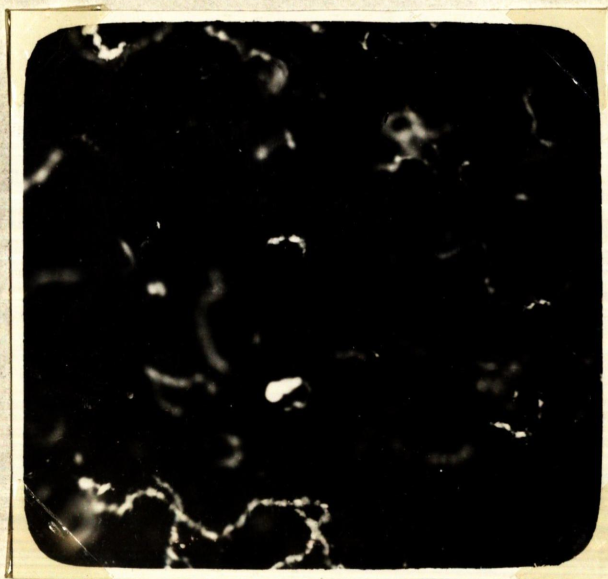
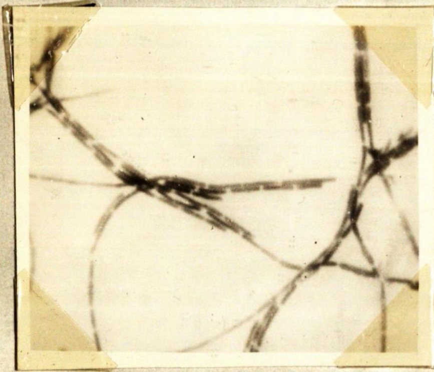


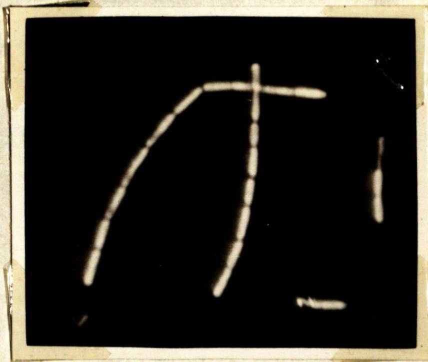
Fig. 1. B. subtilis. Type S colonies on glycerol agar after twelve hours. The colonies have developed from long chains of organisms. X36.

Plain nutrient agar gave rise to the same type of growth after incubation for a like period.

The morphology of the cells from a twelve hour agar slant,

inoculated with the S type of B. subtilis, corresponds exactly with the description as given by Soule (1928). The slender rods and long chain habit of growth is characteristic. (Figs. 2 & 3).





Figs. 2 & 3. B. subtilis. Characteristic long chain habit of growth of type S from glycerol agar after twelve hours. Gram and Congo red negative stain. X1250.

The colonies shown in Fig. 1, develop into typical amoeboid colonies by continued multiplication of the individual cells. (Fig. 4). Development on plain nutrient agar is similar but not as rapid.

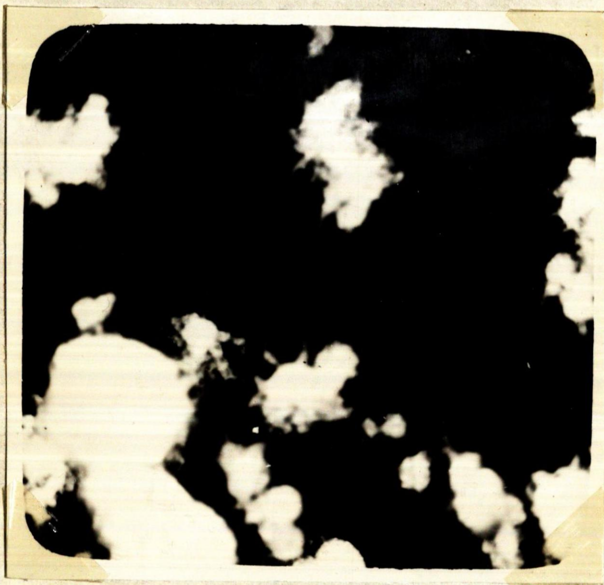
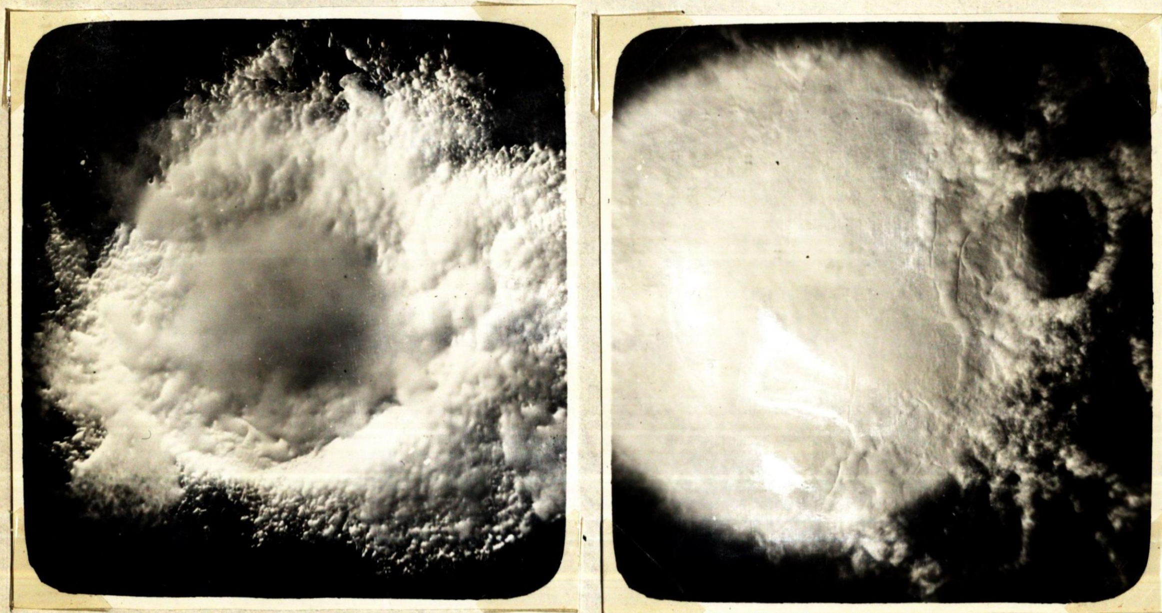


Fig. 4. B. subtilis. Type S amoeboid colonies on glycerol agar after twenty-four hours. X36.

After the organisms had been grown in a 250cc. volume of glycerol broth, and then subcultured on glycerol agar, the morphology differs from that noted above. There is a tendency for the formation of a dense matlike "nucleus" in the colony. While the colony is young, many rhizoidal outgrowths are present, radiating out from the colony. (Fig. 5). As the colony develops further the "nucleus" spreads out and finally nearly envelops all of the rhizoidal outgrowth. (Fig. 6).



Figs. 5 & 6. B. subtilis. Twenty-four and fourty-eight hour growths on glycerol agar showing transformation to a dense matlike colony. X36.

The same colony transformation was noted on a medium free from glycerol, but none of the growth was as luxuriant, and the transformations took place much more slowly.

Cells from a twenty-four hour colony on glycerol agar appeared perfectly normal when stained by Gram's method, but when stained with the Congo red negative stain some deterioration could be noted. (Fig. 7). As the colony became older, more and more

deterioration of the cells could be noted and many of the cells had formed endospores within fourty-eight hours. The formation of endospores occured much earlier on media free from glycerol.

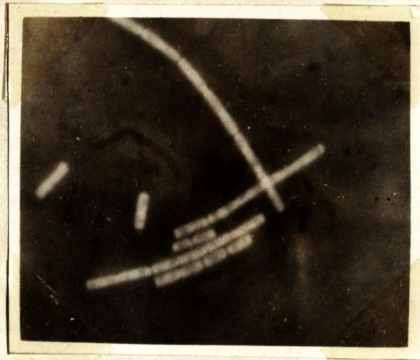


Fig. 7. B. subtilis. Cells from rhizoidal colony on glycerol agar after twenty-four hours. Slight deterioration of the cells is noticeable. Congo red negative stain. X1250.

The tendency for the formation of dense matlike colonies became more pronounced after the organisms had been passed through two 250cc. volumes of glycerol broth and then subcultured on glycerol agar. The typical colony is quite dense, circular, and filamentous. (Fig. 8).

This type of variation did not occur as readily when the organisms were grown in media free from glycerol. The type of colony in Fig. 8 did not predominate over other types until the organisms had been passed through three 250cc. volumes of nutrient broth, and then subcultured for 24 hrs. on nutrient agar.

Simultaneously with the appearance of the filamentous type of colony shown in Fig. 8, there occured a conversion from the filamentous type to the typical R colony. Evidently most of the conversion took place in the individual cells while grown in broth,

but occasionally conversion was noted on agar both in the presence, and in the absence of glycerol. (Fig. 9).

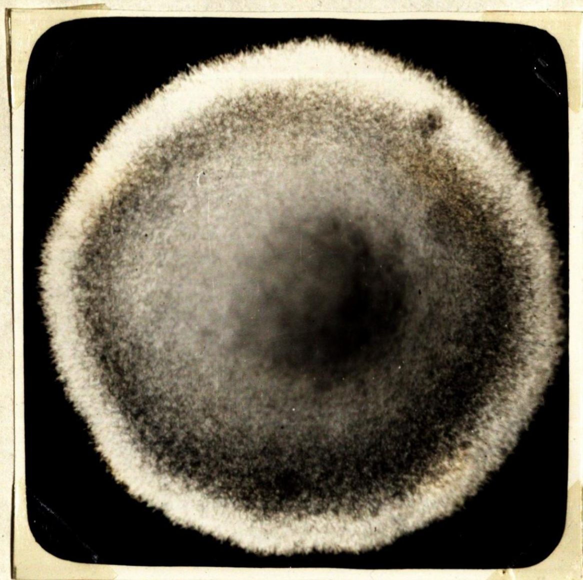


Fig. 8. B. subtilis. Filamentous colony on glycerol agar after 24 hrs. The organisms had previously been passed through two 250cc. volumes of glycerol broth. X36.

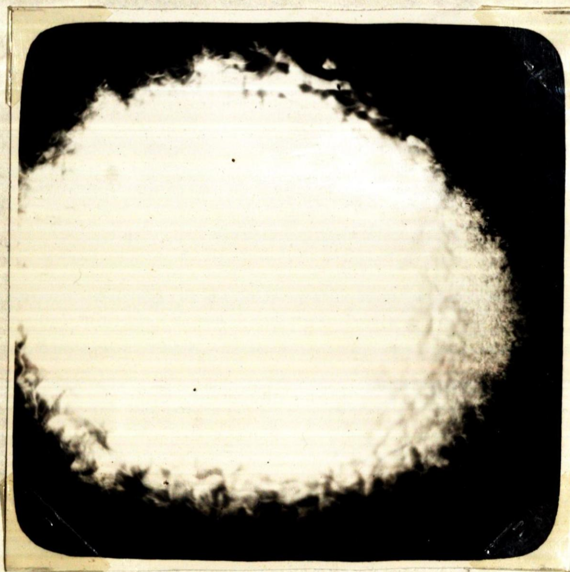


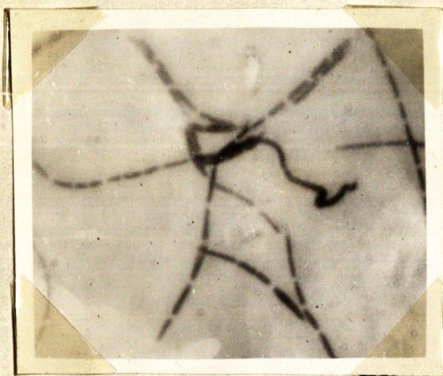
Fig. 9. B. subtilis. Conversion from filamentous to curled R colony on glycerol agar after thirty-six hours. One side of the colony is still filamentous. The organisms had previously been passed through two 250cc. volumes of glycerol broth. X36.

The margin of the R colony has a characteristic stippled appearance under reflected light. This is due to the growth of the organisms in long parallel strands which form folds or curls in the colony. (Fig. 10).

Fig. 10. B. subtilis. Curled margin of typical R colony on glycerol agar after thirty-six hours. X90.

Coincident with the transformation from filamentous to R colonies, variation occurs in the morphology of individual cells. The appearance of enlarged, spiral cells was noted. They were strongly Gram positive and did not seem to deteriorate as quickly as the normal cells. The same variation was met with on media free from glycerol. (Figs. 11 & 12).

Further incubation induced the formation of many of the enlarged, curled cells. The normal cells are considerably lysed but there is little tendency for the formation of endospores on glycerol agar. (Fig. 13). On media free from glycerol most of the cells have formed endospores within thirty-six hours, and consequently there is a smaller proportion of spiral cells present and also a smaller number of the lysed, deteriorated cells.



Figs. 11 & 12. B. subtilis. Appearance of spiral cells in slant culture on glycerol agar after thirty hours. The organisms had previously been passed through two 250cc. volumes of glycerol broth. Gram and Congo red negative stains. X1250.

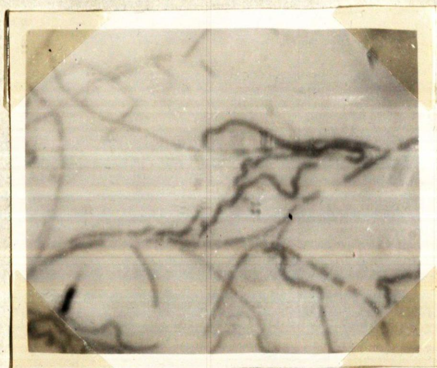
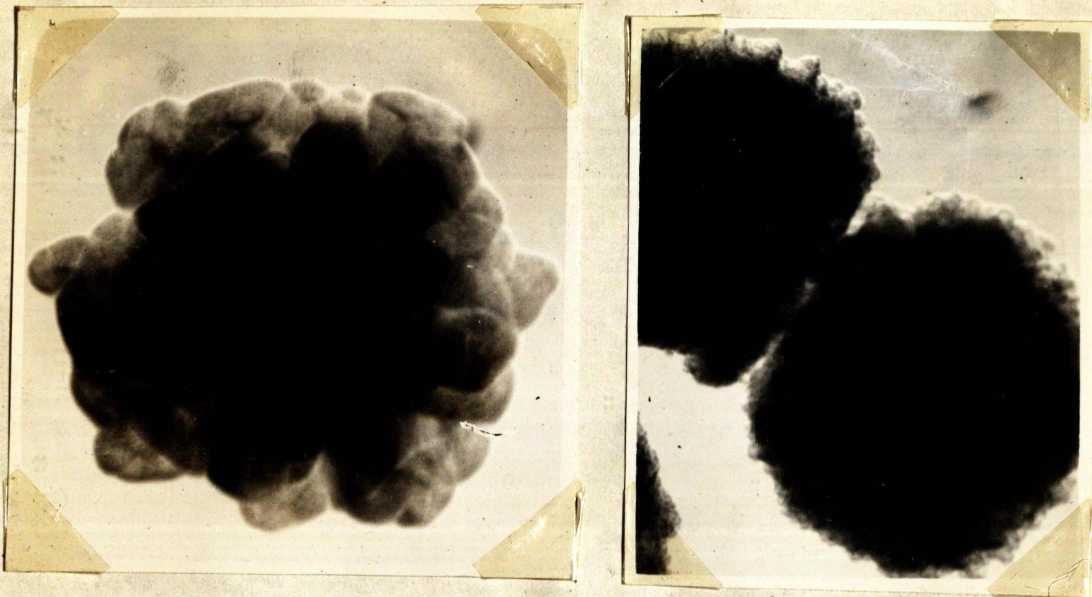


Fig. 13. B. subtilis. Appearance of enlarged, spiral cells and lysed normal cells on glycerol agar after fourty-two hours. Gram stain. X1250.

After the organisms had been passed through three of the 250cc. volumes of glycerol broth and subcultured on glycerol agar, very little transformation in colony morphology was noted. The colonies were all of the typical R type. (Figs. 14 & 15). The type of colonies produced on media in the absence of glycerol was identical, excepting that the growth was not so profuse.



Figs. 14 & 15. B. subtilis. Typical curled R colonies on glycerol agar after four and twenty-four hours respectively. X90, X36.

The morphology of the cells, after passage through three 250cc. volumes of glycerol broth and subculture on glycerol agar, also differed considerably from the original S type. The individual cells were considerably enlarged and the long chain habit of growth had been lost. In young cultures the cells occurred in singles, pairs, and very short chains, and they were strongly Gram positive. (Fig. 16).

Following continued incubation, further morphological variation in cells was noted. The cells became quite spiral and twisted and there was a tendency for the individual cells to become short. Budding also occasionally occurred. (Figs. 17 & 18).

Incubation for longer periods of time yielded even greater morphological variation in cells. The tendency for the cells to become short rods increased and eventually the transformation from short rods to cocci-bacilli was observed. (Fig. 19).

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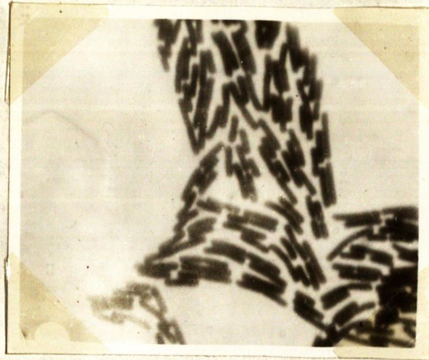
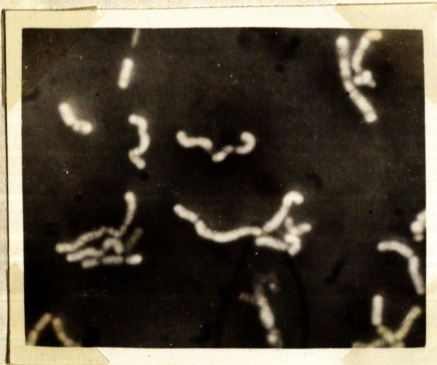


Fig. 16. B. subtilis. Enlarged cells occurring generally in pairs from glycerol agar after six hours. The organisms had previously been passed through three 250cc. volumes of glycerol broth. Gram stain. X1250.



Figs. 17 & 18. B. subtilis. Short, spiral cells with occasional budding, from glycerol agar after thirty hours. The organisms had previously been passed through three 250cc. volumes of glycerol broth. Congo red negative stains. X1250.

The transformation from rods to cocci-bacilli continued until the organisms very closely resembled streptococci in short chains. (Figs. 20 & 21). This transformation also took place to some extent on media free from glycerol, but was not very noticeable since most of the cells formed endospores before the transformation.

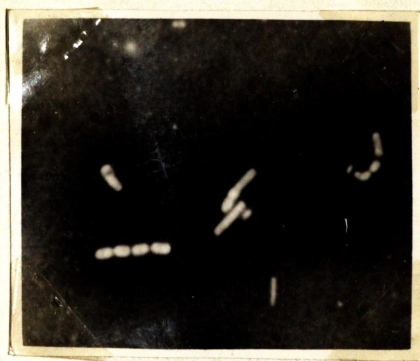
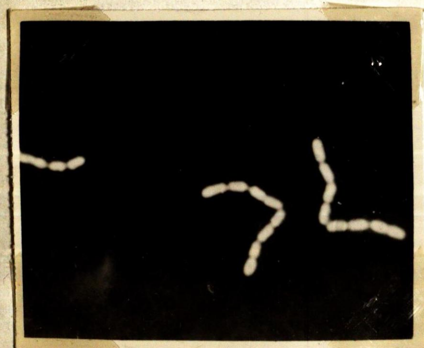


Fig. 19. B. subtilis. Formation of cocci-bacilli on glycerol agar after thirty-six hours. Congo red negative stain. X1250.



Figs. 20 & 21. B. subtilis. Cocci-bacilli from glycerol agar after they were fourty-two and fourty-eight hours old respectively. Congo red negative stain. X1250.

When the agar slant cultures were allowed to incubate for sixty hours practically all of the vegetative cells became senescent, but negative staining with Congo red made visible numerous spore-like bodies contained within the old dead cells. These were at first considered to be fat globules but as all attempts to dissolve them with fat solvents failed, and as they were unstained with Sudan III, it was

concluded that they were in reality minute reproductive spores, and were termed gonidia. (Fig. 22).

Fig. 22. B. subtilis. Dead cocci-bacilli with enclosed gonidia from glycerol agar after sixty hours. Congo red negative stain. X1250.

Continued passage through glycerol broth and plain nutrient broth with subsequent subculture on glycerol agar and plain nutrient agar revealed practically no further morphological variation in the cells. Streak plates prepared from these subcultures yielded only typical R colonies which became very rugose upon aging.

Giant colony bottles inoculated with the normal S strain of B. subtilis gave rise to typical amoeboid S colonies but these proved to be unstable, and after incubation for a few days they were transformed to spreading, curled colonies which were typically R. This conversion took place on both glycerol agar and plain nutrient agar. The giant colony produced on nutrient agar is shown in Fig. 23.

Passage of the organisms through two 250cc. volumes of nutrient broth and subsequent inoculation into nutrient agar giant colony bottles induced growth very similar to that of Fig. 23, only the growth was somewhat more rugose and there was a slight tendency for the central part of the colony to become raised. (Fig. 24).

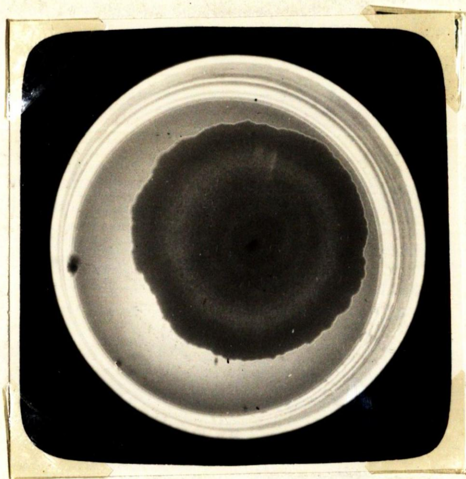


Fig. 23. B. subtilis. Typical R giant colony produced on nutrient agar after twenty-five days. X1.

Fig. 24. B. subtilis. Rugose giant colony with slightly raised center produced on nutrient agar after twenty-three days. The organisms had previously been passed through two 250cc. volumes of nutrient broth. X1.

Greater morphological variation in the giant colony was noted after the organisms had been passed through six of the 250cc. volumes of nutrient broth and then transferred to a nutrient agar giant colony

bottle. There were two distinct zones of growth which differed considerably from each other. The marginal growth was characteristically rough and non-adherent to the surface of the medium while the growth in the central portion was very homogeneous and extremely adherent. Cells from the marginal growth were distinctly cocci-bacilli, while cells from the adherent central portion were normal straight rods in pairs and short chains. This was considered to be a further dissociation to a colony type quite different from either the R or S form and was termed the O form. Upon subculture, the O form proved to be very unstable, reverting to the R form. The giant colony with two zones of growth is shown in Fig. 25.

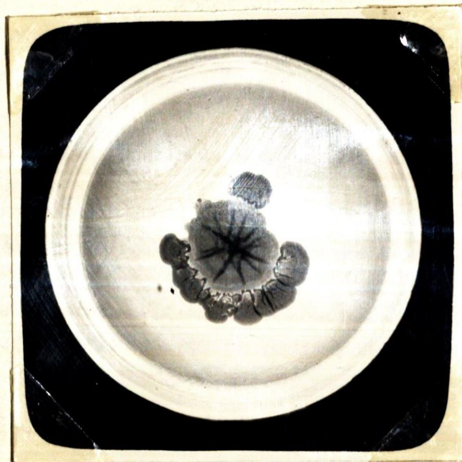


Fig. 25. B. subtilis. Giant colony with two zones of growth, produced on nutrient agar after twenty-two days. The organisms had previously been passed through six 250cc. volumes of nutrient broth. X1.

The adherent O colony type proved to be the climax colony type produced in nutrient agar giant colony bottles. The marginal fringe of R growth completely disappeared after the organisms had been passed through all of the ten 250cc. volumes of nutrient broth. The colonies were quite small in diameter but were extremely raised.

The final adherent colony type is shown in Fig. 26.

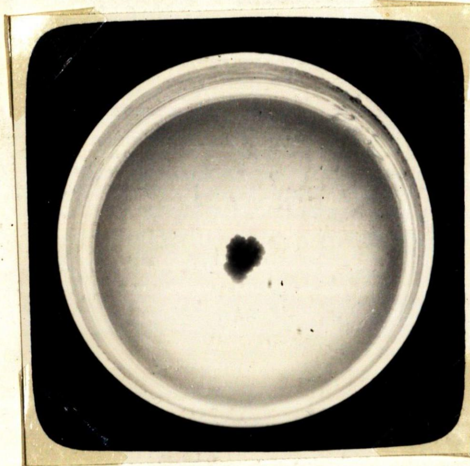


Fig. 26. B. subtilis. Raised, adherent giant colony produced on nutrient agar after twenty days. The organisms had previously been passed through ten 250cc. volumes of nutrient broth. X1.

The cells from the colony shown in Fig. 26 proved to be morphologically identical with cells isolated from the central portion of the colony shown in Fig. 25. They were unstable and reverted back to the R form.

The giant colony produced on glycerol agar without previous passage through glycerol broth was identical with the giant colony produced on plain nutrient agar. However, after the organisms had been passed through two 250cc. volumes of glycerol broth and subcultured in a glycerol agar giant colony bottle, a slightly different type of morphology was noted. The colony was characterized by a rugose surface and was concentrically ringed. (Fig. 27).

The rugose nature of the surface of the giant colonies became more apparent after the organisms had been passed through

glycerol broth for a longer period of time. Also the center of the colony became considerably raised. (Fig. 28).

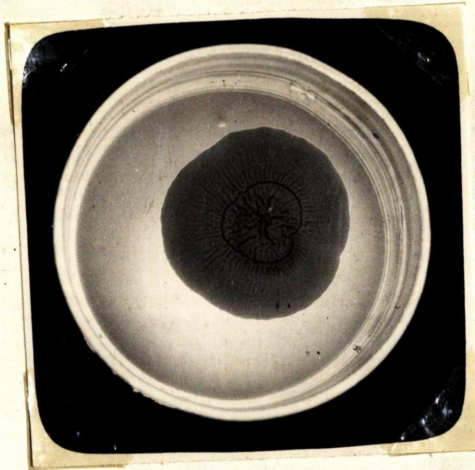


Fig. 27. B. subtilis. Concentrically ringed, rugose giant colony produced on glycerol agar after twenty days. The organisms had previously been passed through two 250cc. volumes of glycerol broth. X1.

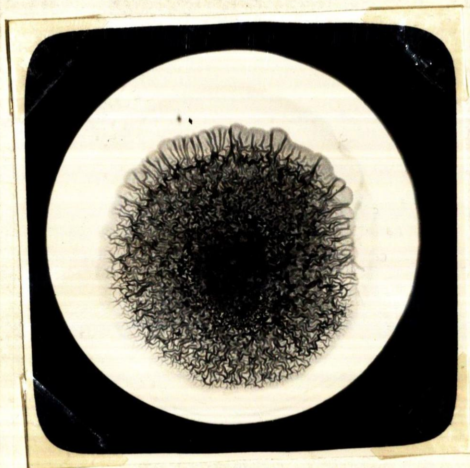


Fig. 28. B. subtilis. Rugose giant colony produced on glycerol agar after twenty-one days. The organisms had previously been passed through four 250cc. volumes of glycerol broth. X1.

The rugose nature of the surface of the colony and the tendency for the center to become raised became even more pronounced

following continued passage of the organisms through glycerol broth. The presence of glycerol in the medium inhibited the formation of spores and thus increased the period of reproduction, which accounts for the luxuriant vegetation noted. (Fig. 29).

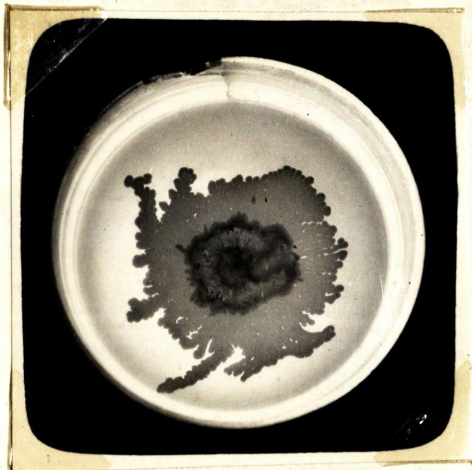


Fig. 29. B. subtilis. Rugose giant colony with considerably raised center produced on glycerol agar after seventeen days. The organisms had previously been passed through eight 250cc. volumes of glycerol broth. X1.

The climax type of vegetation produced on glycerol agar, differed from that shown in Fig. 29 only in that it was still more rugose and considerably raised. The growth was not nearly as spreading as had been noted before. (Fig. 30).

These rugose giant colonies, produced on glycerol agar, are interesting to study under higher magnifications because the surface topography is revealed more clearly. (Fig. 31).

The intensely wrinkled surface of the marginal growth appeared considerably different from the typical R growth in younger colonies. The nature of the surface is shown in Fig. 32.

Cells isolated from this rugose colony were quite short and most of them had produced endospores. There was no great tendency for the production of gonidia in these rugose giant colonies. The morphology of the cells isolated is shown in Fig. 33.

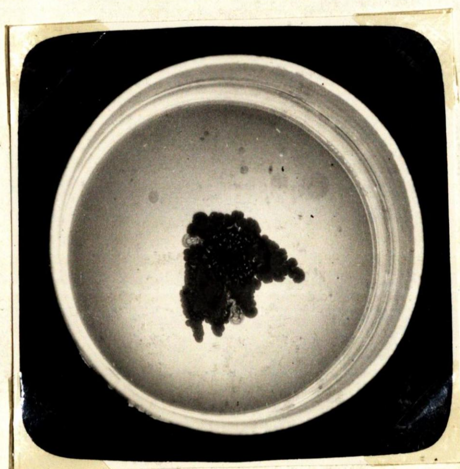


Fig. 30. B. subtilis. Raised, rugose giant colony produced on glycerol agar after fifteen days. The organisms had previously been passed through ten 250cc. volumes of glycerol broth. X1.

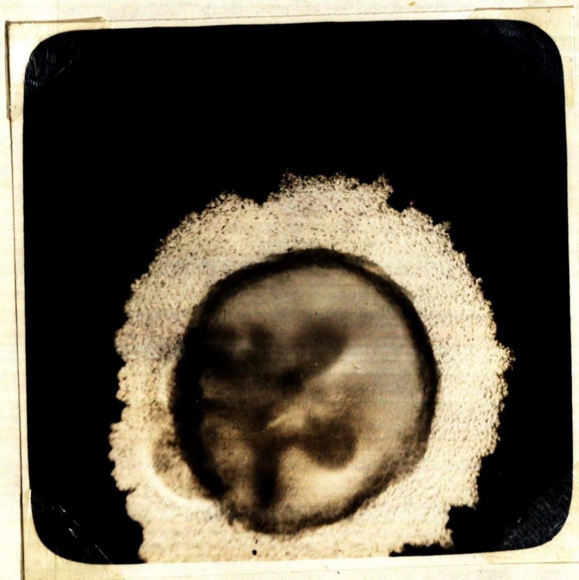


Fig. 31. B. subtilis. Rugose giant colony produced on glycerol agar after four days. The topography of the marginal growth is quite characteristic and the central part is considerably raised. The organisms had previously been passed through seven 250cc. volumes of glycerol broth. X36.

Fig. 32. Fig. 33.

Fig. 32. B. subtilis. Wrinkled surface of rugose giant colony produced on glycerol agar after ten days. X90.

Fig. 33. B. subtilis. Cells from rugose giant colony showing the presence of endospores. Congo red negative stain. X1250.

After these giant colonies had been allowed to incubate for a longer period of time it was noticed that lytic areas were occasionally produced. Microscopical examination revealed these lytic areas to be composed of an amorphous, slimy mass which was made up of the disintegration products of the cells which had been affected by the lytic principle. Daughter colonies could be seen arising from the lysogenic area. (Fig. 34).

The cells isolated from the young daughter colonies were small cocci-bacilli and Gram negative. The cells from the lytic area, however, were obviously different from any observed thus far. Most of the cells were senescent, straight rods of normal size. Occasionally giant cells were found. These were swollen, sausage shaped cells and were strongly Gram positive. (Figs. 35 & 36).

Fig. 34. B. subtilis. Lytic area from rugose giant colony showing the rise of daughter colonies. The giant colony was twenty-six days old. X90.

Figs. 35 & 36. B. subtilis. Giant cells obtained from lytic area of rugose giant colony produced on glycerol agar. Gram stains. X1250.

When cells from the lytic area are examined after staining with the Congo red negative stain, considerable information is gained as to the function of the enlarged cells, for the giant cells become senescent and the stain penetrates the dead protoplasm, and the presence of gonidia in considerable numbers is revealed. (Fig. 37).

Fig. 37. B. subtilis. Senescent giant cells with enclosed gonidia, from lytic area of rugose giant colony produced on glycerol agar. Congo red negative stain. X1250.

When cells isolated from the daughter colonies, which arose from the lytic area, are inoculated into fresh agar, an entirely different type of colony morphology is exhibited. Casual examination reveals no growth at all on the surface of the medium, but by reflected light, minute, bluish colonies are faintly detectable. Upon continued incubation the growth becomes quite spreading and a brownish nucleus is produced in the colony. This phantom growth, as it was termed, is shown in Fig. 38.

Fig. 38. B. subtilis. Transparent margin of a phantom colony on glycerol agar after twenty-four hours. X90.

Upon continued incubation, the phantom colonies become slig-
htly chromogenic and have a light brown appearance by transmitted light.
The colony also becomes denser and is more readily noticeable. (Fig. 39).

Fig. 39. B. subtilis. Margin of a phantom colony on glycerol agar after thirty-six hours. The colony had become slightly raised and chromogenic. X90.

Morphologically the cells were determined to be characteristically small, Gram negative, cocci-bacilli, as is shown in Fig. 40.

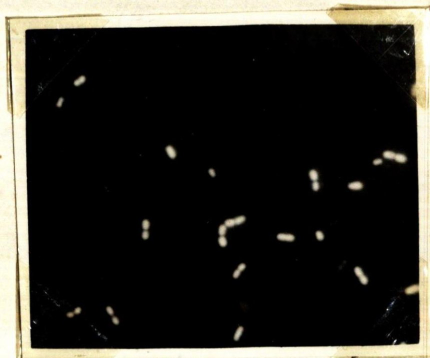
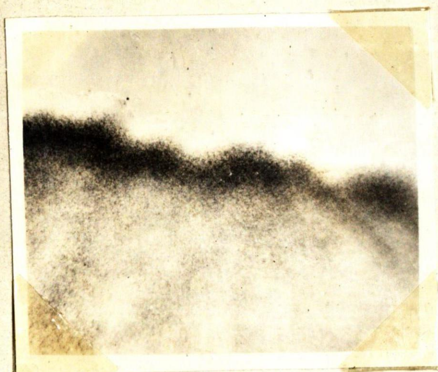


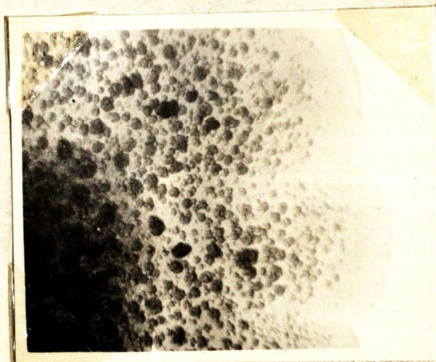
Fig. 40. B. subtilis. Small cocci-bacilli isolated from phantom colony on glycerol agar after thirty-six hours. Congo red negative stain. X1250.

These cells were found to be morphologically identical with the cells produced in the daughter colonies of the lytic area. Their physiological characteristics were studied and were found to agree very closely

with the characteristics of normal B. subtilis, differing from the latter in that they produced acid in glycerol broth.

Upon even further incubation of the phantom colonies, they became finely granular and eventually coarsely granular. Chromogenesis became quite marked in the older colonies. (Figs. 41 & 42).





Figs. 41 & 42. B. subtilis. Development of granular colonies from phantom colonies on glycerol agar after fourty-eight and seventy-two hours. X90.

Upon subculture on agar and in broth the phantom type of growth proved to be very unstable; it reverted to either the S or the R form, and even to the filamentous intermediate form, thus completing the cyclic development from S to R to P(phantom), and from P to the S or R form again.

Demonstration of a Filterable Phase

Löhnis and Smith (1916) reported that the gonidia produced in dissociative strains of bacteria were often small enough to pass through a Chamberland filter. Accordingly an experiment was conducted to determine whether or not this phenomenon could be demonstrated in gonidia-producing cultures of B. subtilis.

Glycerol broth cultures of B. subtilis of varying ages were examined microscopically for the presence of gonidia-producing cells. Practically all broth cultures that had been incubated for a week or longer yielded many of these gonidia-producing cells. These cultures were then filtered through a sterile Berkefeld filter which had previously been checked with a 24 hr. broth culture of B. subtilis and had been found to be absolutely effective in filtering out all normal forms of living organisms. The filtrates from the old glycerol broth cultures were collected under sterile conditions and were then tested for sterility by plating out with agar, both by the pour plate method and by the streak plate method. In every instance resultant growth was obtained and the colonies were characteristically of the phantom type. Conversion of the phantom colonies to S, R or intermediate filamentous forms was very rapid. Fig. 43 shows the type of growth obtained from an agar pour plate inoculated with the filtrate from an old glycerol broth culture of B. subtilis.

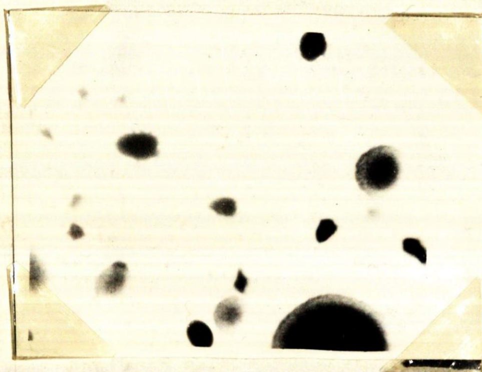


Fig. 43. B. subtilis. S, R, P and intermediate colony forms on glycerol agar inoculated with the filtrate from a glycerol broth culture, after four days. X18.

The results of these experiments led to the conclusion that the minute reproductive bodies, termed gonidia, were intimately connected with a filterable phase in the life cycle of B. subtilis. Whether small gonidia constituted the filterable phase, or whether these gonidia gave rise to, or were produced from, a true virus which passed through the filter, is beyond the scope of this investigation.

Antigenic Properties

Soule (1928) reports a difference in antigenic strength of the R and S strains of B. subtilis. He also reports a lessened agglutinative titer of the S antiserum for the R antigen. In an attempt to confirm his results and also to expand the information to include the antigenic properties of the O and P forms, the following experiments were conducted.

Young rabbits were injected intraperitoneally with antigens prepared from the S, R, O and P forms. They were given three inoculations on alternate days; 2cc. of a saline suspension of the organisms being injected at each inoculation. After a period of nine days they were bled by piercing the marginal vein of the ear and sufficient blood was withdrawn to perform the serological tests. The blood was allowed to clot and then centrifugalized at high speed for one half hour. The clear serum was then withdrawn with a sterile pipette and kept in sterile tubes at $10^{\circ}C$. until used.

The agglutinative titers of the antiserums for the homologous antigens were then determined and the results tabulated. (Table 1).

From the results obtained it was clearly evident that the S antigen was most strongly antigenic; the R and O antigens of equal strength but both weaker than the S antigen; and, the P antigen the weakest of all.

In order to acquire more information about the antigenic properties of the four strains, as concerning agglutinin absorption power, a series of agglutinin absorption tests were performed. The S antiserum was absorbed by each of the different antigens and then the resultant serum was tested for agglutinative power with each of the antigens. The results, which were obtained, are presented in Table 2.

A careful analysis of the results obtained from the agglutinin absorption tests led to the assumption that the P antigen was contained in the O and R antigens; that the R and O antigens are identical; and that the P, O and R antigens are all contained in the S antigen. This furnished considerable information as to the possible phylogenetic relationships existing between the various forms. It is clearly evident that the R and O variants are equally removed from the normal S strain, and it is also evident that the P variant is farther removed from the S form than are the R and O forms. However, no explanation of the conversion of the P form to the S and R forms can be gained from this information. Some factor, as yet undetermined, must bring about this conversion and from the antigenic difference noted it must be one of the strongest factors of the dissociative reaction. Additional work must be done on this phase of the life cycle before the conversion is understood.

TABLE 1

Agglutinative titers of antisera with homologous antigens.

Antiserum	Antigen	Titer
S	S	1:2000
S	R	1:1000
S	O	1:1000
S	P	1:100
R	R	1:1000
O	O	1:1000
P	P	1:500

TABLE 2

Titers of S antiserum after absorption of agglutinins.

Antiserum	Absorption antigen	Agglutinative antigen	Titer
S	S	S	No aggl.
S	S	R	No aggl.
S	S	O	No aggl.
S	S	P	No aggl.
S	R	S	1:500
S	R	R	No aggl.
S	R	O	No aggl.
S	R	P	No aggl.
S	O	S	1:500
S	O	R	No aggl.
S	O	O	No aggl.
S	O	P	No aggl.
S	P	S	1:1000
S	P	R	1:500
S	P	O	1:500
S	P	P	No aggl.

DISCUSSION

The findings of this investigation substantiate to a large extent the findings of Soule (1928) in so far as the occurrence of the S, R and P forms is concerned. In addition the appearance of the O form was noted as one of the cyclostages in the dissociative development of B. subtilis. Various other intermediate forms were also noticed but none of them proved to be stable and were not considered of any great importance. The dissociation of the R strain into adherent and non-adherent substrains was observed. This agrees well with the findings of Lewis (1932) who records the same situation in the dissociation of B. mycoides. His observation on the instability of the adherent form was also confirmed in this investigation.

The formation of spiral cells, and reproduction by budding was found to take place on agar. Cells of this type were definitely reported by Lewis (1932) in his work on B. mycoides. The formation of cocci-bacilli which proved to be quite stable, confirms the recordings of Hadley (1927) who reports that the formation of cocci-bacilli is a characteristic of dissociating bacillus forms. The formation of cocci-bacilli has been specifically reported for B. subtilis by Dr. Evans (1929) and by Löhnis and Smith (1916). They are undoubtedly identical with the C forms (Kokken-Formen) of Kuhn as interpreted by Hadley (1933).

The action of autolysis on giant colonies on glycerol agar was observed. The lytic area was found to be composed chiefly of senescent and lysed cells but a few swollen rods containing reproductive bodies were noted. The reproductive bodies were termed gonidia.

These swollen rods were undoubtedly identical with the gonidangia as reported by Löhnis and Smith (1923). Secondary colonies arising from the lytic area have been found to be composed of small, Gram negative cocci-bacilli. Repeated subculture of this type showed that the characteristic colony morphology of this type was phantom in appearance. These phantom colonies proved to be quite unstable and conversion took place to the S and R forms. This also agrees with the findings of Soule (1928). Cells isolated from the phantom colonies differed from the normal cells of B. subtilis, in their ability to ferment glycerol broth with the production of acid. This physiological adaptation, enabling the organism to utilize some constitutent of the medium not usually attacked, has been reported by Lewis (1932) for the secondary colonies of B. mycoides.

The appearance of typical phantom growth from filtrates of old broth cultures, whose cells showed the presence of gonidia, offered conclusive evidence that the production of phantom growth from gonidia was intimately linked up with a filterable form of the organism. Whether the actual filterable form was a true virus or just extremely small gonidia was undetermined. Dr. Evans (1929) considered the filterable form to be a true virus either identical with or very similar to the virus of encephalitis. She found that normal B. subtilis could be isolated from the brain tissue of rabbits inoculated intracerebrally with the virus. Her work has not been confirmed as yet. Hadley (1933), in his review of Kuhn's work, considers the filterable phase to be composed of the smallest gonidia or cocci forms. Löhnis and Smith (1916) also attach the filterable significance to the smaller gonidia.

The reversion of the phantom type of growth to both S and R forms seems to supply the missing link in a fairly orderly life cycle. The S form has been found to be quite unstable and it might reasonably be concluded that it represents a very transitory stage between the P and the R forms of growth. In pathogenic species the greatest virulence is attributed to the S form, with the one exception of B. anthracis. It is conceivable that the environmental conditions of the animal body would supply the necessary stimulus for the stabilization of the S form.

Serological study has revealed considerable variance in antigenic strength of the various forms. This variation has been reported by Soule (1928), Arkwright (1921), and Hadley (1927). Such variance of antigenic strength seems to be in close correlation with morphological variation, both as to degree of variation from the normal and as to possible phylogenetic relationships of the various forms. Any attempted explanation of the appearance of the various forms as being due to contamination is out of the question after a study of the results of the agglutination and agglutinin absorption experiments.

It has seemed the inevitable conclusion, from a study of the results of this investigation, that there does exist a definite life cycle for the organism B. subtilis. This conclusion was in accord with the findings of Löhnis and Smith (1916), Hadley (1927), and Dr. Evans (1929). Any attempts to explain the conversions on the basis of orthogenic variation as presented by Lewis (1932) or on the basis of a dimorphism as presented by Kuhn and reviewed by Hadley (1933), seem futile.

SUMMARY

1. At least four distinct types of growth have been obtained by passing an S strain of B. subtilis through large volumes of broth, either in the absence or in the presence of glycerol. The types have been considered to be of the S or normal form, the R or rough form, the O or intermediate form, and the P or phantom form.

2. It has been found that there is an apparent orderliness to the variation and a distinctly noticeable tendency for a definite type of growth to be superseded by another distinct type of growth.

3. Variation occurred not only in colony morphology; variation occurred in cell morphology as well.

4. The presence of gonidia-producing, cocci-bacilli has been definitely linked up with the R form of growth.

5. The arising of secondary colonies from lytic areas has been noticed and there seems to be considerable evidence that this growth is produced by the gonidia of the parent cells. The appearance of a new physiological character has been noted in the secondary growth.

6. The presence of a filterable stage in the life cycle of the organism has been noted consistently and is evidently intimately connected with both gonidia germination and phantom colony production. It might even be a connecting phase.

7. The instability of the phantom type and subsequent reversion to the S or R types completes the cyclic development of the dissociating organisms.

8. The stimulus for dissociation is evidently much greater in large volumes of broth than on the surface of solid culture media. The actual morphological variation takes place at a very rapid rate on solid media but the stimulus is undoubtedly received during the rapid growth in the broth.

9. Close antigenic relationships have been noted for the different strains, the difference in antigenic power being comparable with the degree of variation from the normal.

10. The different stages of development have been interpreted as representative of cyclo-stages in a complete life cycle.

In conclusion, I wish to express my gratitude to Dr. W. P. Larson, of the University of Minnesota, for the suggestion of this problem. I also wish to thank Dr. W. L. Miller, Mr. E. A. Walker, and Mr. N. M. Paulson, all of South Dakota State College, for various helpful suggestions and assistance in technique.

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