COLONY ORGANIZATION OF CERTAIN BACTERIA
WITH REFERENCE TO SPORULATION

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Accounts of the study of colonial organization of bacteria and other microorganisms by means of thin sections have appeared sporadically in the literature for nearly fifty years.

Neisser in 1888 devised a procedure for obtaining individual sections of colonies grown in gelatin or agar shake cultures. The plugs of medium were removed from the culture tubes by gentle warming, cleared in a 1 per cent aqueous solution of potassium bichromate, washed, and carried through a graded series of alcohols into 95 per cent alcohol. The plugs were cut into blocks with one colony in each. The blocks were then mounted on pieces of cork, with gum arabic, and placed in absolute alcohol for 24 hours. By means of a sliding microtome, sections were cut, mounted on slides with fixative, stained, and cleared in the usual manner. Neisser's paper is confined to a description of the technique alone, but is of interest since it is apparently the first comprehensive report of a method for sectioning colonies of microorganisms.

Hutchinson in 1907 studied sections of surface and subsurface colonies of Bacillus subtilis, Bacillus megatherium, Saccharomyces cerevisiae, "Mycoderma" cerevisiae, and Oidium lactis. In the case of surface colonies, he poured lukewarm melted agar containing 3 per cent formalin over the colonies, and, when the agar hardened, removed the colonies intact. The colonies were then imbedded in paraffin and sectioned. In the bacterial colonies he found the cells at the margin to be of normal size and ap-

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pearance, while involution forms occurred in the center. In surface colonies spore formation was most profuse at the upper surface. Yeast colonies were more complicated in organization than bacterial colonies. Round or oval cells were found at the colony surface, while the cells next to the substrate were slender and elongated. In some cases an extensive mycelial system developed in the substrate. Mycelium was more abundantly produced in old cultures where the medium presumably contained an abundance of growth products. Hutchinson's extensive paper contains diagrams and photographs of colonies in section, as well as a complete discussion and bibliography of the relevant work of other investigators.

Zikes in 1916 studied colonies of *Schizosaccharomyces pombe*, *Schizosaccharomyces mellacei*, *Zygosaccharomyces priorianus*, and also certain bacteria (species not given) isolated from beer wort. He observed abnormally elevated colonies of these organisms on plates containing a high percentage of gelatin and incubated at a low temperature. Zikes prepared sections from colonies in celluloid. He found that elongated cells predominated at the base of the colonies, but that in the upper portions the cells were progressively smaller. The difference in cell size is ascribed to unequal nutritive conditions.

Legroux and Magrou in 1920 studied wrinkled, atypical colonies of the cholera vibrio in section. By applying differential stains they demonstrated a definite zonation in the complicated internal organization of the wrinkled colonies. The vibrios were localized in well-defined layers and zones, following the outlines of the colony sinuosities.

Truffaut and Bezsonoff (1922) observed on plating soil samples under aerobic conditions oval subsurface colonies which contained *Clostridium pastorianum* in association with aerobic bacilli. Sections showed a central core of sporulating *C. pastorianum* cells surrounded by a layer of aerobic bacilli. The predominant bacillus was gram-negative, and with the gram stain the authors obtained good differentiation between the central mass of gram-positive *C. pastorianum* cells and the overlying bacilli.

Kahn and Nonidez (1936), studying colonies of the tubercle
bacillus, used not only the standard paraffin method, but also the frozen section method and a combined colloidin-paraffin technique.

This paper presents a study of the relation of sporulation to the colony organization of three species of bacteria: *Clostridium acetobutylicum*, *Clostridium pasteurianum*, obligate anaerobes, and *Bacillus acetoethylicum*, a facultative anaerobe. Stock cultures of these bacteria are maintained in the collection of the Department of Agricultural Bacteriology at the University of Wisconsin.

The aim of this study was (a) to follow the course of sporulation in the colony by using a standard medium and a constant incubation temperature combined with varied growth times, and (b) in the case of *B. acetoethylicum* to study also the effect of certain environmental conditions on sporulation in the colony.

Since the results indicate that the sectioning technique may be used in further investigations, it is planned to extend this study of colony organization.

**PROCEDURE**

Inoculum consisted of young, actively growing cultures, containing few or no spores. Usually both surface and subsurface colonies were obtained by ordinary dilution methods. In some cases satisfactory surface colonies could be produced only by inoculating solidified medium with a tiny loop.

The following medium (liter basis) was used as standard in the time-sporulation series: Glucose 5.0 grams, beef extract 3.0 grams, peptone (Parke, Davis & Co. "Bacteriologic") 5.0 grams, agar 15.0 grams, tap water to make to 1 liter. The medium was adjusted to neutrality with NaOH before sterilization. For *C. acetobutylicum* 10 grams of glucose was used since that amount was found to be more favorable for sporulation. All results reported are based on colonies grown at 37°C.

Subsurface colonies were removed in the agar in which they developed. For purposes of orientation a thin layer of agar was poured over the surface of the medium above the colony before removal. The form of surface colonies was preserved by embedding them under a layer of agar so that they could be removed
intact. As a rule, six to eight separately imbedded colonies constituted a sample. If more colonies were desired additional samples were taken. Colonies were fixed in formalin-acetic-alcohol or, where the medium contained excess CaCO₃, in 70 per cent alcohol only, and after dehydration, were imbedded in paraffin. Rapid and satisfactory dehydration was obtained with an alcohol-anilin-oil-of-wintergreen schedule.

A total of approximately 250 colonies were investigated. Sections of 3 μ or 5 μ thickness have usually been most satisfactory.

Two spore stains were tried, a modification of Dorner’s with Ziehl’s carbol fuchsin and nigrosin, and Conklin’s modification of the Wirtz mercurochrome malachite-green stain. Neither was suitable since it proved very difficult to decolorize the colony matrix adequately after staining. Methylene blue, gentian violet, safranin, and Ziehl’s carbol fuchsine were not satisfactory.

Nicolle’s carbol thionin has been almost exclusively used. For staining, the stock solution (0.25 per cent thionin in 50 per cent alcohol) is diluted with an equal volume of 2 per cent phenol. The stain is used at room temperature, and 3 to 5 seconds exposure is sufficient.

Stained mounts were protected with thin films of rapidly drying “Vinylite A” in butyl acetate (Skiles and Georgi, 1937). The “Vinylite” solution is run over the mount, the excess drained off, and a very thin, transparent film remains, giving a permanent mount after an hour’s drying.

RESULTS

Clostridium acetobutylicum. Vegetative cells stain uniformly, young clostridia stain deeply at one end, while clostridia containing mature spores, and free spores, do not stain appreciably with thionin.

In subsurface colonies sporulation is apparently initiated at the periphery of the colony and progresses inward (plate 1, A).

In surface colonies observations were made at time periods ranging from 40 to 144 hours, but sporulation has never been noted under 60 hours. Transitional stages showing the young
colonies immediately preceding sporulation are found only with difficulty. Sporulation is initiated throughout the central and lower portion (that portion next the agar substrate) of the colony. The center portions of older surface colonies show the greatest development of mature spores (plate 1, B), while there is a transition toward the margins from young clostridia to vegetative cells at the extreme outside edges of the colonies.

Dark-field illumination is very useful in giving a clear picture, under the lower powers, of the total extent of sporulation in older colonies of *C. acetobutylicum*, since the highly refractive spores are so imbedded in the colony matrix that it is difficult to discern them with ordinary light, and then only in the restricted oil immersion field (fig. 1).

*Clostridium pasteurianum*. Vegetative cells usually stain uniformly, but have been observed with a pronounced banding. Young clostridia stain deeply at one end, while clostridia containing mature spores, and free spores, do not stain appreciably with thionin.

Subsurface colonies were fixed at representative time periods ranging from 29 through 144 hours. At 29 hours vegetative cells only are present. At 40 hours there is a mixture of vegetative cells and young clostridia, while at 60 hours clostridia with mature spores, and free spores, are also present. At 144 hours young clostridia are not found (plate 1, C, D). A few vegetative cells are always found, even in the most highly sporulated colonies.

It is doubtful whether there is a fixed sporing pattern, but it appears that at certain time periods certain types of cells predominate throughout the colony, but are not noticeably localized.

Surface colonies were fixed at time periods ranging from 44 through 144 hours. According to colony age, zones can be established where certain cell types predominate. Thus, in a 44-hour colony young, terminally-staining clostridia predominate in the central portion (fig. 2) while vegetative cells occur progressively increasing numbers toward the colony margins, where vegetative cells only are found. In a 64-hour colony (plate 1, E) mature clostridia and free spores occur profusely in the center
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(midway between the center and the margins young clostridia are most abundant (BC); and at the extreme margins vegetative cells only are present (AB).

*Bacillus acetoethylicum*. Vegetative cells stain uniformly. Young clostridia stain deeply at one end. This organism is especially favorable for study by the sectioning technique since the colonies, while only moderately compact, section well, and since the conspicuous spores soon lie free and take a pale reddish-purple differential stain with thionin.

Sporulation in the case of *B. acetoethylicum* is strongly favored by aerobic incubation, and has been observed only once under anaerobic conditions.

On the standard medium, the transitional stages leading to sporulation are initiated at about 40 hours. Mature spores appear at from 50 hours onward, and soon lie free within the colony. It is difficult to demonstrate mature spores still within the cells.

In subsurface colonies sporulation is very meagre and spores are found only at the colony periphery (plate 2, A). Experiments with oxidized media failed to produce changes in the manifestations of sporulation in subsurface colonies, under either aerobic or anaerobic conditions.

Surface colonies on dilution plates are often, if not always, initiated subsurface, and then break through, spreading out from a dense central zone. In this connection Orsos (1910), who studied colonial development in various bacteria and yeasts, found that the majority of surface colonies develop from subsurface colonies which form the "nucleus" of the resultant surface colony. In *B. acetoethylicum* the "nucleus" stains a light, uni-

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**Fig. 1. Cl. acetobutylicum. Photomicrograph, Dark Field Illumination**
Profuse sporulation in basal portion of a 90-hour surface colony. ×400

**Fig. 2. Cl. pasteurianum. Photomicrographs**
A. Sector from top central portion of young 44-hour surface colony, showing young clostridia. ×700.
B. Section through entire central portion of same colony showing young clostridia predominating to the practical exclusion of other cell forms.

**Fig. 3. B. acetothyllicum. Photomicrograph**
Sector from colony surface showing profuse, localized sporulation. ×850
form blue with thionin, as opposed to the deeper purplish-blue of the rest of the colony. Such colonies are hence characterized by subsurface growth lobes which may be of greater volume than the surface portion (plate 2, E).

Investigation of the effect of varying environmental conditions on sporulation consisted in (a) use of medium with excess of CaCO₃, as opposed to medium without such addition, (b) varying agar concentrations from 1.5 to 3.0 per cent, (c) varying glucose concentrations from 0.1 to 2.5 per cent, and (d) use of aerobic as opposed to anaerobic conditions. These variables were used in diverse combinations. As a rule, growth periods were from 72 to 85 hours.

Findings concerning the influence of varying conditions on sporulation in aerobic surface colonies are as follows:

Media containing an excess of CaCO₃ strongly favor profuse and generalized sporulation without regard to the concentrations of the other constituents of the media. The effect of the excess CaCO₃ appears to be due, in part at least, to regulation of acid conditions unfavorable to sporulation which are created by the organism in the course of growth, particularly on media with 2.0 per cent or more of glucose (plate 2, B).

The remaining findings for aerobic surface colonies have to do with those produced on media which did not contain excess CaCO₃.

In the early stages of this study plates with unglazed porous clay tops were used. When such plates are incubated anaerobically there is no excessive moisture loss through the porous tops. However, when they are incubated aerobically at 37°C the medium dries down rapidly with resultant concentration of the agar, which is present in the fairly high initial concentration of 1.5 per cent. In ordinary glass top plates the medium is not thus rapidly concentrated. These facts are of significance in that where media do not contain excess CaCO₃, differences in agar concentration appear to be correlated with sporulation variations, as detailed below.

Sporulation is not profuse nor especially localized when low
agar concentration is combined with low glucose concentration. It is even less profuse in the presence of low agar and high glucose concentration (2.0 per cent or more), where the factor of acidity seemingly enters (plate 2, C). The same holds to a lesser degree for high agar in combination with high glucose concentration.

Sporulation is more profuse and also tends to be localized at the colony surface when there is a high agar concentration combined with a relatively low glucose concentration (fig. 3) (plate 1, F; plate 2, D, E).

Thus, in glass top plates, sporulation is not profuse or localized when the initial agar concentration is 1.5 per cent combined with 0.5 per cent glucose. However, with the same medium in clay top plates sporulation is more profuse and localized at the colony surface. On the other hand, with an initial agar concentration of 3.0 per cent, combined with 0.5 per cent glucose in glass top plates, sporulation is profuse and localized, as is also true for the same combination in clay top plates.

Under anaerobic conditions sporulation has not been observed in subsurface colonies. In surface colonies grown anaerobically sporulation has been observed once only. The series included media with and without excess CaCO3, combined with high and low agar concentrations. In all cases sporulation was very slight with no apparent localization.

**SUMMARY**

1. Stained sections of colonies of *Clostridium acetobutylicum*, *Clostridium pasteurianum*, and *Bacillus acetoethylicum* were studied to determine the course of sporulation within the colonies under (a) conditions taken as standard, and (b) in *Bacillus acetoethylicum* under varying as well as standard conditions.

2. In *Clostridium acetobutylicum* subsurface colonies are sporulated principally at the colony periphery. In surface colonies the spores are not found at the upper surface of the colony, but are confined to the central and basal portions.

3. In *Clostridium pasteurianum* subsurface colonies become profusely sporulated, but do not show a regular pattern of spor-
ulation. The older surface colonies show a transition from vegetative cells at the colony margins, to young clostridia in the adjacent portions, to mature spores in the middle region.

4. In *Bacillus acetohexylicum* sporulation is significantly profuse only under aerobic conditions. Subsurface colonies are sporulated only at the colony periphery. In surface colonies sporulation is most profuse and tends to be localized at the upper surface of the colony when a relatively high agar concentration is combined with a relatively low glucose concentration. In surface colonies on media containing excess CaCO₃ sporulation tends to be profuse, but ceases to be localized regardless of the concentrations of the other constituents of the medium.

REFERENCES


PLATE I

A. *Cl. acetobutylicum*. Subsurface colony, sporulated at periphery. ×50.

B. *Cl. acetobutylicum*. Surface colony, sporulated basally. ×40.

C. *Cl. pasteurianum*. Subsurface colony, oval, sporulated throughout. ×125.

D. *Cl. pasteurianum*. Subsurface colony, slender lens-shaped, sporulated throughout. ×60.

E. *Cl. pasteurianum*. Surface colony showing zones of cell types. ×30.


Diagrams were drawn from actual colonies.
(H. C. Greene: Colony Organization and Sporulation)
PLATE II

A. *B. acetoethylicum*. Subsurface colony, slight sporulation at periphery. \( \times 125 \).

B. *B. acetoethylicum*. Surface colony, sporulated throughout, grown on medium with excess CaCO\(_3\). \( \times 60 \).

C. *B. acetoethylicum*. Surface colony, sporulation slight, not definitely localized. \( \times 45 \).

D. *B. acetoethylicum*. Surface colony, sporulation profuse and localized. \( \times 40 \).

E. *B. acetoethylicum*. Surface colony similar to D, but with a large subsurface lobe. \( \times 60 \).

Diagrams were drawn from actual colonies.
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