STRAIN VARIATION AS A FACTOR IN THE SPORULATING PROPERTIES OF THE SO-CALLED BACILLUS GLOBIGII[1]

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For a period of more than fifty years interest has been evidenced in the environmental factors responsible for endospore formation in bacteria. Lehmann (1888) attributed sporulation to the accumulation of metabolites in the medium, but Buchner (1890) held that exhaustion of the nutrients was the cause of spore formation. Subsequent reports in the literature were adequately reviewed by Brunstetter and Magoon (1932). Additional investigations include those of Williams (1930-31), Cook (1931), Bayne-Jones and Petrilli (1933), Fabian and Bryan (1933), Roberts (1934), Roberts and Baldwin (1942), Hayward (1943), and Knaysi (1945).

Our original purpose was to investigate some of the environmental factors which influence the sporulation of "*Bacillus globigii.*" In the course of these investigations we encountered serious difficulty in repeating, or even in duplicating, experimental data. It was noticed that considerable colonial variation took place when this organism was cultured in liquid media. The following experiments were designed to test the premise that various strains, as determined by colonial appearance, possess varying ability to sporulate under similar environmental conditions.

PROCEDURES

The organism used was a strain of *B. globigii*, originally obtained from the University of Wisconisn. This bacillus produces a deep orange pigment and grows well on nutrient agar. However, it was found that pigmentation was enhanced when *B. globigii* was grown on a medium made of corn steep liquor (a by-product of the starch-refining industry); hence this medium was used.

The corn steep liquor was clarified by raising the pH to 8.0 with 50 per cent NaOH, heating for 30 minutes at 70 to 80 C, and filtering through a Buchner funnel precoated with filter-cel no. 540 (a diatomaceous earth filter aid made by the Johns-Manville Company). The filtrate was adjusted to pH 7.2 with H_2SO_4, and the total solids were determined by drying triplicate aliquots in tared dishes at 110 C to constant weight. Sufficient filtrate was diluted with distilled water to make a solution containing 2.5 per cent of total solids. Additional nutrients such as peptones, glucose, and starch were added directly to this solution in the desired concentration, and the medium was sterilized by autoclaving at 125 C for 30 minutes.

Preliminary work was done with liquid media in 500-ml gas-washing bottles equipped with sintered glass spargers through which air was passed by means of a vacuum. This technique, however, was extremely difficult to control and

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recourse was had to agitation as a means of aeration. For this purpose a reciprocal shaking machine was constructed capable of holding six trays, each tray containing thirty-two 125-ml Erlenmeyer flasks. The machine was cam-driven, powered with a $\frac{3}{4}$-hp motor, and delivered 60 complete strokes per minute with a stroke length of 6 inches. The amount of aeration could be controlled approximately by varying the amount of liquid in the flasks. We found that adequate aeration was obtained by using 20 ml of liquid in each flask. The machine was placed in a constant temperature room maintained at 33 ± 1 C.

The use of agitation as a means of aerating liquid cultures proved of great value. Many more experiments could be conducted than was possible with

TABLE 1

Comparison of sporulation of five strains of Bacillus globigii on six different liquid substrates

MEDIA	PERCENTAGE OF SPORULATION OF INDICATED STRAINS, 21, 28, AND 45 HOURS AFTER INOCULATION														
	Strain F_1			Strain F_2			Strain R_1			Strain R_2			Strain S		
	21	28	45	21	28	45	21	28	45	21	28	45	21	28	45
2.5% corn steep liquor	5	20	95	5	20	90	1	5	50	1	2	40	0	2	40
2.0% corn steep liquor 0.5% peptone	40	70	95	25	40	90	1	5	40	1	2	60	0	2	20
2.0% corn steep liquor 0.5% peptone 0.5% glucose	2	5	95	0	2	85	0	2	60	0	2	70	0	2	50
2.0% corn steep liquor 0.5% peptone 0.5% starch	10	15	95	15	15	95	0	2	20	0	2	20	0	2	10
2.0% corn steep liquor 0.5% glucose	2	2	95	0	5	95	0	2	30	0	2	60	0	2	40
2.0% corn steep liquor 0.5% starch	20	30	95	5	10	90	0	2	20	0	2	10	0	2	30

aeration trains, and close agreement between replicate experiments was characteristic.

The percentage of spores was estimated microscopically by the examination of films stained by the Gram technique as well as by examination of wet preparations. This technique had been used previously by one of us (Hayward), and its limitations, which are adequately discussed by Knaysi (1945), are known. Final accuracy is not claimed for the figures in table 1, but the trends shown are considered fully significant.

EXPERIMENTAL

Colonial variants were obtained by smearing a number of plates of a medium composed of 2.5 per cent CSL (corn steep liquor) and 2 per cent agar with

dilutions of an old liquid culture of *B. globigii*. The plates were examined under a dissecting microscope with reflected light at 6× magnification after 20 hours' incubation. It was important to examine the plates while the colonies were relatively young and when the plates were not crowded, otherwise all the colonies had a similar appearance and the variants were difficult to distinguish. Non-pigmented and mucoid colonies were disregarded because it was desired to retain the typical pigmentation of the species. Five different colonial types were isolated and characterized as follows:

(1) S type colony. A smooth, circular, glistening colony with entire edge, umbonate elevation, and with buttery consistency.
(2) R_1 type colony. A rough colony, circular in shape, glistening, with undulate margin, umbonate elevation, and buttery consistency.
(3) R_2 type colony. Differentiated from the R_1 type by deeper serrations and a more coarsely granular appearance.
(4) F_1 type colony. A flat, very coarsely granular colony with curled edge, generally circular, and lacking the glistening qualities of the S and R types.
(5) F_2 type colony. Differentiated from the F_1 type by a comparative lack of pigment and more tenacious adherence to the agar medium.

In the parent culture the R_1 colonies predominated, whereas the F types were encountered less than once in each thousand colonies observed. Although no special effort was made to determine the variation pattern, it was noticed that R types would vary to S. The reverse was not encountered, and the S as well as the F type colonies were relatively stable.

The sporulating qualities of the five strains were determined by inoculating flasks of 2.5 per cent CSL medium supplemented with peptone or carbohydrate, or both, with approximately 1.0×10^5 spores of the respective strains. The flasks were agitated on the shaking machine described above, and examination of the contents was made at periodic intervals. The results of a typical experiment are shown in table 1. It will be seen that the F_1 and F_2 strains sporulated much faster and more completely than the R and S strains on the six media combinations tested. The composition of the substrate exerted some influence, but our data are incomplete. It is hoped that a more complete report on this phase of the problem can be made in the future.

The objection might be raised that the percentage figures quoted do not represent a true picture because of the lysis of vegetative cells in the F strains. This objection is not valid however, because the total spore crop of the F_1 strain in these media was approximately 1.0×10^9 spores per ml, whereas the total count for the R_1 strain rarely exceeded 0.5×10^9 spores per ml. All spore counts were made by averaging the counts of quadruplicate plates having at least 50 colonies per plate.

DISCUSSION

It was determined that various strains of *B. globigii* possessed different degrees of ability to sporulate under a given set of environmental conditions. This fact led to difficulty in duplicating experiments and thus to doubtful interpretation of data. By the selection of a relatively stable strain (F type) having good spor-

ulating ability, together with the use of controlled agitation as a means of aeration, it was possible to duplicate results, so that the standard deviation of replicate experiments was of the same order as the standard deviation of the poured plate and direct microscopic counting procedures used.

SUMMARY

It is shown that variant strains of the so-called *Bacillus globigii* possess varying degrees of ability to sporulate under similar environmental conditions.

Positive correlation between colonial morphology and sporulating ability is shown for *B. globigii*.

The importance of using a pure and stable strain of an organism for studies on physiological factors affecting endospore formation is indicated.

Favorable evidence is presented for the use of agitation as an aeration procedure, and a brief description is given of a machine designed for this purpose.

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