EFFECT OF CULTURE FILTRATES ON SPOROGENESIS IN A SPECIES OF *CLOSTRIDIUM*[1]

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In the course of concerted efforts to produce large crops of spores of the *Clostridium* species known as putrefactive anaerobe (PA) 3679, a sporogenic property of the culture filtrates of the organism was observed. Sporulation induced by culture filtrates was considered to be of sufficient fundamental importance to justify further investigation. This is a preliminary report of a study of the sporogenic property of spent media, presenting evidence of the occurrence of the phenomenon.

The literature on the physiological aspects of sporulation has been comprehensively reviewed by Knaysi (1948), Murrell (1955), Foster (1956), and Stedman (1956). It is evident that much effort has been directed to the study of sporulation in the genus *Bacillus*, but reports of investigations on sporulation in the genus *Clostridium* are notably sparse. The work of Leifson (1931), Kaplan and Williams (1941), and Wynne (1948) are the most comprehensive contributions in this area.

It seems generally agreed that bacteria form spores when the growth of a sporulating species in an adequate medium has made the medium unfavorable for further growth. Some early students of sporulation pointed to the accumulation of metabolic products as the initiator of sporulation, but a larger group has held that the exhaustion of nutrients induces sporulation (Knaysi, 1948). On the basis of published evidence, one cannot conclude that either of these is the specific condition inducing sporulation.

There have been several reports of enhanced sporulation in the presence of culture filtrates of bacteria. Mellon (1926) noted the improved sporulation of a bacillus in the presence of a symbiont with which it was isolated. Culture filtrates of the symbiont organism also increased the sporulation of the bacilli without inhibiting growth. Beck *et al.* (1956) found that a streptomycin resistant strain of *Bacillus anthracis* was unable to sporulate in the presence of streptomycin. However, when grown in the presence of the culture filtrate of *Bacillus cereus* var. *terminalis* in a medium containing streptomycin, the resistant strain of *B. anthracis* sporulated. Buston and Rickard (1956) reported that sporulation of the fungus *Chaetomium globosum* was enhanced by the metabolic products of its own growth.

MATERIALS AND METHODS

The organism used in this study is a variant of PA 3679 which arose spontaneously in the course of work with PA 3679 (ATCC 7955), and it has been designated in this paper as PA 3679h. The only significant difference observed between the two strains is that PA 3679h sporulates under conditions which allow no sporulation of the parent strain. For example, on solid medium (brain-heart infusion agar, Difco), the parent strain did not sporulate although it grew well, but the variant yielded a high percentage of spores. Neither strain sporulated in the identical fluid medium. The morphological and biochemical characteristics of both strains were identical with those described by Gross *et al.* (1946) for three strains of PA 3679. The heat resistance of the spores of these two strains grown and heated in the glucose brain broth of Stumbo *et al.* (1945) was equal. Three heat resistance determinations on PA 3679 yielded an average D value[2] of 19.3 min at 110 C, and the average of four measurements of the heat resistance of PA 3679h showed D equal to 19.0 min at 110 C.

Unless otherwise indicated, the growth medium in these experiments was trypticase broth which consisted of 2 per cent trypticase (BBL), a tryptic

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[2] D = time in min at the given temperature necessary to accomplish a 90 per cent reduction in number of spores. (Stumbo *et al.*, 1950.)

digest of casein, and 0.1 per cent sodium thioglycolate, adjusted to pH 7.2 to 7.4 before sterilization. Spent medium refers to the culture filtrates from 72 hr cultures, and refortified spent medium refers to the same medium refortified by addition of all the ingredients of the original medium, with a readjustment of the pH. For example, spent trypticase broth was refortified by adding 2 g trypticase and 0.1 g sodium thioglycolate per 100 ml spent medium, and readjusted to pH 7.2 to 7.4.

The routine inoculum in these experiments was 0.1 per cent by volume of a refrigerated stock spore suspension of PA 3679h. This stock suspension was a refortified spent medium culture containing 1 to 5 $\times$ 10^7 spores/ml.

The cultures were grown in 16 by 150 mm screwcap culture tubes with no special means of insuring anaerobiosis other than the inclusion of sodium thioglycolate in the medium. Control studies with cultures in anaerobic jars showed no advantage in using the anaerobic jar, so its use was discontinued. The media were prepared and sterilized immediately before inoculation. The tubes were inoculated by means of a pipette, the caps were screwed tight, and the tubes were inverted once to distribute the inoculum throughout the medium. When a spore inoculum was used, it was heated for 10 min at 80 C, either as a spore suspension or in the tube after inoculation.

Populations were measured by direct microscopic counting using dark contrast-medium phase microscopy, and the Petroff-Hausser counting chamber. Only highly refractile spores were counted as spores. Sufficient squares were counted to include at least 200 cells or spores, and replicate counts on the same culture fell within the theoretical range for the Poisson distribution. The range in the data presented is indicated by the actual range of the data observed on replicate cultures, or by the theoretical 95 per cent confidence interval based upon the mean $\pm 1.96 \times$ the square root of the number of cells counted, if this range was greater than the observed range.

In this paper the term sporogenic medium arbitrarily refers to a medium which will produce in excess of 1 $\times$ 10^7 spores per ml, and nonsporogenic media are those which produce less than 1 $\times$ 10^7 spores per ml of medium.

RESULTS

When PA 3679h was grown in brain-heart infusion broth (Difco) or trypticase broth, the maximum spore population attained rarely reached 1 $\times$ 10^6 spores/ml unless thiamin was added to the medium; then the spore population averaged slightly in excess of 1 $\times$ 10^6 spores/ml. However, when the growth was interrupted, and the medium reinoculated at a low level and reincubated, sporulation occurred in the same media under similar conditions. The growth could be interrupted either by heating the culture sufficiently to kill the growing cells, or by removal of the growing cells before the medium was exhausted of its nutrients. Table 1 contains typical data showing this effect. Continuous growth of the culture for 77 hr produced less than 1 $\times$ 10^6 spores/ml, but the interrupted growth cultures yielded 6.1 $\times$ 10^7 spores/ml. The growth was interrupted by heating the cultures for 20 min at 80 C at the indicated time intervals after inoculation. The cultures were then reinoculated at the original level, and again incubated for a sufficient time to make the total growth period 77 hr. Maximum sporulation occurred when the initial growth period had been 18 hr. The decrease in spore production in those cultures interrupted after 21 and 24 hr probably occurred because the growth was limited by the diminished nutrients in the medium after the

TABLE 1

The effect of simple interruption of growth of PA 3679h culture in 2 per cent trypticase broth containing 0.1 μg/ml added thiamin

Course of Growth*	Spore Population
	10^6/ml
Continuous for 77 hr	0.5 ± 0.4
Interrupted after	
15 hr	5.5 ± 1.7
18 hr	61.0 ± 8.5
21 hr	26.0 ± 3.8
24 hr	13.5 ± 1.8

* After the initial growth was stopped by heating for 20 min at 80 C the medium was reinoculated with spores so that the initial population was 5 $\times$ 10^5 spores/ml. Combined periods of growth equal 77 hr.

prolonged initial growth period. Such variations in treatment as removal of the cells instead of heating the culture, employing a constant 48 hr second growth period, and using a vegetative inoculum instead of spores had no effect on the results except that, where inoculations were made with vegetative cells at the same level, maximum sporulation occurred when the initial growth period was 8 hr.

When the initial growth period was 72 hr, the resulting culture filtrate supported little or no growth upon reinoculation. This culture filtrate or spent medium was routinely used in the remainder of this study unless otherwise specified. When the spent trypticase broth was refortified as described above, luxuriant growth and sporulation occurred. The refortified spent medium represented a total of 4 per cent trypticase; therefore the sporogenicity of the spent medium was compared to that of 4 per cent trypticase broth. Such a comparison showed that maximum spore populations in both media were attained in 48 hr and that sporulation began earlier and reached higher levels in the refortified spent medium. In a typical experiment the 48 hr spore population of refortified spent medium was 1.32 (±.17) × 10^8 spores/ml and that of the 4 per cent fresh medium was 3.2 (±.8) × 10^7 spores/ml. On incubation beyond 48 hr, the spore populations diminished, indicating that germination was taking place in the medium.

When the sporogenicity of 4 per cent trypticase broth was first compared with that of the refortified spent trypticase broth, no sporulation was observed in the fresh medium, which was in sharp contrast to the good sporulation in the refortified spent medium which consistently yielded about 5 × 10^7 spores/ml. Subsequently when another lot of trypticase was used, relatively good sporulation occurred in the 4 per cent fresh medium, and the refortified spent medium yielded only about three times as many spores as fresh medium. Several lots of the peptone were tested, with highly variable results. When vitamin supplements were added to these media, only thiamin had any effect. Addition of thiamin to the 2 per cent medium produced numerous swollen rods resembling immature sporangia. The culture had all the appearance of incipient sporulation, but no refractile spores developed. Instead the swollen rods diminished in number with extended incubation. Addition of thiamin to the 4 per cent media removed the variability among the different lots and all the media were sporogenic. Supplementation with 0.1 μg/ml of thiamin gave maximum response both in growth and sporulation.

Table 2 shows the results when 2 per cent, 4 per cent, and 2 per cent refortified trypticase broths were prepared from two lots of trypticase with and without thiamin. Even the sporogenic lot of trypticase was deficient in thiamin, inasmuch as both fresh media prepared from this lot exhibited significant increases in growth upon the

TABLE 2

Comparison of growth and sporulation in 2 per cent, 4 per cent fresh, and 2 per cent spent refortified trypticase broth prepared from 2 lots of trypticase, with and without added thiamin

Lot	Broth	No Thiamin Added			Thiamin Added*			Increase	
		Total cells	Spores		Total cells	Spores		Total cells	Spores
		10⁶/ml	*10⁶/ml*	%	*10⁶/ml*	*10⁶/ml*	%	%	%
a	2%	103 ± 10	> 0.1	> 0.1	203 ± 16	> 0.1	>0.1	97	0
	4%	149 ± 12	> 0.1	> 0.1	539 ± 49	35 ± 8	6	262	—
	2%†	99 ± 9	34 ± 3	34	261 ± 22‡	148 ± 14	57	163	336
b	2%	218 ± 21	> 0.1	> 0.1	331 ± 38	4 ± 3.9	2	51	—
	4%	410 ± 58	86 ± 12	21	538 ± 47	115 ± 29	21	31	34
	2%†	152 ± 32	106 ± 12	70	417 ± 40‡	257 ± 31	61	174	142

* 0.1 μg/ml.

† Spent refortified broth.

‡ Thiamin added only to the refortified spent medium; none added to the 2% medium for the initial growth.

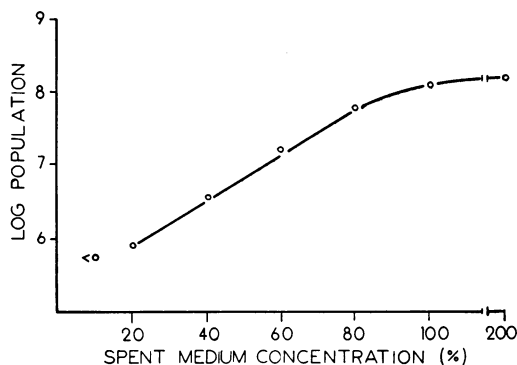

Figure 1. The effect of the concentration of spent medium upon spore production. Concentration indicated represents percentage of the original concentration of the spent medium in 2 per cent fresh typticase broth.

addition of 0.1 μg of thiamin/ml. The addition of thiamin to lot *a* media caused large growth increases and allowed sporulation to occur in the 4 per cent medium. The media from both lots produced the same amount of growth with thiamin supplementation. It seems apparent that the thiamin has little direct effect on the sporulation other than producing an adequate nutritional environment for growth, since the percentage of sporulation was not affected in the 4 per cent lot *b* medium, and only 6 per cent sporulation occurred in the lot *a* medium with added thiamin.

Inasmuch as the 4 per cent medium was relatively sporogenic, the effect of trypticase concentration upon sporulation and growth of the organism was examined. This revealed that the percentage of sporulation of PA 3679h increased with the concentration of trypticase in the broth up to 6 per cent, where 40 per cent sporulation occurred. Above this concentration the degree of sporulation was constant and the increased population reflected the linear increase in total population of the culture up to 10 per cent trypticase.

Antisporulation factors for certain bacilli have been reported in complex organic media (Roberts and Baldwin, 1942; Foster *et al.*, 1950; Hardwick *et al.*, 1951). When trypticase media or their dry ingredients were treated in the manner these workers describe for the removal or inactivation of antisporulation factors, the resulting media yielded no enhanced sporulation. On the basis of these observations, and the sporogenicity of the increased concentrations of trypticase, it appears that antisporulation factors are not complicating elements in this system.

An evaluation of the effect of concentration of the spent medium in which PA 3679h has grown is shown in figure 1. Spent 2 per cent trypticase broth was serially diluted to 10 per cent of its original concentration. A portion of spent medium was concentrated by vacuum distillation, and made up to volume with distilled water to yield 200 and 500 per cent of its original concentration. Each concentration level from 10 through 500 per cent was refortified with 20 mg/ml of trypticase and 1 mg/ml of sodium thioglycolate, and the pH was adjusted to 7.2. The media were then sterilized, inoculated, and incubated in the usual manner, and the spore populations were measured after 48 hr incubation. The 500 per cent concentration was inhibitory to the growth of PA 3679h. The 10 per cent and 200 per cent points on the curve represent the mean of 2 separate experiments; all other points are the mean of 3 or 4 separate experiments. The plot of the log of the spores produced against the concentration of spent medium shows that the sporulation is proportional to concentration through 80 per cent of the original concentration of the spent medium.

Preliminary experiments indicated that inocu-

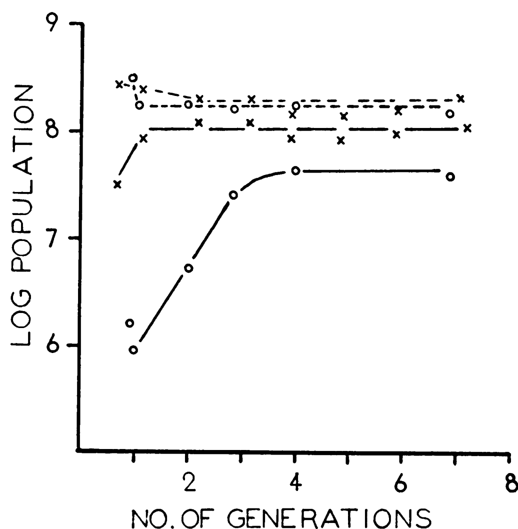

Figure 2. The effect of the number of generations of growth in single and double strength spent medium upon growth and sporulation of PA 3679h (o = spent medium, x = double strength spent medium, —— = spores, - - - = total cells).

lation of the spent medium with more than 10 per cent of the terminal population which the medium would support resulted in no sporulation. This suggested that for sporulation the cells must grow for several generations in the environment supplied by the spent medium. In order to examine this more thoroughly, a study was made of the effect upon spore production of inoculum size—and thereby the number of generations of growth—in single and double strength spent trypticase broth refortified at a constant level. The double strength spent medium was prepared as previously described. Both the single and double strength trypticase broth were refortified with 20 mg trypticase and 1 mg sodium thioglycolate per ml of spent medium, both were adjusted to pH 7.2, then distributed in tubes and sterilized in the usual manner.

A 21 hr 2 per cent trypticase broth culture of PA 3679h containing 1.75×10^8 cells per ml was used as the inoculum. Aliquots of 10, 5, 2.5, and 1.25 ml of the culture were centrifuged and the supernatant fluids were discarded and replaced with 10 ml of single and double strength spent trypticase broth refortified with sporogenic trypticase. Additional cultures receiving smaller inoculations were prepared by adding 0.63, 0.31, 0.15, and 0.08 ml aliquots of the whole culture to tubes containing 10 ml of the two test media. All cultures were incubated for 40 hr, refrigerated, and observed microscopically for sporulation and growth. Results of this experiment, showing the spore population as a function of the number of generations of growth in the medium, are seen in figure 2. The number of generations was calculated by the formula

$$g = \frac{\log P_t - \log P_i}{\log 2}$$

in which g is the number of generations, P_i is the initial population, and P_t is the terminal population.

These results show that in the single strength spent medium cultures, sporulation increased as the growth in the medium increased up to four generations, beyond which additional generations were of no significant effect. In double strength spent media, maximum sporulation was attained after about one generation and this medium yielded twice as many spores as the single strength medium, but the two media yielded essentially the same total population. It must be

TABLE 3

Sporulation of PA 3679h when grown in the refortified culture filtrates of several species of Clostridia

Source of Culture Filtrate	Spores/ml
	10^6
PA 3679h	63 ± 11
Clostridium sporogenes (ATCC 3584)	83 ± 13
Clostridium parasporogenes (ATCC 693)	95 ± 14
Clostridium aerofoetidum (ATCC 4894)	103 ± 14

pointed out that the size of the inoculum was varied to produce cultures yielding variations in the number of generations of growth in otherwise identical cultures. The calculated values for the number of generations were based on the assumption of 100 per cent viability of the inoculum, but this is possibly in error. An appreciable oxygen damage may have been incurred by the vegetative cell inoculum for each of these anaerobic cultures, especially in those receiving the large inocula, or, in other words, in those cultures showing the fewer generations of growth. These cells were subjected to more aerobic manipulation than is usual in anaerobic culture methods. If damage did occur, the number of generations would have been greater than the calculated values indicate.

Further evidence for the inability of cells from a nonsporogenic medium to sporulate in a sporogenic medium unless growth occurred was obtained by resuspending different concentrations of cells from fresh trypticase broth cultures in spent medium to which no additional nutrients had been added. In this case, cells equivalent to 100 per cent of the population of the culture, suspended in equivalent volumes of 1×, 2×, 3×, and 5× concentrations of spent medium, yielded no more than 0.8 per cent spores. This is equal to what would have been obtained if the cells had been left in the growth medium.

The sporogenicity of culture filtrates is not peculiar to those of PA 3679h. *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus cereus* were 3 of the aerobic species of bacteria examined whose culture filtrates were significantly more sporogenic for PA 3679h than the corresponding fresh medium when both the filtrates and the fresh medium were supplemented with trypticase. Comparison of the sporulation of PA 3679h

in its own refortified spent trypticase broth and in refortified spent broth from 3 other *Clostridium* species is shown in table 3. These results indicate that the culture filtrates from *C. sporogenes* strain ATCC 584, *C. parasporogenes* strain ATCC 693, and *C. aerofoetidum* strain ATCC 4894 were equal or superior to those of the homologous species.

DISCUSSION

Growth of PA 3679h through a normal culture cycle yielded a very low degree of sporulation, but when the growing population was replaced by a new small population before the medium was exhausted, good sporulation ensued. This sporogenic property acquired by the medium during growth of the culture must be the result either of the removal of some component(s) from the medium, or the addition of some substance(s) to the medium during vegetative growth. Evidence is presented which indicates that the removal of substances specifically inhibiting sporulation is probably not the mechanism for the development of sporogenicity. This is shown by the inability to enhance sporulation of the fresh medium by methods which others have successfully used to remove or inactivate specific antisporulation factors. It is further supported by the facts that refortification does not depress the spore production, and that sporogenicity of the fresh medium increases with the concentration of the medium up to 6 per cent.

The greatly enhanced sporulation in trypticase broth culture filtrates as compared to that in the fresh medium, the precipitous decrease in sporogenicity of the culture filtrate upon dilution, and the observation that sporulation occurs only if the culture is permitted to grow for several generations in a single strength medium (but will occur after only one generation in the concentrated culture filtrate) suggest the following explanation for the sporogenicity of the culture filtrates. During the vegetative phase of the culture, something is produced in the medium which is necessary for sporulation. This material is present in sufficient concentration so late in the culture cycle that large-scale sporulation will not occur in the original culture. If, however, the means are provided for additional growth in this environment, either by removal of the cells before the medium is exhausted, or by the addition of nutrients to the exhausted filtrate, and reinoculation, sporulation occurs. Under these conditions several generations of growth occur in an environment where the ratio of the sporogenic component to the cell population is much greater than in the continuous growth of the culture from the fresh medium. To be consistent with this explanation, the higher concentrations of.trypticase must either allow a greater production of the sporogenic material, or sporulation occurs by a different mechanism.

The exhaustion of nutrients would appear to be excluded as the cause for this sporogenic property of the culture filtrate because the refortification of the spent medium yielded an exceedingly sporogenic medium as compared to the fresh 2 per cent trypticase broth. It should not be overlooked, however, that the altered ratio of nutrients which occurred as the result of the initial growth before refortification might play a role in the sporogenicity.

The effect of the addition of thiamin to the 4 per cent trypticase broth is consistent with the thesis that sporulation occurs only in an adequate growth medium. The growth stimulation which occurred upon the addition of thiamin showed that thiamin was present in suboptimal concentration. The incipient sporulation which occurred upon the addition of thiamin to the 2 per cent trypticase broth, and the failure of these abortive spores to mature, show that the 2 per cent broth is deficient in something required for the maturation of the spores.

In addition to the usual difficulties and limitations inherent in anaerobic methods, two problems which could not be adequately controlled limit the accuracy of data obtained in studying sporulation of the Clostridia. The tendency for lysis of the vegetative cells to occur almost simultaneously with the initiation of visible sporulation in some media makes comparisons of percentages of sporulation in various media deceiving unless one has rather exact knowledge concerning the amount of lysis which occurs. The germination of spores in the medium in which they are formed requires careful observation of the cultures to evaluate sporogenicity under a given set of conditions.

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SUMMARY

Culture filtrates of several species of bacteria are more sporogenic for a variant strain of putrefactive anaerobe no. 3679 than is fresh medium. Evidence has been presented which suggests that the growth of the organism produces something in the medium which is required for sporulation.

Spore formation in tryptic digest of casein broths increases with the increase in the concentration of peptone, through 6 per cent of the casein digest.

Addition of thiamin to casein digest broths stimulates the growth of PA 3679h. When added to the 2 per cent casein digest broth, thiamin causes the initiation of sporulation in a large proportion of the vegetative cells but does not permit the maturation of the spores. In broths containing higher concentrations of the peptone, the addition of thiamin enhances sporulation in proportion to the growth stimulation it effects.

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