THE IMPORTANCE OF ENRICHMENTS IN THE CULTIVATION OF BACTERIAL SPORES PREVIOUSLY EXPOSED TO LETHAL AGENCIES

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Although the elemental food requirements of all microörganisms may be considered identical, the form in which the essential elements can be utilized by the various species is subject to almost infinite variation. Literally hundreds of different culture media have been proposed, many of which find application because they favor or prevent the growth of particular species or related groups. That the individual organisms which make up a pure culture may also vary widely in their metabolic requirements is apparently not recognized by many bacteriologists. The published reports of many investigators dealing with bacterial resistance and the cultural methods employed by a large number of our control laboratories show how little this fundamental fact affecting the nutrition of bacteria is understood.

It is the purpose of the present paper to show that bacterial spores which survive destructive physical or chemical influences are much more exacting in their nutritional requirements than spores not subjected to such treatment. Evidence is presented which indicates that this phenomenon is not restricted to spores but applies to vegetative forms as well.

EXPERIMENTAL

Cultures and methods

Of the cultures used *Bacillus subtilis*, *Bacillus cohaerens*, and *Bacillus albolactis* were obtained from The American Type Culture Collection, CC is an unidentified species isolated from spoiled evaporated milk. The spores were produced on infusion agar

slopes. After one to six weeks' incubation, the growth was washed off with distilled water, filtered through cotton, and centrifuged. The supernatant fluid was then completely poured off and the spores resuspended in distilled water, following which they were again centrifuged and resuspended as before. To insure thorough removal of the medium and culture products, the washing process was performed three times. The suspensions were then examined microscopically, and if spore clumps were present these were broken up by vigorous shaking with sterile washed sea sand. The concentrated stock suspensions thus prepared were plated for purity and enumeration and held at room temperature until used. The test spore suspensions in distilled water contained from one to two million viable spores per ml.

The suspensions were heated in pyrex tubes in a stirred glycerin bath equipped with a thermo-regulator which maintained a temperature of 98°C. ± 0.5°C. The heated suspensions were cooled to 20°C. immediately after their removal from the bath.

The spores were exposed to $HgCl_2$ in aqueous solution containing 0.05 per cent of the salt. After their exposure the spores were recovered by centrifugation and washed twice with sterile distilled water.

Ultra-violet light was supplied by a cold quartz mercury-vapor lamp of the orificial applicator type. The power consumption of this burner is about 9 watts and approximately 95 per cent of the rays emitted are at the line 2537A. Four milliliters quantities of the spore suspension at 30°C. were irradiated in a small quartz flask placed four inches from the burner. The flask was equipped with a quartz stirrer which provided rapid and uniform agitation. After exposure to light, heat or $HgCl_2$, equal portions of the suspension were poured with 15 ml. of agar of the desired composition. The plates were counted after 48 hours' incubation at the optimum temperature of the organism. A magnifying glass was used in counting and the figures represent the averages of triplicate plates. After the initial count was recorded, the plates were returned to the incubator and examined one week later to preclude the possibility that observed differences were due to rate of development rather than capacity to develop.

Six media of the following compositions were used:

1. Nutrient agar: 0.5 per cent peptone (Difco), 0.3 per cent beef extract (Liebig's), 0.5 per cent sodium chloride, 1.5 per cent agar (granulated).

2. Nutrient agar reinforced with one drop of sterile defibrinated cows' blood per plate.

3. Nutrient agar reinforced with 0.3 cc. of sterile 10 per cent glucose solution per plate.

4. Nutrient agar reinforced with 0.3 cc. of sterile 10 per cent yeast extract (Difco) solution per plate.

5. Infusion agar in which the beef extract of the above nutrient agar was replaced by beef infusion made by extracting 500 grams of lean beef with 500 ml. water, heating, filtering and using at the rate of 500 ml. per liter.

6. Tomato-juice milk-powder agar containing in addition to the standard quantities of peptone and beef extract, 5 per cent of tomato juice, 0.5 per cent milk powder, and 0.15 per cent Sörensens' air-dried phosphate. ($Na_2HPO_4 \cdot 2H_2O$).

INFLUENCE OF ULTRA-VIOLET LIGHT UPON THE GERMINATION OF SPORES

In table 1 is shown the number of survivors germinating on different nutrient media when the spores of four species were exposed to ultra-violet light sufficient to kill all but a relatively small proportion. In the first column under each species is also shown the number of colonies developing from the untreated spores plated on the same series of media, at the same time. From this table it may be seen that nutrient agar was much less favorable to the germination of the irradiated spores than to the germination of those not so treated. With two species, namely, *B. subtilis* and CC, enrichments had considerable influence upon the viability of the untreated spores but this effect was always much less than that observed after ultra-violet irradiation. Blood seems to be particularly effective as enrichment for irradiated spores. The effect of yeast extract not only in this but in nearly all experiments was to somewhat reduce rather than to increase the number of germinating spores. The toxicity of yeast

extract was further shown by the fact that combinations of this extract with glucose, blood and other enrichments materially reduced the viable count. It was not possible to increase the count significantly by increasing the quantity of glucose or blood, or by combination of the more effective enrichments. Increasing the concentration of the peptone and beef extract in the nutrient agar formula, likewise failed to increase the total count obtained with this medium.

TABLE 1

The ability of bacterial spores to germinate in different nutrient media before and after lethal exposures to ultra violet light

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER							
	B. cohaerens		*B. subtilis*		CC		*B. albolactis*	
	Untreated	Irradiated	Untreated	Irradiated	Untreated	Irradiated	Untreated	Irradiated
	0000 omitted		*0000 omitted*		*0000 omitted*		*0000 omitted*	
Nutrient agar	134	22	88	55	45	62	47	86
Nutrient agar plus blood	140	225	123	315	122	379	51	239
Nutrient agar plus glucose	120	64	218	154	137	153	47	83
Nutrient agar plus yeast	131	16	47	51	25	54	37	84
Infusion agar	142	75	237	171	148	187	41	156
Tomato juice milk powder agar	149	25	232	88	145	124	34	49

INFLUENCE OF HEAT UPON THE GERMINATION OF SPORES

When the spores of the same species were exposed to destructive heat and subsequently cultivated on different media, the results shown in table 2 were obtained. As in the preceding experiment, spores which outlived the treatment were much usually more fastidious in their growth requirements than those derived from the same culture protected from destructive action. The differences observed here in favor of enrichments were much greater than those noted with irradiated spores. Glucose was usually more effective than blood in the cultivation of heat-surviving spores, although the reverse was true in the subculture of irradiated spores. Glucose enrichment not infrequently increased

the number of viable heated *B. cohaerens* spores one hundred fold. Infusion or tomato milk powder may under some conditions equal or excel glucose as enrichments for the cultivation of heated spores.

The need for the application of the enrichment principle to resistance studies is obvious. Under the conditions described above, the thermal death time of *B. cohaerens* spores was 25 minutes when nutrient agar was used as the subculture medium;

TABLE 2

The viability of bacterial spores in different nutrient media before and after lethal exposures to heat

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER							
	B. cohaerens		*B. subtilis*		CC		*B. albolactis*	
	Untreated	Heated	Untreated	Heated	Untreated	Heated	Untreated	Heated
	0000 omitted		*0000 omitted*		*0000 omitted*		*0000 omitted*	
Nutrient agar	134	40	88	80	45	15	47	157
Nutrient agar plus blood	140	345	123	350	122	160	51	320
Nutrient agar plus glucose	120	2100	218	521	137	511	47	210
Nutrient agar plus yeast	131	34	47	70	25	6	37	135
Infusion agar	142	1350	237	480	148	440	41	390
Tomato juice milk powder agar	149	1320	232	400	124	420	34	280

with glucose agar the thermal death time was 39 minutes, an extension of over 50 per cent.

Table 3 shows the results obtained when *Escherichia coli* was cultivated with and without enrichment, before and after exposure to irradiation and heat. The organisms were recovered from an 18-hour broth culture prepared and exposed as were the spore suspensions previously described. Enrichment in one instance increased the number of colonies which developed on nutrient agar almost nine fold, although such growth-promoting substances were practically without effect on the unexposed organisms. Other enrichments were somewhat less effective, although all except yeast extract yielded a much higher count than plain nutrient agar.

Table 4 shows that many spores which survive rigorous chemical treatment, in common with irradiated and heated spores, require enrichments for their germination.

The relative proportion of *B. cohaerens* spores previously irradiated or heated which grow on different media depends largely on the relative proportion of spores which survive the destructive

TABLE 3

Viability of E. coli in different media before and after lethal exposures to irradiation and heat

MEDIA	UNTREATED	IRRADIATED	HEATED
	0000 omitted		
Nutrient agar	57	20	27
Nutrient agar plus blood	57	65	102
Nutrient agar plus glucose	60	45	105
Nutrient agar plus yeast	61	25	27
Infusion agar	61	38	189
Tomato juice milk powder agar	54	69	237

TABLE 4

The viability of bacterial spores before and after lethal exposures to $HgCl_2$

MEDIA	NUMBER OF VIABLE SPORES PER MILLILITER			
	B. cohaerens		*B. subtilis*	
	Untreated	$HgCl_2$	Untreated	$HgCl_2$
	0000 omitted		*0000 omitted*	
Nutrient agar	134	10	88	52
Nutrient agar plus blood	140	24	123	129
Nutrient agar plus glucose	120	1200	218	230
Nutrient agar plus yeast	131	43	47	42
Infusion agar	142	351	237	230
Tomato juice milk powder agar	149	414	232	219

action which in turn depends on the severity and duration of the treatment. This fact is illustrated in table 5. When 50 per cent of the culture survived, the survival ratios closely resembled those obtained with untreated spores. As the mortality is increased, however, these ratios increase rapidly and progressively. If the results with this organism are representative for

other species the effectiveness of enrichment substances in subculture media will depend on the intensity of the killing influence and may be quite limited in the presence of mild killing agents.

In the next experiment, consideration was given to the time at which enrichment was supplied. In this experiment spores of *B. cohaerens* were so heated that direct subculture on glucose agar yielded after incubation a convenient number of colonies, while similar inoculations poured with nutrient agar permitted no ger-

TABLE 5

The development of irradiated and heated B. cohaerens spores in different media with changes in the proportion of survivors

MEDIA	PERCENTAGE SURVIVAL							
	50		25		10		1	
	Irradiated	Heated	Irradiated	Heated	Irradiated	Heated	Irradiated	Heated
Nutrient agar	111	121	101	37	31	3	10	3
Nutrient agar plus blood	147	173	278	128	180	403	130	748
Nutrient agar plus glucose	108	185	146	300	99	1000	94	6000
Nutrient agar plus yeast	72	104	60	10	21	3	7	1
Infusion agar	141	176	174	231	123	500	80	3167
Tomato juice milk powder agar	118	177	151	226	49	667	42	2650
Ratio nutrient: glucose agar		1:1+		1:8		1:333		1:2000
Ratio nutrient: blood agar	1:1+		1:2+		1:5		1:13	

mination. A large series of nutrient agar plates were poured immediately after exposure. As soon as the agar became solid the glucose solution was spread uniformly over the surface of three plates with a sterile applicator. All the plates were then incubated and at desired intervals three plates were treated with the glucose solution as described. All plates containing spreaders were discarded. These were troublesome only when the enrichment was added to the freshly poured plate. The results obtained are presented in table 6. With *B. cohaerens* the results are rather surprising. Within three hours there is a definite

decline in the number of spores able to germinate. This trend increases so rapidly that after 24 hours only an occasional spore is viable. Thereafter glucose was practically without effect as a means of inducing the germination of these spores. With CC and *B. subtilis* little or no loss of viability occurred during the first 24 hours, but from this time on CC spores rather rapidly lost their power to germinate, so that 8 to 10 days after the initial seeding they were practically non-viable. *B. subtilis* spores retained their viability unchanged for the first five days and then declined rapidly. The reason for this rather rapid loss of via-

TABLE 6

The growth of heated spores with changes in the time at which glucose enrichment is supplied

TIME AFTER PLATING ENRICHMENT WAS ADDED	B. COHAERENS	B. SUBTILIS	CC
Immediately	206	36	78
3 hours	161	38	82
5 hours		35	79
24 hours	3	36	70
2 days	0	41	47
3 days	0	35	26
4 days	0	50	10
5 days	0	39	6
8 days	0	40	1?
10 days	0	6	0

Suspensions contained approximately 1,000,000 viable spores per milliliter before exposure.

bility of spores seeded on an inadequate medium is not entirely clear. When *B. cohaerens* spores heated as specified above were examined microscopically on nutrient agar blocks, it was observed that a large number of the cells became swollen rather rapidly and lost their characteristic refractive property, but the germination process did not continue visibly beyond this stage.

In view of these observations, the logical deduction would be that heated spores undergoing incomplete germination (because of an inadequate medium) have thereby assumed the characteristics of the vegetative form, and consequently die rather rapidly in the absence of a favorable nutrient environment. This view

is in harmony with some of our unpublished data in which it was observed that susceptibility to heat in germinating spores occurs a considerable time before visible rupture in the spore wall.

DISCUSSION

The foregoing observations show clearly that organisms which survive drastic killing factors are more fastidious in their food requirements than the less resistant individuals which predominate in an unexposed portion of the culture. While the reaction is apparently common to all organisms, it is not surprising that different species vary considerably in the degree to which they exhibit it. The advantages of a fermentable carbohydrate in the bacteriological examination of milk were pointed out by Sherman (1916). It is interesting to note that lactose enrichment yielded a higher count with both raw and pasteurized milk, but the percentage increase was much greater with the latter. More recently Ayers and Mudge (1920), Safford and Stark (1935), and others, confirmed this but it is apparent that the broader implications to be drawn from these observations were not considered. Morrison and Rettger (1930) made the significant observation that enrichment substances present in the heating or subculture media entirely eliminated dormancy and skips exhibited by heated spores subcultured in plain nutrient broth. Williams (1936) recently reported the superiority of casein digest as a basic plating medium for the detection of flat-sour spores in sugar. The fact that the sugar solutions were boiled before they were used is of interest in view of the results obtained.

In seeking an explanation for the dissimilar food requirements of resistant and non-resistant spores, two theories suggest themselves neither of which can easily be proved or refuted. The relatively small proportion of resistant spores which make up every culture may by inheritance possess metabolic requirements different from the vast majority. If this were true destructive influences by eliminating all the weak and moderately resistant individuals would serve to concentrate those cells more exacting in their growth requirements and consequently more responsive to growth-promoting substances. This view is not necessarily

incompatible with the findings that different enrichment substances may be required depending on the nature of the destructive action, for proof is lacking that spores which are resistant to one influence are necessarily those resistant to other influences, and there is some evidence to the contrary (Duggar and Hollaender, 1934). The possibility exists, also, that spores surviving destructive influences have sustained some form of injury, impairment of certain enzymic functions perhaps, which has rendered them unable to metabolize nutrients formerly utilizable. Oster (1934) has shown that monochromatic ultra-violet light may cause different degrees of damage with yeast, and that certain metabolic functions of the cells are affected before reproduction is stopped. Rahn and Barnes (1933), however, reported that loss of reproductive ability preceded loss in fermentation ability for the majority of yeast cells exposed to irradiation. The second hypothesis seems somewhat less likely in view of the fact that significant differences in the germination of heated *B. cohaerens* spores in the different media were not noted until more than 50 per cent of the cells were killed. If injury were the cause it is difficult to understand why this should not be reflected in the less resistant half of the culture.

SUMMARY

The results of experiments are reported which indicate that bacterial spores which survive drastic killing influences are much more exacting in their nutritive requirements than the less resistant individuals which comprise the bulk of the viable population before treatment. Heat-surviving *Escherichia coli* show the same reaction.

Enrichment substances incorporated in culture media are essential for the accurate enumeration of bacteria previously exposed to highly lethal factors.

The enrichment substances to be used will vary with the species of organism and the nature of the destructive action.

The effectiveness of enrichments in the determination of viability varies with the extent of the killing action. The favorable influence of growth-promoting substances increases as the per-

centage mortality increases and may be negligible in the presence of low mortality.

Spores which survive destructive influences when seeded in inadequate media lose their viability rather rapidly.

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