REQUIREMENTS FOR PRODUCTION AND GERMINATION OF SPORES OF ANAEROBIC BACTERIA[1]

DONALD O. HITZMAN,[2] H. ORIN HALVORSON, AND TYUNESIA UKITA[3]

Department of Bacteriology, University of Illinois, Urbana, Illinois

Received for publication December 17, 1956

The important role of amino acids in spore germination was established by Hills (1949) and Stewart and Halvorson (1953). Their studies, however, were limited to the aerobic forms because the anaerobes could not be made to produce sufficient quantities of spores in media within a specified time. Using an autoclaved 1 per cent solution of glucose phosphate, Wynne *et al.* (1954) germinated anaerobic spores in 1 hr, but rapid germination in a medium of well-defined nutrients has not been reported.

The investigation reported here had three phases: (1) the development of media and methods for growing and harvesting anaerobic spores, (2) the determination of the optimum conditions and minimal nutritional requirements for their rapid germination, and (3) a study of the effect of inhibitors on germination.

MATERIALS AND METHODS

Organisms. Cultures of *Clostridium butylicum* strain 6014, *Clostridium roseum* strain 6012 (both of the American Type Culture Collection), and *Clostridium acetobutylicum* were tested for their ability to sporulate in various media and under different conditions. Stock cultures maintained on brain heart infusion medium (Difco) were used to inoculate 150 ml of clear sporulation medium in 6 oz bottles.

The sporulation medium (TS-5) giving optimum spore production contained trypticase (BBL), 1.5 per cent; glucose, 0.2 per cent; sodium chloride, 0.5 per cent; and dipotassium phosphate, 0.25 per cent; the whole adjusted to pH 7.2.

Spore germination. Spore germination was measured by determining loss of heat resistance and change in optical density of the cells, as well as by direct count of stainable cells. For heat resistance measurements, a known quantity of a spore culture was added to each of a series of tubes containing a germination medium. The tubes were suspended in a water bath held at 80 C. Aliquots were withdrawn at intervals (up to 15 min), cooled and then transferred to Prickett tubes containing trypticase soy agar (BBL). These dilution tubes were incubated for 1 week at 37 C after which counts were made. The ungerminated spores survived exposure to 80 C for 20 min, whereas germinated cells were killed in 1 min.

As the spores germinate, the optical density of the suspension will change. Two ml of a germination medium were added to 0.05 ml of a spore suspension (approx 5×10^8 spores/ml) in a 10 by 75 mm tube. At pH 7 the germination medium should be clear and in a reduced state, and can be so maintained by the addition of sodium thioglycolate. The tube was immediately inserted into a Bausch and Lomb colorimeter (640 mμ filter) and the change in light transmission observed continuously or at intervals.

Staining with 1 per cent aqueous crystal violet for 30 sec will distinguish germinated cells from spores since the latter do not absorb the stain. Spores and cells were counted in at least 20 fields and the ratio of stained to unstained cells established the percentage germination.

EXPERIMENTAL RESULTS

Production of anaerobic spores. A basal medium consisting of yeast extract (Difco), 0.5 per cent; glucose, 0.2 per cent; K_2HPO_4, 0.25 per cent; NH_4Cl, 0.3 per cent; and NaCl, 0.4 per cent; adjusted to pH 7.5, gave excellent growth of vegetative cells of all three anaerobes, but did

[1] This research was supported in part by Swift and Company, Office of Naval Research, and taken in part from a dissertation presented by the senior author in partial fulfillment of the requirements for the Ph.D. degree at the University of Illinois, Urbana, Illinois.

[2] Present address: Research Division, Phillips Petroleum Company, Bartlesville, Oklahoma.

[3] Present address: Institute for Infectious Disease, University of Tokyo, Shirokane-Damachi, Shiba, Minata-Ku, Tokyo, Japan.

TABLE 1

Sporulation of Clostridium roseum in the presence of trypticase

Medium	Composition of Medium (pH 7.2)						Sporulation after 48 Hr at 37 C
	Trypticase	Glucose	NaCl	K_2HPO_4	Phytone	Proteose peptone #3	
	%	%	%	%	%	%	%
TSB	1.7	0.25	0.5	0.25	0.3		99.0
TS1	1.7	0.20	0.5	0.25			99.0
TS2	1.0	0.25	0.5	0.25			95.0
TS3	0.5	0.25	0.5	0.25		0.3	97.0
TS4	2.0	0.20	0.5	0.25		0.3	80.0
TS5	1.5	0.20	0.5	0.25			99.9
TS6	1.8	0.20		0.50		.	70.0
TS7	1.8	0.20		0.10			60.0

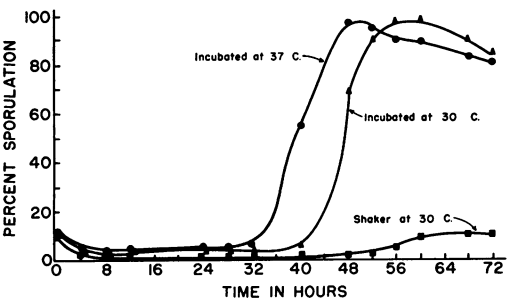

Figure 1. The effect of time, temperature and agitation on the percentage sporulation of *Clostridium roseum* in medium TS 5.

not produce more than 1 per cent spores even after an incubation period of 2 weeks. Various materials were therefore added to this basal medium in an attempt to stimulate sporulation.

Sporulation was not increased by the addition of vitamins, amino acids, purine, or pyrimidines. As with mesophilic aerobes (Stewart and Halvorson, 1953; Curran and Evans, 1954), manganese gave some indication of increasing sporulation but the greatest increase occurred in media containing materials derived from casein, such as casamino acids (acid hydrolyzed casein, Difco), casitone (pancreatic digest of casein, Difco), and trypticase (peptones derived from casein by pancreatic digestion, BBL). Of the three organisms tested, *C. roseum* consistently produced the most spores and was used in all subsequent tests.

Because the trypticase concentration had been found to influence the percentage sporulation, media containing various concentrations of trypticase were used to determine the conditions for optimum sporulation (table 1).

With a 10 per cent inoculum of a 24 hr culture (600 ml medium in a 1 L flask, held at 37 C) medium TS 5 consistently gave essentially complete sporulation in 48 hr with a yield of approx 2×10^9 spores/ml. Except for a slight shaking after 24 hr to disperse the clear top layer of unused medium the flask should remain stationary for 48 hr. Spore formation was directly affected by the time, temperature, and method of incubation (figure 1). Sporulation reached a peak at 48 hr, after which there was a gradual decline due to germination in the growth medium. Similar results were obtained at 30 C, but only after a longer incubation time (56 hr). The cells germinated in the growth medium did not have the typical elongated rod shape of vegetative cells, but retained the spore shape and stained with a simple stain. These stainable "spore" forms did not retain the heat resistance of spores, and so were "germinated spores." Thus the presence of vegetative cells in the culture indicates that the sporulation peak has not yet been reached; the presence of stainable "spores" indicated the peak has passed.

Harvesting clean anaerobic spores. Any handling of the medium, such as the adjustment of pH, precipitation of spores, or centrifugation, resulted in some spore germination. Various attempts were made to prevent germination from occurring in the growth medium. The addition of inhibitors failed because some germination occurred before the changed conditions became uniform throughout the culture. However, if the culture was allowed to stand at 4 C for approx 2 weeks the spores settled and the clear supernatant fluid causing germination could be siphoned off, leaving a precipitate of ungerminated spores. Adding cold distilled water to the

spore precipitate will dilute any remaining nutrients to the point of insignificance. The suspension was again allowed to stand in the cold, and the supernatant fluid removed by siphon. The spore mass was washed repeatedly with cold water, and recovered by centrifugation. Upon centrifugation at low speed in a clinical centrifuge any vegetative cells appear as a black precipitate and the spores remain suspended. Differential centrifugation produced spores, free of vegetative cells and debris, which could be stored and handled at room temperature without showing any further evidence of germination.

Germination of anaerobic spores. A suspension of spores containing 5×10^8 spores/ml, prepared by the above method, was used to determine the minimum requirements and conditions fostering germination. The optimum conditions of spore germination in 5 per cent trypticase were determined by measuring the decrease in heat resistance, the increase in stainability (table 2), and the change in optical density. As the measurement of heat resistance involved a longer incubation period and more manipulations, this method was used only as a check on other methods. The change in optical density of the germinating spores was measured on a Bausch and Lomb colorimeter. A known number of spores was added to a solution of 5 per cent trypticase. As the spores germinated a change in light transmission was noted. Under optimum conditions, germination was essentially complete after 15 min. That the change in light transmission (figure 2) during this time was due to germination and not to the growth of cells was confirmed by microscopic examinations of samples (0.05 ml) removed at intervals and stained to determine the percentage germination.

Germination occurred only under anaerobic conditions (figure 3) and could be stopped at any time by the introduction of air. The anaerobic state was produced by heating the medium to remove the oxygen, and maintained by addition of sodium thioglycolate (table 3) which is nontoxic and plays no role in germination. The optimum pH for germination was 7. A great many substances were tested for their ability to cause rapid germination of anaerobic spores. Trypticase and certain other digests, especially thiotone (peptic digest of animal tissue, BBL) and fresh pork liver extract produced the best germinations. Therefore, they were fractionated

TABLE 2

Heat resistance and stability of spores germinated in 5 per cent trypticase

Exposure Time	Spores Suspended in Phosphate Buffer		Spores Suspended in 5 Per Cent Trypticase plus Phosphate Buffer	
	Heated to 80 C: viable cells/ml	Unheated: % cells staining	Heated to 80 C: viable cells/ml	Unheated: % cells staining
min				
0	1.0×10^8	0	1.0×10^8	0
1	0.5×10^8	0	0.8×10^8	0
2	0.6×10^8	0	8.0×10^7	40
5	0.5×10^8	0	5.0×10^4	85
7	0.8×10^8	0	3.0×10^3	95
10	0.5×10^8	0	1.2×10^2	98

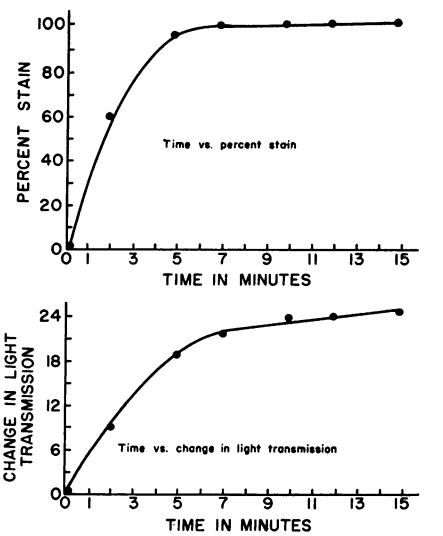

Figure 2. A comparison of the change in optical density and stainability which occurs during germination of *Clostridium roseum* spores in 5 per cent trypticase.

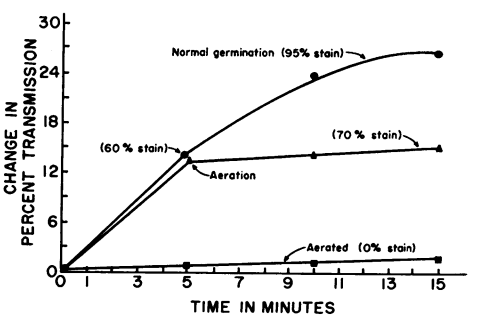

Figure 3. The effect of aeration on the germination of anaerobic spores in the presence of 5 per cent trypticase.

TABLE 3

Effect of various conditions on anaerobic germination in 5 per cent trypticase

Composition of Test Solution	Treatment*	pH	Change in Light Transmission after 15 Min	Cells Absorbing Stain after 15 Min
			%	%
5% Trypticase		7	8	10
5% Trypticase + 2% sodium thioglycolate		7	10	20
5% Trypticase + 2% sodium thioglycolate	Heated and cooled	7	23	99
5% Trypticase + 2% sodium thioglycolate	Heated, cooled and shaken	7	3	5
5% Trypticase	Heated and cooled	7	20	90
2% Sodium thioglycolate		7	0	0
5% Sodium thioglycolate	Heated and cooled	7	0	0
5% Trypticase + 2% sodium thioglycolate	Heated and cooled	5	8	15
5% Trypticase + 2% sodium thioglycolate	Heated and cooled	10	7	15

* Treatment performed on test solution prior to addition of spore suspension.

TABLE 4

Stock solutions of amino acids used in the germination of anaerobic spores*

Solution	Amount in Germination Medium
	mg/ml
Solution A	
L-Arginine·HCl	0.285
L-Lysine·HCl	0.285
DL-Threonine	0.285
L-Leucine	0.114
DL-Isoleucine	0.285
L-Histidine	0.114
DL-Methionine	0.285
DL-Phenylalanine	0.285
DL-Valine	0.285
Solution B	
DL-Alanine	1.440
DL-Aspartic acid	1.440
L-Glutamic acid	1.440
DL-Serine	0·285
L-Proline	0.144
L-Tyrosine	0.144
Glycine	0.144
L-Cystine	0.285

* Solutions prepared in distilled water.

by various methods to isolate the active material, which was found to be heat stable, soluble in water and in 70 per cent ethyl alcohol, but insoluble in absolute alcohol or organic solvents. Enough material for adequate testing could not be isolated by the ion exchange method, by the zone electrophoresis starch column, nor by Durrum's (1951) method of paper electrophoresis. However, it was suspected that more than one substrate is necessary for germination. The fraction isolated from fresh pork liver was unstable and gradually lost its activity under various conditions. A casein "D" digest, in which the concentrations of amino acids were known[4] showed full germination. To determine whether the active material was present in the fraction of known amino acids or in the unknown portion of the casein digest, a stock solution with a similar concentration of amino acids was tested. This solution of 17 known amino acids gave complete germinations indicating that amino acids alone could cause germination. Solutions containing different concentrations and ratios of amino acids that had failed previously to cause germination were retested and once again found to be inactive indicating that the ratio and concentration of amino acids is critically important.

Two new solutions (A and B) were prepared duplicating the components found in the complete amino acid solution that caused germination in 15 min (table 4). Neither solution showed full activity, indicating that at least one amino acid

[4] Sample submitted by Dr. L. M. Henderson, Department of Biochemistry, University of Illinois, Urbana, Illinois.

TABLE 5

Effect of known concentrations of amino acids on germination of anaerobic spores

Amino Acids Present*	Change in Light Transmission after 15 Min	Cells Staining after 15 Min
	%	%
Complete amino acid solutions	29	95
Solution A	1	0
Solution A plus:		
DL-alanine	13	20
DL-aspartic acid	−1	0
L-glutamic acid	−5	0
DL-serine	0	0
L-proline	−1	0
L-tyrosine	−2	0
glycine	0	0
L-cystine	0	0
Solution B	7	10
Solution B plus:		
L-arginine	20	40
L-lysine	10	5
DL-threonine	4.5	0
L-leucine	11	5
DL-isoleucine	0	0
L-histidine	8	5
DL-methionine	10	10
DL-phenylalanine	17	20
DL-valine	3	0

* Concentrations are the same as reported in table 4.

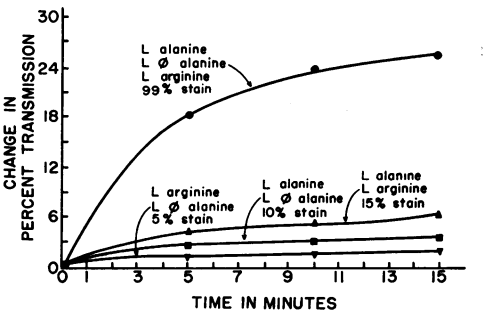

Figure 4. The effect of amino acids on germination of anaerobic spores.

TABLE 6

Effect of various concentrations of three amino acids on germination of anaerobic spores

Concentration of Amino Acid Germination Medium			Change in Light Transmission after 15 Min	Cells Staining after 15 Min
L-Alanine	L-Phenylalanine	L-Arginine		
mg/ml	*mg/ml*	*mg/ml*	%	%
1.44	0.285	0.285	38	98
1.44	0.570	0.285	38	98
2.16	0.285	0.285	27	85
2.88	0.570	0.570	26	75
2.88	0.285	0.570	31	85
1.44	0.570	0.570	7	20
1.44	0.285	0.570	34	95
0.72	0.285	0.285	4	15
0.36	0.285	0.285	0	0
2.88	0.285	0.285	25	75

from solution A and one from solution B were necessary. These same concentrations of amino acids were used in the studies described with exceptions as noted.

Germination studies were performed by the addition of single amino acids to solutions A and B (table 5). Other tests involving the elimination of one or several amino acids from the test solution indicated that full activity for the germination of anaerobic spores was present only in a combination of three amino acids: DL-alanine, L-arginine, and DL-phenylalanine. It is known that D-alanine strongly inhibits the activity of L-alanine in the germination of spores of bacilli. The action of D-alanine on anaerobic spore germinations was studied and the D form was found to be completely inactive. Adenosine played no role in anaerobic germination, as had been observed with aerobes (Stewart and Halvorson, 1953). Only L-alanine, L-phenylalanine, and L-arginine were necessary for complete germination (figure 4). The addition of other amino acids caused interference with germination, but as each combination resulted in a change, the relationship is obscure. Decreased concentrations of L-alanine resulted in sharp decreases in germination while an increase in concentration also resulted in a decreased germination. An increase or decrease in L-arginine or L-phenylalanine resulted in similar though smaller decreases. The optimum concentrations for rapid germination were found to be: L-alanine, 1.44 mg/ml; L-phenylalanine, 0.285 mg/ml; and L-arginine, 0.285 mg/ml (table 6). Thus the concentration of the three amino acids is as important as their presence. The sequence of action of the three amino acids on spore germination revealed that all three must be present at the same time for complete germination. Sublethal heating of

spores before the addition of amino acids did not have any effect on the rate or extent of anaerobic spore germination.

Inhibition of anaerobic spore germination. Various materials were tested for their ability to inhibit germination in the presence of optimum concentrations of thiotone (BBL). The normal germination procedure was followed and the inhibitory materials were added to the culture just before the addition of the spore suspension. The final pH in all cases was adjusted to 7. The following materials inhibited germination completely: sodium chloride, 10 per cent; diabasic potassium phosphate, 8 per cent; potassium nitrate, 6 per cent; sodium nitrate, 6 per cent; and potassium sulfate, 6 per cent. As the concentration of these chemicals increased, the germination decreased. No inhibition occurred with 5 per cent monobasic or 5 per cent dibasic sodium phosphate, or with a combination of the two.

DISCUSSION

It has been shown that complete sporulation of *C. roseum* is possible in a clear medium. Sporulation was complete at the end of 48 hr and continued incubation resulted in some germination in the sporulation medium. Such germination can be halted simply by exposing the culture to adverse conditions (4 C) with no agitation of the medium. Once the spores are separated from the sporulation medium by differential centrifugation they will not germinate without the addition of known germination factors.

The factors for optimum germination were found to be pH 7.0 and anaerobic conditions. Any change in these factors resulted in decreased germination. A germinating culture will show decreased heat resistance and a change in optical density; the germinated spore will retain a simple stain. All three characteristics were used to measure the degree of germination and the results paralleled each other. With such measurements, any material may be tested for germination factors. The best germinations were obtained with casein digests and fresh liver extract.

Wynne and Foster (1948*a*, 1948*b*) studied anaerobes but the only work directly concerned with minimal nutritional requirements for anaerobic spore germination is that of Wynne *et al.* (1954). These workers found maximum germination after 1 hr in a 1 per cent glucose solution, autoclaved for 80 min at 121 C in the presence of 0.1 per cent K_2HPO_4 and 0.1 per cent KH_2PO_4. In the work reported here, substantially complete germination was achieved in 15 min with only three amino acids: L-alanine, L-phenylalanine, and L-arginine.

The role of L-alanine in aerobic spore germination has been reported by several investigators: Hills (1950), Stewart and Halvorson (1953), Powell (1950) and Church *et al.* (1954). However, this is the first report presenting the role of L-alanine in the germination of anaerobic spores.

Substituting any other amino acid for one of the three mentioned above or changing their concentration results in decreased or no germination. The D forms of these amino acids were inactive, but did not inhibit germination. Although the exact role of these three amino acids in anaerobic spore germination is unknown, L-alanine appears to be the key to the germination process. Zoha (personal communication) has investigated the effect of these three amino acids on the spores of *Clostridium botulinum*, types A and B, and has found that germination in this species is exactly the same in *C. roseum*. Whether these three amino acids will cause germination of aerobic spores is doubtful.

SUMMARY

Germination of spores of *Clostridium roseum* is stimulated by amino acids, as has been reported by a number of investigators for spores of aerobic bacilli. When the anaerobe was grown in a medium containing trypticase, 1.5 per cent; sodium chloride, 0.5 per cent; K_2HPO_4, 0.25 per cent; and glucose, 0.2 per cent, nearly complete sporulation was obtained in 48 hr. The anaerobic spores must be harvested soon after sporulation is complete to prevent germination in the medium in which they were formed. Spores are harvested by cooling the culture to 4 C, and centrifuging. The precipitates of sedimented spores are washed with water until free from absorbed nutrients, and can then be suspended in distilled water or phosphate buffer at room temperature without danger of germination, and can be separated from any remaining vegetative cells by fractional centrifugation.

When a mixture of three amino acids: L-alanine, L-phenylalanine, and L-arginine, is added to a suspension of these spores in distilled water containing 0.05 per cent sodium thioglycolate at pH 7–8, germination takes place in from 2 to 5 min

under anaerobic conditions. Germination can be measured by determining the change in light transmission by means of a spectrophotometer, as has been done with spores of aerobic organisms. The change in light transmission parallels the loss of heat resistance and the susceptibility of the cells to staining.

REFERENCES

CHURCH, B. D., HALVORSON, H., AND HALVORSON, H. ORIN. 1954 Studies on spore germination: its independence from alanine racemase activity. J. Bacteriol., **68,** 393–399.

CURRAN, H. R. AND EVANS, F. R. 1954 The influence of iron or manganese upon the formation of spores by mesophilic aerobes in fluid organic media. J. Bacteriol., **67,** 489–497.

DURRUM, E. L. 1951 Continuous electrophoresis and ionophoresis on filter paper. J. Am. Chem. Soc., **73,** 4875–4880.

HILLS, G. M. 1949 Chemical factors in the germination of spore-bearing aerobes. Effects of amino acids on germination of *Bacillus anthracis*: some observations on the relation of optical forms to biological activity. Biochem. J. (London), **45,** 262–270.

HILLS, G. M. 1950 Chemical factors in the germination of spore-bearing aerobes. Observations on the influence of species, strain, and condition of growth. J. Gen. Microbiol., **4,** 38–47.

POWELL, J. F. 1950. Factors affecting the germination of thick suspension of *Bacillus subtilis* spores in L-alanine solution. J. Gen. Microbiol., **4,** 330–338.

STEWART, B. T. AND HALVORSON, H. ORIN. 1953 Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol., **65,** 160–166.

WYNNE, E. S. AND FOSTER, J. W. 1948*a* Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of a quantitative method. J. Bacteriol., **55,** 61–68.

WYNNE, E. S. AND FOSTER, J. W. 1948*b* Physiological studies on spore germination with special reference to *Clostridium botulinum*. II. Quantitative aspects of the germination process. J. Bacteriol., **55,** 69–73.

WYNNE, E. S., MEHL, D. A., AND SCHMIEDINY, W. R. 1954 Germination of Clostridium spores in buffered glucose. J. Bacteriol., **67,** 435–437.