Rapid Methods of Staining Bacterial Spores at Room Temperature

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Received for publication 30 October 1964

ABSTRACT

LECHTMAN, M. D. (University of Southern California, Los Angeles), J. W. BARTHOLOMEW, A. PHILLIPS, AND M. RUSSO. Rapid methods of staining bacterial spores at room temperature. *J. Bacteriol.* **89**:848-854. 1965.—Spores of *Bacillus subtilis* var. *niger* were stained in 2 min at room temperature, after suitable pretreatment, with a dye reagent composed of 2% crystal violet in 1% phenol and 26% ethanol. Pretreatments included heat fixation to 260 C, mechanical rupture, and hydrolysis at room temperature in 44 N H₃PO₄ for 5 min, 33.4 N H₃PO₄ for 10 min, 12 N HCl for 5 sec, 6 N HCl for 2 min, 12 N HNO₃ for 5 sec, and 6 N HNO₃ for 60 sec. Acid hydrolysis at 60 C enabled the lowering of both acid concentration and time: 33.4 N H₃PO₄ for 15 sec, 25.9 N H₃PO₄ for 60 sec, 2 N HCl for 30 sec, 1 N HCl for 30 sec, 2 N HNO₃ for 15 sec, and 1 N HNO₃ for 30 sec. After acid treatment, 1 N NaOH was used as a neutralization agent. The cytological manifestations of these pretreatments, examined in an electron microscope after replication, showed definite degradation of spore coats, which probably explains the increase in dye permeability. The pretreatments were evaluated for use in a differential staining procedure for spores and vegetative cells. They were found to be too drastic in that they resulted in replacement of the primary dye by the 0.25% safranine counter stain in both vegetative cells and endospores. Less drastic pretreatments, such as 6 N HNO₃ for 10 sec at room temperature, gave good differential stains, but failed to stain some free spores. The staining techniques above were evaluated with six species of *Bacillus* and were found to apply to all.

The classical methods for staining bacterial spores involve exposure to heated staining solutions for long periods of time (Dorner, 1922; May, 1926; Schaeffer and Fulton, 1933). A procedure for rapidly staining bacterial spores at room temperature would be of great help in the determination of free spores in cultures, in the rapid detection and counting of airborne bacterial spores, and to classroom instructors who find that students have some difficulty with procedures involving steaming dye reagents.

Bacterial spores are difficult to stain, because they are not permeable to aqueous dye reagents. It has been demonstrated (Hashimoto, Black, and Gerhardt, 1959) that this impermeable characteristic appears at the same time as the cytological differentiation of the cortex region of the spore. Many treatments are known which destroy the permeability barrier, such as severe heat fixation (Bartholomew and Mittwer, 1950), acid hydrolysis (Robinow, 1951), ultraviolet light (Bartholomew and Mittwer, 1952), and mechanical rupture (Fitz-James, 1953; Rode and Foster, 1960a, b). After such treatments, bacterial spores are easily

stainable at room temperature. In this paper, we report on studies of the specific conditions needed to produce spore stainability. In addition, we found that we could correlate certain cytological changes in the spores with the conversion to a state of stainability.

MATERIALS AND METHODS

Most of the work reported in this paper was conducted on free spores of *Bacillus subtilis* var. *niger*. These spores were obtained in the frozen state from Bioferm Corp., Wasco, Calif., and were washed twice in saline before being placed on glass slides. Vegetative cells were obtained from these spores by plating out in nutrient agar, and subinoculation from single colonies into nutrient broth. Nutrient broth cultures were incubated in 250-ml Erlenmeyer flasks, with 10 ml of medium, at 34 C, in a reciprocal shaker water bath, operated at a speed of 100 cycles per min (Eberbach Corp., Ann Arbor, Mich.). Vegetative cells were placed on glass slides after an incubation period of 10 hr, and endospore preparations required 20 to 24 hr. The results of the experiments with this *B. subtilis* strain were checked against two other strains of *B. subtilis*

(ATCC 6633 and 6051) as well as against cultures of *B. megaterium*, *B. cereus*, *B. coagulans*, *B. polymyxa*, and *B. stearothermophilus*. These cultures were obtained from the stock culture collection of the Microbiology Section, Department of Biological Sciences, University of Southern California. Spores from these cultures were obtained by growth for 24 to 48 hr on agar slants at 37 C, except for *B. stearothermophilus* for which 55 C was used.

The temperatures obtained during heat fixation of smears on glass slides were followed with a Leeds and Northrup automatic temperature-time recording instrument, using a chromel-alumel thermocouple sealed to the slide near the smear. Heat-fixation treatments were obtained by placing the slides (smear side up) on the surface of a hot plate (Temco Stir Plate, Thermo Electronics Co., Dubuque, Iowa). The slides were removed when the desired temperature had been held for the desired time, and allowed to air cool. In the final experiments, a chemical indicator (Tempilaq, Tempil Corp., New York, N.Y.) was used to show when 260 C had been reached. This temperature could be realized by holding the slide at the tip of a blue flame in a Bunsen burner, for a period of 6 to 8 sec.

The spore-replication technique used for electron microscopy was essentially that of Bradley and Williams (1957). The replicas were observed in a RCA-EMU electron microscope.

The acids used were reagent grade, and the normalities reported were on the basis of the usually accepted approximate normalities for concentrated acids; that is, 44 N for H_3PO_4 (85%), 36 N for H_2SO_4 (96%), 12 N for HCl (36%), and 16 N for HNO_3 (70%). The acid solutions were placed in Coplin dishes (75 by 25 mm), and all slide exposures to acid were carried out in these dishes.

Crystal violet, basic fuchsin, and malachite green were compared in aqueous solutions ranging from 1 to 4%. Similar formulations were made to which phenol was added in concentrations of 1 to 6%, and ethyl alcohol in concentrations up to 52%. When phenol was added to a dye such as crystal violet, a gel was formed. This could be prevented by using the proper concentration of ethyl alcohol. For example, 2 to 4% crystal violet with 1% phenol would not form a gel if 10% ethyl alcohol was included in the formula; with 6% phenol, gel formation was prevented if 52% ethyl alcohol was included. The compounding procedure was to add the dye to absolute ethyl alcohol, then add the proper quantity of an aqueous solution of phenol, slowly, and with constant stirring. The required aqueous phenol solution was prepared from 90% phenol solution. This was obtained by melting phenol crystals and adding 10% water; such a 90% phenol solution remains liquid at room temperature.

Of the three dyes studied, none was greatly superior or inferior to the others. The choice would be made primarily on the basis of the spore color desired. It was found, however, that the

addition of phenol (with the necessary ethyl alcohol) resulted in deeper and more rapid spore staining. For the data reported in the present paper, a 2% crystal violet, 1% phenol, in 26% ethyl alcohol formula was used.

RESULTS AND DISCUSSION

Effect of heat fixation on spore stainability. Spore smears were prepared on glass slides, and various degrees of heat fixation were applied. The temperatures of heat fixation obtained were followed as described in Materials and Methods section. It was found (Table 1) that heat fixation could result in spore stainability, and that a correlation existed between the temperatures obtained and their time of application. That is, 215 C for 6 min could produce the same effect on spore stainability as 260 C for 10 sec. In both instances, spores were stained with our dye reagent in 2 min at room temperature after such heat-fixation procedures. From a practical point of view, expensive thermocouple devices, as used in these experiments, were not necessary to assure sufficient heat treatment for stainability of spores. A chemical heat indicator melting at 260 C could be placed near the smear, and the slide could be heated over a Bunsen burner (adjusted to a hot blue flame) to the instant of melting of the indicator. This usually took only 6 to 8 sec, and the slide was then air-cooled. Figures 1 and 2 compare *B. subtilis* spores which were fixed by the usual heat-fixation procedure (which attains a maximal temperature of only about 135 C) with spores after heat fixation at 260 C. Spores were not stainable after ordinary heat fixation, but they were deeply stained after fixation at 260 C. The

TABLE 1. *Stainability of Bacillus subtilis* var. *niger* spores after various heat treatments*

Time	Temp (C)			
	215	225	240	260
10 sec	No	No	No	Yes
1 min	No	No	No	Yes
2 min	No	No	Yes	Yes
4 min	No	Yes	Yes	Yes
6 min	Yes	Yes	Yes	Yes

* A hot plate was adjusted to the temperature indicated, and a spore smear on a glass slide was placed on this surface for the time indicated, and then air-cooled to room temperature before staining. Slides were stained for 2 min at room temperature with 2% crystal violet in 1% phenol and 26% ethyl alcohol.

Yes = Spores uniformly stained.

No = Spores not stained, or stained only on the outer edges.

FIG. 1-6. Spores of *Bacillus subtilis* var. *niger*. (1) Spores stained for 2 min at room temperature after a normal heat-fixation period. $\times 2,000$. (2) As in Fig. 1, except heat fixation was at 260 C for 10 sec. $\times 2,600$. (3) As in Fig. 1, except spores broken by mechanical rupture with 0.2-mm glass beads and thumb pressure. $\times 200$. (4) Replica staining of normal spores. Electron micrograph. $\times 26,000$. (5 and 6) As in Fig. 4, except spores heat fixed at 260 C for 10 sec. $\times 26,000$.

latter heat fixation was adequate to produce stainability of free spores of the other organisms included in this study. Of the organisms studied, only one, *B. stearothermophilus*, failed to give good spore stains. These spores apparently were more heat resistant. They stained well, however, if slightly higher temperatures, or longer periods of heat treatment, were used. The use of a chemical heat indicator, and the Bunsen burner, to achieve 260 C results in a very easy method for the detection of free spores in bacterial cultures.

Effect of mechanical pressure on spore stainability. It has been known for some time that the mechanical rupture of spores resulted in their becoming stainable (Rode and Foster, 1960a, b; Hashimoto et al., 1959; Fitz-James, 1953). Usually, these procedures involved the use of spore suspensions and glass beads in a Mickle apparatus; therefore, the results of such procedures would not be applicable to spore-staining procedures applied to smears on glass slides. However, one could reason that, if the spore coats and cortex could be ruptured, this would produce a change in permeability that would result in spore stainability. Spore rupture was achieved as follows; a thick smear of spores was made on a glass slide and allowed to air-dry. About ten 0.2-mm glass beads (#16-220 VirTis Co., Inc, Yonkers, N.Y.) were placed on the smear, and either a no. 1 cover slip or a glass slide was placed over the beads. Thumb pressure was applied, and the cover slip was moved back and forth to give a rolling motion to the beads. The result of the subsequent 2-min staining at room temperature is shown in Fig. 3. The spores within the tracks of

the beads were heavily stained, those outside of the tracks were uncrushed and remained unstained. The track pictured is about 60- μ wide. Within this track, the spores appeared large, but were otherwise cytologically normal. They were heavily stained when observed with the oil-immersion objective. Vegetative cells, when subjected to this treatment, were visibly crushed, but were still stainable.

That such mechanical pressure would rupture spores was demonstrated by Monk, Hess, and Schenk (1957). These workers reported that, when *B. subtilis* var. *niger* spores were placed under pressures of 4 to 8 tons per square inch, they flattened out, the spores became ruptured, and viability was lost. If a 1-lb thumb pressure were placed on ten glass beads, each with a 60 μ^2 surface contact with the slide, the resultant pressure applied would be about 8 tons per square inch. The thumb pressure used in the present experiments would be sufficient, therefore, to rupture the spores and hence render them permeable to dye.

Effect of acid hydrolysis on spore stainability. That spores could be stained at room temperatures after acid hydrolysis has been known for some time (see Robinow, 1951). However, very little work has been done comparing the effectiveness of different acids at different concentrations. Such comparisons are made in Table 2. When similar normalities were compared (12 N), the effectiveness of the acids studied was in the order, from slowest to fastest, of phosphoric, sulfuric, hydrochloric, and nitric acid. Other acids, such as acetic, formic, and boric (12 N solutions), were

TABLE 2. *Effect of acid hydrolysis at room temperature on the stainability of spores and vegetative cells of Bacillus subtilis* var. *niger**

Time of acid hydrolysis	H ₃ PO ₄				H ₂ SO ₄						HCl				HNO ₃			
	44 N		33.4 N		36 N		28.4 N		12 N		12 N		6 N		12 N		6 N	
	S	V	S	V	S	V	S	V	S	V	S	V	S	V	S	V	S	V
5 sec	0	4	0	5	5	0	4	3	0	5	<u>5</u>	<u>5</u>	1	5	<u>5</u>	<u>5</u>	4	5
15 sec	0	4	0	5	5	0	4	3	0	5	<u>5</u>	<u>4</u>	4	5	<u>5</u>	<u>4</u>	4	5
30 sec	0	4	0	5	4	0	4	0	0	5	4	4	4	4	5	4	4	4
60 sec	3	4	0	5	2	0	3	0	0	5	4	4	4	4			<u>5</u>	<u>4</u>
2 min	5	4	0	5					0	5			<u>5</u>	<u>4</u>			<u>5</u>	<u>4</u>
5 min	<u>5</u>	<u>4</u>	1	5					1	4			<u>5</u>	<u>4</u>			5	4
10 min	<u>5</u>	<u>4</u>	<u>4</u>	<u>5</u>					4	4			4	4				

* S = spores; V = vegetative cells. For spores, 0 = no stain; 1 = 20% of spores evenly stained; 2 = 40%; 3 = 60%; 4 = 80%; 5 = 100%. For vegetative cells, 0 = ghost; 1 = just visible; 5 = normal staining. All acid treatments, neutralizations, and staining were done in Coplin dishes (75 by 25 mm) at room temperature. Procedure: expose to acid for the time indicated, wash, expose to 1 N NaOH for 30 sec, wash, stain for 2 min at room temperature with 2% crystal violet in 1% phenol and 26% ethyl alcohol, wash, dry, and examine. The acid concentrations and hydrolysis times which produced the most satisfactory spore stains are italicized.

without effect on spore stainability within the 10-min maximal time allowed for these experiments. The times of hydrolysis, and the concentrations of acids which gave the most satisfactory results, are italicized in Table 2.

Each of the acids had its own advantages and disadvantages. Phosphoric acid was the most pleasant to handle and least injurious to vegetative cells, but it also was the slowest acting. Sulfuric acid was least acceptable, because it was the most injurious to vegetative cells. Hydrochloric and nitric acids were fast and effective; however, the fumes of hydrochloric acid were very irritating, and nitric acid produced skin discoloration.

Temperature had a significant effect on the time requirements for acid hydrolysis (Table 3). If the acids were held in a water bath at 60 C, most of the above disadvantages of the acids were eliminated. Not only could the concentrations of the acids be lowered to where they were no longer objectionable, but the time of hydrolysis also could be greatly reduced. At 60 C, the concentration of nitric or hydrochloric acid could be reduced to 1 or 2 N, and the hydrolysis time could be shortened to 30 sec. If a 33.4 N (60%) phosphoric acid was used, the hydrolysis time could be shortened to 15 to 30 sec. The acid concentrations and hydrolysis times giving the most satisfactory results are italicized in Table 3.

Cytological changes produced by heat fixation or acid hydrolysis. The replica technique of Bradley and Williams (1957) was used to determine whether the change in dye permeation after heat fixation or acid hydrolysis could be correlated with any observable cytological effects on the spores. Figure 4 shows untreated spores of *B. subtilis*. Figures 5 and 6 show similar spores which have been exposed, on a glass slide, to 260 C. The spore coats have collapsed, exposing the internal

spore area, and possibly cytoplasm, which then was easily stained. This effect of dry heat on bacterial spore coats resembles the changes reported by Hunnell and Ordal (1961) for the effects of lethal temperatures on *B. coagulans* spores in aqueous suspensions.

Acid hydrolysis produced two different cytological effects. Hydrolysis with hydrochloric or nitric acids resulted in small ruptures on the spores which extruded spore material (Fig. 7 and 8), as reported by Robinow (1951). On exposure to a stain, not only did this extruded material stain easily, but the internal areas of the spore also stained evenly. On exposure to phosphoric or sulfuric acids, the spores became enlarged and flattened, and collapsed in the center (Fig. 9 and 10). The cytological effect of these acids was similar to that reported by Hunnell and Ordal (1961) for *B. coagulans* spores after digestion with pepsin, trypsin, and ribonuclease. After staining, spores treated with phosphoric or sulfuric acid appeared larger than did those exposed to hydrochloric or nitric acid.

Rode and Foster (1960a, b) reported that, as spores begin to germinate, almost all of the calcium dipicolinate (CaDPA) present is secreted into the medium, and that at the same time the spore becomes permeable to aqueous solutions of stains. Perry and Foster (1955) reported that dilute mineral acid at boiling temperatures can remove the CaDPA from spores. This suggested the possibility that the action of the acids reported above might be primarily to remove CaDPA from the spores and thus change their permeability. If this were true, then procedures (other than acid treatment) known to extract CaDPA should have a similar effect on stainability. Such a procedure would be treatment with 80% ethyl alcohol at 56 C for 2 hr (Rode and Foster, 1960b). When such an extraction procedure was tried on spores on glass slides, stainability did not result. Either the extraction method used did not remove CaDPA under these conditions, or the effect of acid hydrolysis as used in our experiments produced other effects in addition to the removal of CaDPA. More extensive studies of this phenomenon are planned.

Differential staining of endospores and vegetative cells. The procedures presented above stain free spores and vegetative cells. However, endospores in vegetative cells could not be differentially stained in a satisfactory manner. The staining method of Schaeffer and Fulton (1933) does give such differentiation. It uses a high temperature to drive the primary dye into the stain-resistant endospore. Once so stained, a different color counterstain is used at room temperature. The second dye easily replaces the primary dye in the

TABLE 3. Effect of acid hydrolysis at 60 C on the stainability of spores and vegetative cells of *Bacillus subtilis* var. *niger**

Time of acid hydrolysis	H ₃ PO ₄				HCl				HNO ₃			
	33.4 N		25.9 N		2 N		1 N		2 N		1 N	
	S	V	S	V	S	V	S	V	S	V	S	V
5 sec	1	5	0	5	1	5	0	5	4	5	0	5
15 sec	<i>4</i>	<i>5</i>	1	5	4	5	3	5	<i>5</i>	<i>5</i>	0	5
30 sec	<i>3</i>	<i>5</i>	3	5	<i>5</i>	<i>5</i>	<i>4</i>	<i>5</i>	<i>5</i>	<i>4</i>	<i>5</i>	<i>5</i>
60 sec	0	4	<i>5</i>	<i>5</i>	<i>4</i>	<i>5</i>	<i>3</i>	<i>5</i>	4	4	<i>4</i>	<i>5</i>
2 min	0	4	<i>5</i>	<i>5</i>			2	5				

* Conditions were the same as described in Table 2, except exposures to acids were at 60 C.

FIG. 7-10. Electron micrographs of replica of normal spores of *Bacillus subtilis* var. *niger*. $\times 26,000$. (7) Spores exposed to 12 N HCl at room temperature for 5 min. (8) Spores exposed to 12 N HNO₃ at room temperature for 5 min. (9) Spores exposed to 44 N H₃PO₄ at room temperature for 10 min. (10) Spores exposed to 28.4 N H₂SO₄ at room temperature for 10 min.

TABLE 4. Procedures for the differential staining, at room temperature, of endospores and vegetative cells*

Acid	Concn	Hydrolysis time		Remarks
		<i>N</i>	<i>sec</i>	
Hydrochloric..	6	15		
Nitric.....	6	10		Best procedure
Sulfuric.....	18.75	12-15		Very sensitive, little or no leeway in time
Phosphoric....	44	120		Second-best procedure

* Procedure: after acid hydrolysis, wash, expose for 30 sec to 1 N NaOH, wash, stain for 2 min with 2% crystal violet in 1% phenol and 26% ethyl alcohol, wash, expose for 5 sec to 95% ethyl alcohol, wash, counterstain with 0.25% safranin for 1 min, wash, dry, and examine. Endospores blue, vegetative cells red.

vegetative cell, but it does not penetrate into the endospore; therefore, no dye replacement occurs in this structure (Bartholomew, Roberts, and Evans, 1950). The acid and heat treatments reported in Tables 1, 2, and 3, were found to have affected endospores to the extent that they no longer resisted staining, and, therefore, they took the color of any counterstain used. However, if the acid or heat treatments were made less severe, then differential staining could be accomplished. Procedures which could be used for good differential staining are presented in Table 4, and were satisfactory for cultures of *B. subtilis* (ATCC 6633 and 6051), *B. megaterium*, *B. cereus*, *B. coagulans*, *B. polymyxa*, and *B. stearothermophilus*. The reduced acid treatment required, however, often left a good percentage of the free spores inadequately stained. Therefore, if the procedures presented in the present paper are used, they must be selected according to whether one wishes to demonstrate endospores in vegetative cells, or free spores in cultures.

ACKNOWLEDGMENT

Part of the work reported in this paper was supported by a Biological Stain Commission Student Fellowship to Max Lechtman, and part was supported by the Physical Defense Division of

the U.S. Army Biological Laboratories, Fort Detrick, Md., under contract with Douglas Aircraft Co., Inc.

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