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Resistance of *Bacillus subtilis* Spores to Inactivation by Gamma Irradiation and Heating in the Presence of a Bactericide

I. Suitability of Viable Count Procedures

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A statistical evaluation of viable count procedures utilized for obtaining treatment survival curve data for *Bacillus subtilis* NCTC 8236 spores is described. Within the various recovery conditions tested, incubation on nutrient agar containing 1% dextrose for 48 hr at 37 C was found to promote the highest count of viable spores surviving a variety of bactericidal treatments involving gamma irradiation, heat, and chlorocresol. The count of viable spores on the medium was not significantly altered when the dextrose was added to the nutrient agar either before autoclaving or aseptically at 50 to 55 C from a solution sterilized by filtration. The volume of medium which promoted the highest count of viable spores was 20 ml per 85 mm of diameter in disposable plastic plates. Counts of viable spores were reproducible on successive batches of media. The carry-over of variable concentrations of chlorocresol into the medium from serial dilutions affected the count of viable spores. Spores in the aqueous stock suspension used for all experiments were uniformly distributed after shaking and did not diminish significantly in viability after 16 months of storage at 5 C. Grouping of indexes of dispersion, calculated from quintuplicate plate colony counts, indicated that the suitability of the viable count procedures, employed for the enumeration of spores surviving the various bactericidal treatments, tended to diminish as the level of spore inactivation exceeded 95%.

Bacillus subtilis spores have been reported to be resistant to inactivation by separate treatment with gamma irradiation, moist heat, or phenolic disinfectants (3, 9). Accordingly, such spores were used as a convenient test organism for evaluating the bactericidal efficiency of the combined effect of these treatments. From observations on treatment survival curves, the effect of gamma irradiation on the rate of spore inactivation by heating in the presence or absence of a phenolic disinfectant was studied. However, before a reliable inference could be drawn from such survival curves, the suitability of experimental procedures, utilized for obtaining curve construction data, was established. This communication describes the evaluation of the experimental procedures. Spore inactivation or loss of viability was determined by the inability of the spore to form a visible and countable colony on a medium.

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MATERIALS AND METHODS

The preparation and storage of the stock spore suspension of *B. subtilis* NCTC 8236, the dosimetry of the cesium-137 gamma irradiation facility, and the utilization of the heat source have been described (5). The count of viable spores in the stock suspension used was ca. 5×10^8 organisms per ml.

Viable count procedure. A suitable serial dilution of a spore suspension to be counted was made in glass-distilled sterile water. A Whirlimixer (Fisons Scientific Apparatus Ltd.) was used to mix the dilution tubes. Volumes (0.3 ml) of the appropriate serial dilution were spread with a small L-shaped sealed capillary tube on the surface of the dried medium in disposable plastic plates (85 mm in diameter). Colonies were counted after suitable incubation, by using a colony illuminator (Spencer Ltd.) and a Markounter (Scientifica Instruments Ltd.). An estimate of the total

count in the spore suspension was determined by reference to the mean of the quintuplicate plate colony count and to the serial dilution employed.

RESULTS AND DISCUSSION

Effect of different media and incubation conditions. Spores were subjected to a variety of bactericidal treatments in later experiments and therefore suitable recovery conditions were determined beforehand. The composition of the medium and the temperature and duration of incubation were considered, because damaged or weakened spores are known to be more exacting in their nutritional requirements (4, 10).

The following general purpose media were investigated: A, nutrient agar (Oxoid); B, nutrient agar containing 1% dextrose; C, nutrient broth no. 2 (Oxoid) solidified by 1.2% agar no. 3 (Oxoid). The inclusion of dextrose in medium B was found by Richardson (8) to give increased viable cell counts of heat-treated *B. subtilis* spores. The media were sterilized by autoclaving at 10 psi for 15 min, and 20-ml volumes of each were used to prepare plates.

The following bactericidal treatments, representative of those used in later experiments, were applied to samples of the stock spore suspension: (i) heating at 90 C for 15 min, (ii) heating at 80 C for 1.5 hr with 0.2% chlorocresol in phosphate buffer (*p*H 7), (iii) exposure to 150,000 rad of gamma irradiation while sealed under air. A count of viable spores in each of the three treated suspensions and in an untreated stock suspension was made by using a group of 15 prepared plates from each medium per viable count. Each group was divided into three sets of five plates; one set was incubated at 32 C, a second set was incubated at 32 C for 24 hr followed by further incubation at 37 C, and a third set was incubated at 37 C.

Table 1 shows the means and indexes of dispersion (χ^2) of the quintuplicate plate colony counts obtained after 48 hr of incubation. Within treatments, maximal mean counts were obtained by incubation at 37 C on medium B, and no increase in colony count was detected by prolonging incubation to 7 days. The indexes of dispersion associated with these maximal mean counts were compared with those to be expected if the variation was assumed to involve only normal sampling variance. The probability was in each case satisfactory at the level of significance ($P = 0.05$) used throughout this communication, i.e., degrees of freedom = 4, $\chi^2 = 4.37$, $P = >0.30 - <0.50$; $\chi^2 = 1.36$, $P = >0.80 - <0.90$; $\chi^2 = 1.05$, $P = >0.90 - <0.95$; $\chi^2 = 1.13$, $P = >0.80 - <0.90$. This observation indicated that the incubation on this batch of medium resulted in the development of the same number of colonies in the parallel platings within the limits of sampling variance.

Effect of concentration and method of incorporation of dextrose into the medium. A 20-ml volume of nutrient agar containing 0.1, 0.5, 1.0, or 2.0% dextrose was used to prepare plates. One series of five plates containing each of the concentra-

TABLE 1. *Effect of different media and incubation conditions on the count of viable spores surviving various treatments*[a]

Medium	Incubation temp and duration	Treatment 1		Treatment 2		Treatment 3		Untreated	
		Mean	χ^2	Mean	χ^2	Mean	χ^2	Mean	χ^2
A	32 C for 48 hr	110.8	9.56	62.8	6.83	159.4	4.80	132.6	13.60
	32 C for 24 hr followed by 37 C for 24 hr	122.6	14.89	56.6	6.31	161.4	3.88	132.2	0.90
	37 C for 48 hr	302.4	4.41	63.4	4.12	199.2	7.08	153.6	5.20
B	32 C for 48 hr	259.8	7.22	82.0	8.10	222.6	1.72	159.6	6.45
	32 C for 24 hr followed by 37 C for 24 hr	253.0	6.23	78.8	4.53	236.0	5.17	169.8	3.69
	37 C for 48 hr	548.0	4.37	82.6	1.36	253.8	1.05	199.4	1.13
C	32 C for 48 hr	423.8	8.16	42.6	3.87	170.4	6.58	156.6	1.00
	32 C for 24 hr followed by 37 C for 24 hr	401.2	20.79	47.0	3.62	163.8	1.85	167.2	1.74
	37 C for 48 hr	479.4	13.93	46.2	6.00	162.2	3.94	165.6	3.80

[a] Results show the means and indexes of dispersion of quintuplicate plate colony counts. Values of the index of dispersion (χ^2) were calculated from the equation given by Fisher et al. (6).

tions of dextrose was prepared from nutrient agar containing dextrose added before sterilization by autoclaving. A second series was prepared from nutrient agar sterilized by autoclaving to which was added aseptically, at 50 to 55 C, dextrose solution sterilized by filtration. Stock spore suspension was heated at 80 C for 1.5 hr, and a count of surviving viable spores was made by using both series of plates so prepared. The heat treatment chosen was representative of that employed in later experiments.

Table 2 records the results and statistical analysis of the colony counts appearing on the various media after 48 hr of incubation at 37 C. Extension of the incubation period did not produce any increase in colony count. Inspection of the data shows that it was not possible to establish from this limited experiment a significant difference between mean colony counts on both series of prepared plates at each concentration of dextrose tested. However, as the highest mean colony count was recorded on the nutrient agar containing 1% dextrose added before autoclaving and as the method of incorporation of the dextrose into the nutrient agar was simple and not likely to introduce contamination, this medium and method of preparation were used in all further experiments.

Effect of variation in the volume of growth medium per plate. Sets of five plates containing 10-, 15-, 20-, 25-, or 30-ml volumes of medium per plate were prepared and used for a count of viable spores in the stock suspension.

The colony counts obtained after 48 hr of incubation at 37 C are recorded in Table 3. No increase in colony count was observed by extending the incubation period to 7 days. A significant difference was noted between the mean colony count of the set of plates containing 20 ml of medium per plate and the mean colony count of each of the other sets of plates, except where the volume of medium per plate was 15 ml. The mean colony counts of sets of plates containing 15 or 10 ml of medium per plate were progressively less than the mean colony count of the set of plates containing 20 ml of medium per plate. A possible explanation for this finding is that there was insufficient nutrient in 15 ml of medium per plate and even less in 10 ml for the growth of all developing colonies. It was also observed that, as the volume of medium per plate was increased above 20 ml, such plates exhibited progressively increased colonial spreading and opacity, which tended to reduce the distinct appearance of individual colonies. These observations could explain the progressive reduction in the mean colony counts obtained from such plates. Accordingly, plates containing 20 ml of medium were used and colony counts were determined after 48 hr of incubation at 37 C, in all further experiments.

Reproducibility of viable counts on successive batches of medium. The two different batches of medium, used to obtain treatment survival curve data, were tested for reproducibility of counts.

TABLE 2. *Effect of concentration and method of incorporation of dextrose into medium on the count of viable spores surviving heat treatment*[a]

Concn of dextrose added	Dextrose autoclaved						Dextrose added aseptically						*d*	*P*
	Colony counts					Mean	Colony counts					Mean		
%														
0.1	147	126	127	135	122	131.4	154	134	118	121	118	129.0	0.333	>0.70–<0.80
0.5	136	146	117	119	114	126.4	152	140	142	136	107	135.4	1.244	>0.20–<0.30
1.0	146	144	143	127	130	138.0	138	142	125	125	133	132.6	0.734	>0.40–<0.50
2.0	133	146	135	133	118	133.0	134	158	133	126	127	135.6	0.355	>0.70–<0.80

[a] Values of the statistic *d* were calculated from the equation given by Bailey (1).

TABLE 3. *Effect of variation in the volume of medium per plate on the count of viable spores*

Volume of medium per plate	Colony counts					Mean	*d*	*P*
ml								
10	244	280	264	258	277	264.6	4.929	<0.001
15	314	312	330	282	297	307.0	0.966	>0.30–<0.40
20	312	315	322	323	317	317.8		
25	275	318	297	314	276	296.0	1.968	>0.02–<0.05
30	282	302	288	272	290	286.8	2.820	>0.002–<0.01

TABLE 4. *Reproducibility of the count of viable spores on successive batches of medium*

Spore sample	Batch 1						Batch 2						d	P
	Colony counts					Mean	Colony counts					Mean		
a	148	155	158	165	159	157.0	164	161	151	146	155	155.4	0.202	>0.80–<0.90
b	318	317	305	341	323	320.8	281	315	297	330	289	302.4	1.648	>0.05–<0.10

TABLE 5. *Effect of concentration of chlorocresol carry-over on the count of viable spores*

Concn of chlorocresol carry-over	Colony counts					Mean	d	P
%								
0.02	107	103	94	94	92	98.0	6.923	<0.001
0.002	122	122	142	122	127	127.0	2.624	>0.001–<0.01
0.0002	125	133	134	128	144	132.8	1.820	>0.05–<0.10
0.00002	116	117	123	118	126	120.0	3.617	<0.001
0.000002	107	120	123	124	125	119.8	3.646	<0.001
0.0	147	133	146	161	145	146.4		

TABLE 6. *Count of viable spores in 10 samples of the stock suspension*

Sample no.	Colony counts					Total colony count	Mean colony count
1	157	156	143	142	157	755	151.0
2	143	156	143	146	158	746	149.2
3	142	145	155	160	143	745	149.0
4	148	140	151	140	141	720	144.0
5	144	158	146	147	158	753	150.6
6	159	147	152	150	155	763	152.6
7	149	138	146	148	161	742	148.4
8	150	135	148	151	151	735	147.0
9	157	156	149	143	150	755	151.0
10	137	152	144	146	145	724	144.8

TABLE 7. *Analysis of variance of colony counts*

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio	P
Difference between samples	349.92	9	38.88	1.153	>0.20
Difference within samples	1,793.20	40	44.83		

The results are recorded in Table 4. Comparison of mean colony counts derived from equivalent count procedures showed no significant difference between the two batches of medium for supporting the growth of identical numbers of colonies arising from the stock spore suspension.

Effect of concentration of chlorocresol carry-over. To follow the rate of inactivation of spores treated with 0.2% chlorocresol, viable counts of the inoculated disinfection mixture were made at intervals as inactivation proceeded. The effect of different concentrations of chlorocresol carry-over from appropriate serial dilutions was subjected to prior investigation. Quintuplicate platings of equivalent dilutions of the stock spore suspension containing variable concentrations of added chlorocresol were made.

The results are recorded in Table 5. A significant difference was noted between the mean colony count of the spore suspension containing no chlorocresol and the mean colony count of each of the spore suspensions containing decreasing concentrations of chlorocresol, except where the carry-over concentration of chlorocresol was 0.0002%.

It was desirable to follow the inactivation of spores treated with chlorocresol through several log cycles of the surviving fraction if necessary and yet to minimize the chlorocresol carry-over effect. In all such experiments, the spore suspension initially had a high total count of viable cells and the appropriate serial dilutions used for the enumeration of surviving spores were adjusted to contain 0.002% chlorocresol.

Distribution and viability of spores in stock suspension. To test whether the distribution of spores in the suspension could be rendered uniform, 10 samples were withdrawn at intervals from the suspension immediately after dispersal of sedimented spores by shaking and subjected to an equivalent viable count procedure.

TABLE 8. *Effect of storage on the count of viable spores in the stock suspension*

Period of storage	Colony counts	Mean	*d*	*P*
months				
0	169 165 170 168 162	166.8	1.445	>0.10–<0.20
16	158 148 167 149 154	155.2		

TABLE 9. *Goodness-of-fit of the indexes of dispersion obtained from counts on sets of quintuplicate plates used in experiments on the rate of inactivation of spores by various bactericidal treatments*

Value of index of dispersion	Expected frequency *m*	Observed frequency *m* + *x*	*x*	x^2/m
0–<1	28.84	39	10.16	3.58
1–<2	47.48	47	−0.48	0.00
2–<3	51.48	43	−8.48	1.40
3–<4	41.87	41	−0.87	0.02
4–<5	27.24	20	−7.24	1.92
5–<6	14.77	10	−4.77	1.54
6–<7	6.86	12	5.14	3.85
>7	13.46	20	6.54	3.18

$\chi^2 = \sum x^2/m = 15.49$, degrees of freedom = 7, P = >0.02 – <0.05

TABLE 10. *Goodness-of-fit of the indexes of dispersion obtained from counts on sets of quintuplicate plates used in experiments on the rate of inactivation of spores by various bactericidal treatments without phenolic disinfectants*

Value of index of dispersion	Expected frequency *m*	Observed frequency *m* + *x*	*x*	x^2/m
0–<1	6.97	7	0.03	0.00
1–<2	11.74	9	−2.74	0.64
2–<3	13.17	14	0.83	0.05
3–<4	11.09	11	−0.09	0.00
4–<6	11.66	10	−1.66	0.24
>6	5.37	9	3.63	2.45

$\chi^2 = \sum x^2/m = 3.38$, degrees of freedom = 5, P = >0.50 – <0.70

TABLE 11. *Goodness-of-fit of the indexes of dispersion obtained from counts on sets of quintuplicate plates used in experiments on the rate of inactivation of spores by various bactericidal treatments with phenolic disinfectants*

Value of index of dispersion	Expected frequency *m*	Observed frequency *m* + *x*	*x*	x^2/m
0–<1	22.44	31	8.56	3.27
1–<2	35.74	39	3.26	0.30
2–<3	38.28	29	−9.28	2.25
3–<4	30.75	30	−0.75	0.02
4–<5	19.76	14	−5.76	1.68
5–<6	10.59	6	−4.59	1.99
6–<8	6.81	14	7.19	7.59
>8	7.63	9	1.37	0.25

$\chi^2 = \sum x^2/m = 17.35$, degrees of freedom = 7, P = >0.01 – <0.02

The colony count data are recorded in Table 6 and the analysis of variance of these counts is recorded in Table 7. The variance ratio test showed that the between-samples mean square was not significantly different from the within-samples mean square. It was therefore assumed that the observed variances in the colony count on quintuplicate sets of plates were adequate to account for the observed variance between mean colony counts and hence that the distribution of spores in the stock suspension was uniform.

The spore suspension was stored for 16 months at 5 C, and during this period samples of the suspension were used in experiments on the rate of spore inactivation by various bactericidal treatments. Colony counts obtained from the suspension by equivalent viable count procedure at the beginning and end of the period are recorded in Table 8. The mean colony counts were compared, and no significant change in the viability of the spores in the suspension was detected.

Overall assessment of the suitability of the viable count procedures. Samples of stock spore suspension and the viable count procedures, adopted as the result of the evaluation described in this communication, were used in an extensive series of experiments on the influence of gamma irradiation on the rate of spore inactivation by heating in the presence or absence of phenolic disinfectants. The 232 indexes of dispersion calculated from the quintuplicate plate colony counts performed in these experiments were used for an overall assessment of the viable count procedures employed. The method described by Fisher et al. (6) was used for comparing the distribution of these indexes of dispersion with the hypothetical distribution. Since the goodness-of-fit is recorded in Table 9

and is only slightly unsatisfactory, it indicated that the viable count procedures used in these experiments were suitable for obtaining reliable mean colony counts.

In an attempt to trace why better agreement with the hypothetical distribution was not obtained for the total grouping of the indexes of dispersion, the 232 values were further analyzed. Table 10 records that the goodness-of-fit of the 60 indexes of dispersion, obtained from plate counts in the experiments performed in the absence of phenolic disinfectants, was satisfactory. The level of spore inactivation in such experiments rarely exceeded 95%. Table 11 records the unsatisfactory goodness-of-fit of the remaining 172 indexes of dispersion, obtained from plate counts in the experiments performed in the presence of phenolic disinfectants and in which the level of spore inactivation regularly exceeded 95%.

Jordan and Jacobs (7) noted an unsatisfactory goodness-of-fit of indexes of dispersion from replicate plate counts when *Escherichia coli* was exposed to phenol. Berry and Michaels (2) observed an unsatisfactory goodness-of-fit for the distribution of indexes of dispersion from counts on sets of replicate roll-tubes when *E. coli* was treated with disinfectant, although indexes of dispersion obtained from counts on sets of replicate roll-tubes not treated with disinfectant showed satisfactory agreement with the theoretical distribution.

A possible explanation for the unsatisfactory goodness-of-fit in Table 11 is heterogeneity in the viability of *B. subtilis* spores under the recovery conditions provided after surviving treatment with phenolic disinfectants. This suggestion is supported by the findings of Jordan and Jacobs (7), who observed that 75% of excessively discrepant sets of plates occurred among the counts where the inactivation level was greater than 95% and where viability of surviving organisms on the medium might be expected to be more variable.

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