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Replacement Sporulation of *Bacillus subtilis* 168 in a Chemically Defined Medium

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A replacement sporulation technique (i.e., the sporulation of vegetative cells upon suspension in an appropriate medium) has been developed for *Bacillus subtilis* 168 (a transformable Marburg strain of *B. subtilis*). The replacement sporulation medium used is composed of inorganic salts and 10 mM ammonium lactate or glutamate. The requirement for ammonium lactate or glutamate could also be satisfied by other compounds that are metabolized via the tricarboxylic acid cycle. Sporulation of the suspended vegetative cells was completed by 8 to 10 hr after suspension, and the resulting spores were indistinguishable from spores produced in a conventional growth and sporulation medium. Various physiological changes previously reported to be associated with sporulation (e.g., increase in the level of tricarboxylic acid cycle enzymes and changes in the rates of synthesis of deoxyribonucleic acid, ribonucleic acid, and protein) could also be demonstrated during replacement sporulation.

Physiological studies on bacterial sporulation in which conventional "batch" growth and sporulation media are used are complicated by the lack of a clear transition between vegetative growth and sporulation (12, 21). Even though the time of transition to sporulation can be identified experimentally [e.g., by the rise in the *p*H as organic acid utilization commences (4, 22)], it is desirable to be able to initiate sporulation by some experimental manipulation and also to be able to increase the synchrony of sporulation (27).

This study reports the development of a chemically defined medium that allows the sporulation of suspended vegetative growth phase cells of *Bacillus subtilis* 168 without permitting appreciable further vegetative growth. This technique is called a "replacement technique" (27), and thus we refer to the subsequent process as "replacement sporulation." The physiological changes that occurred during replacement sporulation of *B. subtilis* 168 are essentially the same as those previously reported from studies in which "batch" growth and sporulation media were used. The similarities include changes in enzyme levels, radioactive isotope incorporation patterns, and the production of normal heat-resistant spores.

B. subtilis 168 was chosen for this work because its ability to be transformed (1) will facilitate eventual genetic analysis of the early events in sporulation (28, 31, 32).

MATERIALS AND METHODS

Organisms. *B. subtilis* 168 wild type (wt) and *B. subtilis* 168 carrying *trp-2* and *thy* (i.e., requiring tryptophan and thymine) were obtained from F. Young and J. Spizizen of the Scripps Clinic, La Jolla, Calif.

Media. The basal inorganic salt medium used was a modification of the medium reported by Bernlohr and Novelli for *B. licheniformis* (4) and contained, per liter of distilled-deionized water: 85% H_3PO_4, 0.33 ml; $MgSO_4 \cdot 7H_2O$, 1.0 g; NaCl, 0.4 g; KCl, 0.4 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 25 mg; $CaCl_2$, 10 mg; and $MnSO_4 \cdot H_2O$, 6.0 mg. Salts sufficient for 1 liter of medium were dissolved in 800 ml of water, the *p*H was adjusted to 7.2 with concentrated KOH, and the solution was sterilized by autoclaving. The final medium for growth was prepared by addition of 100 ml of 0.2 M glucose and 100 ml of either 0.5 M glutamate or ammonium lactate (sterilized by filtration through a 0.45 μm membrane filter; Millipore Corp., Bedford, Mass.). The "replacement sporulation medium" differed only in that it contained no glucose and only 20 ml of the 0.5 M glutamate or ammonium lactate solution; 180 ml of sterile distilled-deionized water was added to achieve the final volume of 1 liter. This medium becomes slightly turbid upon autoclaving. However, this does not interfere to any appreciable extent with the subsequent determination of either growth or sporulation.

Growth conditions. Cells were grown at 37 C in 125-ml flasks with Morton stainless-steel closures. These contained 25 ml of growth medium and were agitated by shaking in a New Brunswick reciprocal water-bath shaker at 120 strokes per min. Cultures were also grown in 1-liter baffled flasks (Bellco Glass,

Inc., Vineland, N.J.) with Morton stainless-steel closures containing 300 ml of the growth medium and were agitated by rotation at 150 rev/min on a New Brunswick three-tiered rotary shaker at 37 C in a constant-temperature room.

"Replacement sporulation" technique. Vegetative growth phase cultures (grown to less than 3×10^8 cells/ml) were centrifuged for 5 to 10 min in 50-ml sterile glass centrifuge tubes (full speed in an International model HN centrifuge) at room temperature. The medium was discarded, the cells were suspended in the original volume of prewarmed (37 C) replacement sporulation medium, and the cells were centrifuged again. The cells were then suspended in the replacement sporulation medium to a density of 100 Klett units (#66 filter) (equivalent to 2.5×10^8 colony-forming units per ml). The cultures were agitated in the manner described above under growth conditions.

The same procedure was followed for the 300-ml cultures except that 300-ml sterilized polycarbonate centrifuged bottles were used for the centrifugation of the cells in a Servall RC-2B centrifuge with a prewarmed GSA rotor (35 C). (The centrifuge motor was turned off as soon as the rotor speed reached 8,000 rev/min.) As previously reported in studies of cell resuspension with *B. licheniformis* (24), it was essential that the resuspension be carried out quickly and with prewarmed medium to prevent extensive lysis. In some cases, especially with the 300-ml culture, the washing and centrifugation of the vegetative cells with the sporulation medium prior to the final suspension in the sporulation medium gave rise to cellular lysis (10 to 20%), and this step was omitted in some of these studies.

Determination of growth and sporulation. Cultures were serially diluted for total viable count in 0.9% NaCl containing 0.01% Nutrient Broth. Samples were either mixed with molten Nutrient Agar (55 C) or spread on prepoured plates of Nutrient Agar. Thymine at a concentration of 20 μg/ml was added to the Nutrient Agar for experiments with the thymine-requiring auxotroph. Spore counts were determined in a similar fashion after samples had been heated at 70 C in 0.9% NaCl containing 10 mM potassium phosphate (*p*H 7.2) for 30 min. Sporulating cells in the medium containing glutamate tended to clump and were separated for viable counting by the use of a 15-sec treatment in a Waring Blendor under aseptic conditions. Culture turbidity was determined with a Klett-Summerson photometer (#66 filter), and the total cell count was determined with a Petroff-Hausser bacteria counter.

Determination of the rates of incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-phenylalanine into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. Samples (1.0 ml) were removed from the culture at the indicated times during "replacement sporulation," and 10 μc of ^{3}H-thymidine (120 μc/μmole), ^{3}H-uridine (20 μc/μmole), or ^{3}H-phenylalanine (10 μc/μmole) was added. Portions (0.1 ml) of the 1-ml culture sample were removed after 1, 5, 10, 15, 20, 25, and 30 min and placed in 5 ml of cold 5% trichloroacetic acid. The suspensions were filtered through 0.45-μm Millipore filters (25 mm) and washed with cold 5% trichloroacetic acid. The filters were placed in scintillation vials and dried at 110 C for 20 min. A 10-ml amount of a scintillation counting solution containing 0.7% 2,5 diphenyloxazole and 0.03% 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in spectroanalyzed toluene was added to each vial, and the radioactivity present was measured with a Packard Tri-Carb scintillation counter (model 3325) or a Beckman scintillation counter (model LS-150).

The values for the initial velocity of radioisotope uptake reported in Fig. 2 were derived from plots of uptake versus time by use of the 1-, 5-, 10-, 15-, 20-, 25-, and 30-min sample points for each 1-ml sample withdrawn from the original culture. The rates of uptake were linear over the 30 min at the specific activities of each of the radioisotopes used, and chemical analysis of the incorporated radioisotope (16) showed the incorporation into DNA, RNA, or protein.

Preparation of extracts for enzymatic assays. At various times prior to and during replacement sporulation, 75- to 300-ml cultures of cells were removed. The cells were chilled, centrifuged, and washed with 0.10 M potassium phosphate (*p*H 7.2); they were then frozen and stored at −20 C until disrupted. Cells which had been thawed and suspended in 5 to 10 ml of 0.10 M potassium phosphate (*p*H 7.2) were disrupted by sonic treatment for a total of 5 to 10 min with a 75-w Branson Sonifier (half maximal power, 30 sec on and 30 sec off to allow cooling of the sample.) The extracts were centrifuged at 105,000 × *g* (Spinco ultracentrifuge) for 90 min at 4 C, and the supernatant fraction was used for the enzyme assays. Extracts were also prepared by the corrected lysozyme method of Hanson and Cox (13). [The original report of Hanson and Cox calls for a 70-min incubation with 100 μg of lysozyme. The correct incubation time should have been 7.0 min (Hanson, *personal communication*)].

Aconitase, isocitric dehydrogenase (nicotinamide adenine dinucleotide phosphate), and fumarase were assayed by the methods reported by Hanson and Cox (13), and glucose dehydrogenase was assayed by the method of Sadoff et al. (26). Protein was estimated by the procedure of Lowry et al. (17) on trichloroacetic acid-washed precipitates that were subsequently heated in 0.5 ml of 1.0 N NaOH (100 C) for 30 min before addition of the remaining Lowry reagents at room temperature. Failure to heat the trichloroacetic acid-washed crude extract samples in NaOH caused erratic results. Bovine serum albumin, fraction V (also heated in 1 N NaOH), was used as the protein standard.

Determination of the germination requirements for spores produced in replacement sporulation. Spores were isolated by use of Renografin gradients as described by Tamir and Gilvarg (33) or by use of the two-phase polyethylene glycol-potassium phosphate system of Sacks and Alderton (25) as reported by Donnellan et al. (9). Germination of the spores was followed by the loss in refractility (decrease in optical

absorbancy at 625 nm) with a Gilford recording spectrophotometer (36). The samples were mixed frequently to prevent any settling of the spores.

Chemicals. All inorganic salts used were of reagent grade. The addition of trace minerals or the use of tap water (4) was not required for sporulation when medium constituents from the following commercial sources were used: $MgSO_4 \cdot 7H_2O$, from J. T. Baker Chemical Co., Phillipsburg, N.J.; H_3PO_4 and glucose from Fisher Scientific Co., Pittsburgh Pa.; and $MnSO_4 \cdot H_2O$, KCl, NaCl, KOH, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and lactic acid from Mallinckrodt Chemical Works, St. Louis, Mo. The glutamic acid, adenosine triphosphate (ATP), D,L-isocitric acid, fumaric acid, malic acid, and nicotinamide adenine dinucleotide phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., and the radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.

The distilled-deionized water used for all media and solutions was building distilled water passed through two Continental Deionizing Water Service deionizers.

RESULTS

Conditions for the replacement sporulation of B. subtilis 168. Figure 1 shows the time course of replacement sporulation of the wild-type strain of *B. subtilis* 168 and of a tryptophan-thymine auxotrophic strain of *B. subtilis* 168 (*trp-2 thy*). Sporulation, as measured by the appearance of heat resistance, was completed by 9 to 10 hr after resuspension of the vegetative cells into the sporulation medium. The lower level of spores found in vegetative cultures of the strain requiring tryptophan and thymine has been consistently observed, even upon repeated transfers in growth medium. However, no explanation for this difference between the two strains can be offered at this time.

The effect of different concentrations of ammonium lactate or glutamate in the replacement sporulation medium is demonstrated in Tables 1 and 2. In the absence of these compounds, there was eventual lysis of the resuspended cells. Optimal sporulation occurred in cells resuspended in media containing 10 mM ammonium lactate or glutamate, whereas at concentrations much in excess of 10 mM there was a continuation of growth. With the higher substrate concentrations, an appreciable amount of clumping was observed microscopically, especially in the glutamate medium.

The effect of ammonium lactate or glutamate in promoting replacement sporulation could also be observed with such compounds as ammonium succinate, ammonium acetate, or ammonium pyruvate at concentrations of 10 mM. A number of other compounds (e.g., fructose) were tested for their ability to support sporulation while not permitting further vegetative growth, and the results seem to support an earlier report (J. Mandelstam, W. M. Waites, and S. C. Warren, 7th Int. Congr. Biochem., Tokyo, Symp. V-2, vol. 1, p. 253, 1967) that the most satisfactory carbon sources are those that can be metabolized via the tricarboxylic acid cycle.

The percentage of conversion of vegetative cells to spores (i.e., sporulation efficiency) given in Tables 1 and 2 was calculated as follows: number of heat resistant cells/*highest* number of viable cells during sporulation × 100. By this method, the data in Fig. 1 and Tables 1 and 2 indicate that there is between 90 and 100% sporulation efficiency under optimal conditions. However, this method of calculating sporulation efficiency

TABLE 1. *Effect of ammonium lactate concentration on the "replacement sporulation" of Bacillus subtilis 168 (wt)*[a]

Ammonium lactate concn	Viable cells after							Sporulation efficiency[b]
	0 hr	1 hr	2 hr	3 hr	5 hr	17 hr	17 hr (spores)	
mM								%
0	2.8×10^8	5.4×10^8	4.3×10^8	2.3×10^8	1.0×10^8	0.45×10^8	0.19×10^8	3.5
5	3.1×10^8	4.4×10^8	4.5×10^8	4.6×10^8	4.1×10^8	2.2×10^8	0.98×10^8	21.3
10	3.3×10^8	4.6×10^8	4.5×10^8	4.9×10^8	4.5×10^8	4.0×10^8	4.5×10^8	91.2
15	3.3×10^8	4.3×10^8	4.8×10^8	6.8×10^8	6.1×10^8	4.2×10^8	4.7×10^8	69.1
20	3.4×10^8	5.3×10^8	7.5×10^8	—	17.1×10^8	8.1×10^8	6.6×10^8	38.6
25	3.2×10^8	4.6×10^8	8.0×10^8	12.2×10^8	17.0×10^8	5.1×10^8	1.8×10^8	10.6

[a] Vegetative cells (grown in a medium containing 20 mM glucose-50 mM ammonium lactate) were grown to 3×10^8 viable cells per ml (120 Klett units), centrifuged, and suspended in 25 ml of the sporulation medium containing the indicated concentrations of ammonium lactate. The number of viable cells and spores (heat-resistant cells) was determined at the times indicated.

[b] Per cent sporulation efficiency = no. of viable heat-resistant spores/highest no. of viable cells obtained × 100.

TABLE 2. *Effect of glutamate concentration on the "replacement sporulation" of Bacillus subtilis 168 (wt)*[a]

Glutamate concn	Viable cells after						Sporulation efficiency[b]
	0 hr	1 hr	3 hr	5 hr	19 hr	19 hr (spores)	
[mM							%
0	2.1×10^8	5.2×10^8	2.6×10^8	0.25×10^8	0.24×10^8	0.19×10^8	3.7
5	2.4×10^8	4.0×10^8	2.7×10^8	2.2×10^8	2.4×10^8	2.3×10^8	57.5
10	2.8×10^8	4.3×10^8	3.5×10^8	2.9×10^8	3.3×10^8	3.4×10^8	79.1
15	2.4×10^8	5.4×10^8	4.4×10^8	4.7×10^8	3.4×10^8	2.6×10^8	48.3
20	2.8×10^8	4.3×10^8	3.6×10^8	3.1×10^8	3.1×10^8	1.8×10^8	41.9
25	2.6×10^8	5.4×10^8	7.1×10^8	4.5×10^8	3.2×10^8	1.4×10^8	19.8

[a] Vegetative cells (grown in a medium containing 20 mM glucose-50 mM glutamate) were grown to 2.5×10^8 viable cells per ml (100 Klett units), centrifuged, and suspended in 25 ml of the sporulation medium containing the indicated concentrations of glutamate. The number of viable cells and spores (heat-resistant cells) was determined at the times indicated.

[b] Calculated as indicated in Table 1.

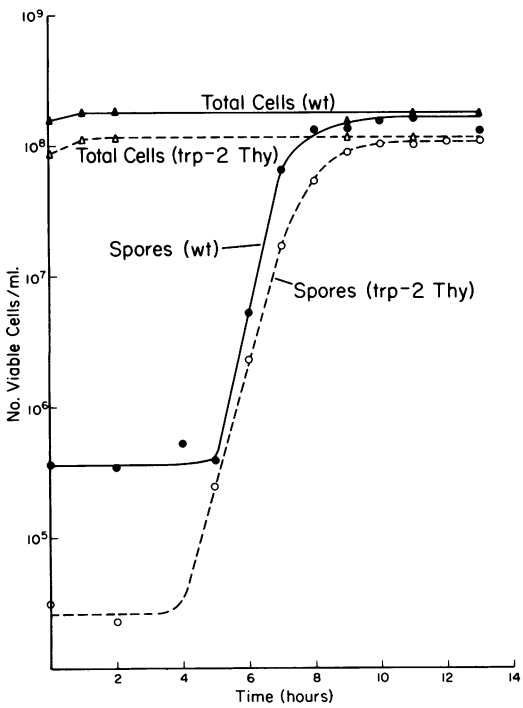

FIG. 1. *"Replacement sporulation" of B. subtilis 168 (wt) and B. subtilis 168 (trp-2 thy). Vegetative cells of the wild type or the tryptophan-thymine requiring auxotroph were grown in the medium containing 20 mM glucose-50 mM ammonium lactate. The cells were centrifuged and suspended in the sporulation medium containing 10 mM ammonium lactate. The media for growth and for "replacement sporulation" of the tryptophan-thymine auxotroph contained 40 μg of thymine and 40 μg of tryptophan per ml. The number of viable cells and spores present was determined at the indicated times.*

does not take into account that up to 50% of the cells undergoing replacement sporulation exist in pairs, even up to the time of formation of sporangia (appearance of heat resistance). For example, if the data presented in Table 1 are calculated from direct microscopic observation, the overall sporulation efficiency at an ammonium lactate concentration of 10 mM is 73% rather than 91.2%.

Changes in enzyme levels during replacement sporulation. Since an increase in various tricarboxylic acid cycle enzymes has been reported during sporulation in glutamate batch cultures (14, 30), the levels of aconitase, fumarase, and isocitric dehydrogenase were determined during replacement sporulation in glutamate media or ammonium lactate media. The specific activities of these enzymes reached their maxima 2 to 3 hr after suspension in the sporulation media (Table 3). Table 3 shows the specific activities obtained after either sonic treatment or lysozyme disruption. Consistently higher specific activities and more reproducible results were obtained when sonic treatment was used. The lower level of aconitase and isocitric dehydrogenase found during sporulation in the glutamate culture may reflect partial repression of these enzymes by glutamate (13, 30).

A similar experiment was done with the thymine-tryptophan auxotroph of *B. subtilis* 168 (Table 4). In this experiment, the tricarboxylic acid cycle enzymes had reached a maximum at 2 hr and then decreased to approximately half of their maximal value after 3 to 4 hr. The small increase in specific activity after 6 hr may be due to partition of the developing spore rather than synthesis of enzyme. Glucose dehydrogenase, which has been identified as a spore component (26),

TABLE 3. *Effect of the ammonium lactate or glutamate in the "replacement sporulation" medium on the levels of aconitase, fumarase, and isocitric dehydrogenase in B. subtilis 168 (wt)*[a]

Medium	Time (hr)	Specific activity (units/mg of protein)					
		Aconitase		Fumarase		Isocitric dehydrogenase	
		Sonic extract	Lysozyme extract	Sonic extract	Lysozyme extract	Sonic extract	Lysozyme extract
Ammonium lactate	0	429	75	1,180	207	.069	.020
	1	620	151	1,340	294	.134	.100
	2	1,030	1,000	3,380	299	.205	.125
	3	2,240	894	4,620	378	.173	.095
	4	1,980	733	2,140	378	.170	.090
Glutamate	0	415	131	1,770	828	.080	.040
	1	512	150	2,190	1,880	.082	.045
	2	638	172	2,810	2,600	.090	.070
	3	888	449	4,120	2,370	.093	.065
	4	624	390	3,200	1,290	.075	.035

[a] Cells (300 ml of culture ~3.0 × 10^8 cells/ml) were harvested at the indicated time after suspension in the "replacement sporulation" medium. The vegetative cells for the suspension were grown to 3.0 × 10^8 cells/ml in their respective medium (e.g., cells suspended in 10 mM ammonium lactate were grown in 20 mM glucose and 50 mM ammonium lactate).

appeared approximately 4 to 5 hr after suspension of the vegetative cells in the sporulation medium, as previously reported (35).

Synthesis of DNA, RNA, and protein during replacement sporulation. The synthesis of DNA, RNA, and protein during replacement was estimated from the rates of incorporation of ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-phenylalanine into the cold trichloroacetic acid-insoluble cell fraction. Figure 2 shows the incorporation of these isotopes at various times during replacement sporulation. These results indicate a steady decrease in the rate of thymidine incorporation in vegetative cells suspended in the sporulation medium, suggesting a decrease in DNA synthesis prior to sporulation. At the same time, however, there was a slight increase in the incorporation of ^{3}H-uridine and ^{3}H-phenylalanine upon suspension, showing a continued synthesis of RNA and protein during sporulation (29).

Examination of the spores produced by replacement sporulation. The purified spores produced by replacement sporulation have the same requirement for L-alanine for rapid germination as do the spores produced from batch cultures (11). Heat-shock of the spores from either the replacement sporulation cultures or the batch cultures resulted in a faster germination rate (36) for both types of spores (Table 5). Microscopic examination of the cells after germination showed greater than 99% germination.

TABLE 4. *Specific activities of aconitase, fumarase, isocitric dehydrogenase, and glucose dehydrogenase during "replacement sporulation" of B. subtilis 168 (trp-2 thy) in 10 mM ammonium lactate*[a]

Time (hr)	Specific activity (units/mg of protein)			
	Aconitase	Fumarase	Isocitric dehydrogenase	Glucose dehydrogenase
(vegetative)	800	2,200	.190	<5
0	712	2,470	.210	<5
1	1,480	2,910	.505	<5
2	1,400	3,300	.592	<5
3	860	2,640	.449	10
4	655	1,790	.287	102
5	349	1,300	.172	127
6	573	1,830	.386	—
7	520	2,340	.299	144
8	528	2,240	.304	137
9	646	2,860	.253	64

[a] Cells (100 ml of culture ~2.5 × 10^8 cells/ml) were harvested at the indicated times after suspension in the "replacement sporulation" medium containing 10 mM ammonium lactate. Extracts were prepared by sonic extraction, and the enzyme activities were determined at the times indicated. The media for growth and for "replacement sporulation" of the thymine-tryptophan auxotroph contained 40 μg of thymine and 40 μg of tryptophan per ml.

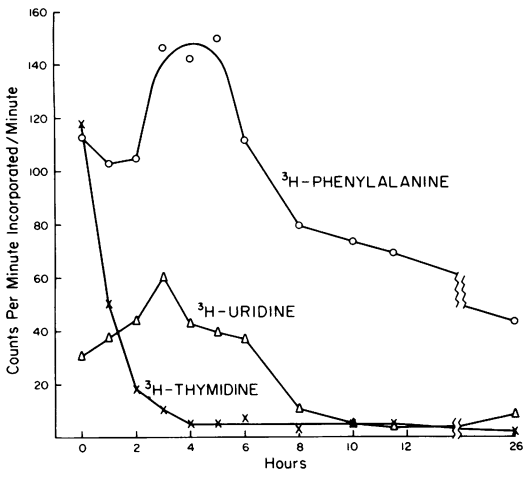

FIG. 2. *Rate of ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-phenylalanine incorporation into the cold TCA insoluble fraction of cells during "replacement sporulation." At the indicated times after resuspension of the vegetative cells into the sporulation medium containing 10 mM glutamate, 1 μc of ^{3}H-thymidine (120 μc/μg), ^{3}H-uridine (20 μc/μg), or ^{3}H-phenylalanine (10 μc/μg) was added to 1 ml of a culture (~2.0 × 10^8 cells/ml). The initial rates of incorporation were determined as indicated in Materials and Methods.*

Freshly prepared spores from "replacement sporulation" cultures had a slightly faster germination rate than freshly prepared spores from batch culture (Table 5). The comparison was made with freshly prepared spores because there is a considerable difference in the germination rate of spores upon storage (6, 23). Thus, there appears to be no difference between the spores produced by replacement sporulation and the spores produced by conventional batch culture.

DISCUSSION

Our results indicate that vegetative cells of *B. subtilis* 168 suspended in a minimal salts medium containing 10 mM ammonium lactate or glutamate give rise to normal spores without further appreciable vegetative cell growth or cell lysis. The requirement for the additional energy source is an indication that *B. subtilis*, unlike *B. megaterium* (5), does not contain sufficient endogenous storage compounds to permit endotrophic sporulation. This requirement of an additional substrate for replacement sporulation of *B. subtilis* presents an experimental difficulty. If the substrate is supplied at too high a level or if too few cells are suspended in the sporulation medium, the cells continue vegetative growth for a short while. On the other hand, if the substrate is supplied at too low a level or the cells are suspended at too high a density for the substrate concentration, the cells lyse. Probably the success of compounds like ammonium lactate and glutamate is due to the fact that they are metabolized rather slowly by *B. subtilis*, as evidenced by the slow growth rates obtained with asporogenous cultures (10). Thus, these compounds can serve as a type of "exogenous storage compound," providing enough energy for sporulation but not enough for growth.

The studies of the changes in enzyme levels and the patterns of DNA, RNA, and protein synthesis show close similarities to the results previously reported in studies of sporulation following the end of vegetative growth (7, 8, 13, 21, 29). Although an increase in the incorporation of uridine and phenylalanine during the early stages of replacement sporulation was observed in the present study, this cannot be considered as evidence for an increased synthesis or turnover of RNA or protein. The results of the ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-phenylalanine incorporation

TABLE 5. *Rates of initiation for germination of B. subtilis 168 (wt) spores produced by "replacement sporulation"*

Additions to	Rates of initiation[a] for germination of	
	"Replacement sporulation" spores	"Batch culture" spores[b]
Heat-shocked spores (30 min, 70 C)		
L-Alanine (100 μg/ml)	2.62	2.05
L-Alanine (100 μg/ml), glucose (20 mM), and ammonium lactate (50 mM)	2.61	2.10
Glucose (20 mM) and ammonium lactate (50 mM)	<0.04	<0.04
No additions	<0.04	<0.04
Non-heat-shocked spores		
L-Alanine (100 μg/ml)	0.91	0.72
L-Alanine (100 μg/ml), glucose (20 mM), and ammonium lactate (50 mM)	0.93	0.71
Glucose (20 mM) and ammonium lactate (50 mM)	<0.04	<0.04
No additions	<0.04	<0.04

[a] The rate of initiation is the maximal rate at which the observed optical density at 625 nm decreases per hour divided by the initial optical density (36). (The initial optical density at 625 nm was 0.500.)

[b] Batch culture spores were obtained from the growth and sporulation medium containing 20 mM glucose-50 mM ammonium lactate and were harvested 16 hr after the end of vegetative growth.

are relative because the wild-type *B. subtilis* 168 was used and there was no correction for changes in pool size due to endogenous synthesis of amino acid and protein turnover (2) or changes in permeability during sporulation (3). In preliminary experiments with thymine, uracil, and phenylalanine auxotrophs of *B. subtilis* 168, incorporation patterns were similar to those reported in the present paper, suggesting that the suspension of vegetative cells in the sporulation medium does not introduce any obvious artifacts.

Although studies previously reported have utilized the replacement sporulation technique with other *Bacillus* species (e.g., *B. megaterium* and *B. cereus*; 5), this report represents nearly quantitative replacement sporulation obtained with *B. subtilis* 168. Similar results have also been reported by Mandelstam et al. (19). Although the actual percentage of conversion of the suspended vegetative cells to spores may vary in any one experiment, the average percentage of conversion in more than 50 experiments was greater than 90%. Mandelstam (*personal communication*) found that, using the Donnellan medium (19), the percentage of conversion was at least 50%.

The technique of "microcycle sporulation" (formation of a spore from a spore without intervening division), first reported by Vinter and Slepecky with *B. cereus* (34) and extended to the use of chemically defined medium by Holmes and Levinson for *B. megaterium* (15) and by MacKechnie and Hanson for *B. cereus* strain T (18), has been investigated for use with *B. subtilis*. However, no single medium has been obtained, to date, that will allow outgrowth (formation of a vegetative cell from the germinative spore) while permitting reasonable efficiencies of "resporulation" without several additional rounds of cell division. Some success has been obtained by germination and out-growth in medium supplemented with 0.01% Difco Casamino Acids followed by suspension in the "replacement sporulation" medium (R. Ramaley, *unpublished observations*); these results will be reported at a later date.

It is hoped that the technique of replacement sporulation will aid in the biochemical and the eventual genetic analysis of sporulation in *B. subtilis* 168.

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