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Study of *Bacillus subtilis* Endospores in Soil by Use of a Modified Endospore Stain†

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The Schaeffer-Fulton endospore stain was modified so that it would stain *Bacillus subtilis* endospores in soil smears. The modified stain differentiated among dormant spores, spores undergoing activation, and spores which had germinated but had not yet shown outgrowth. These differentiations were seen for spores in soil and for pure spore preparations in the laboratory. This stain was used to show reversible *B. subtilis* spore activation promoted by an *Ensifer adhaerens*-like indigenous bacterium in soil and by pure cultures of *E. adhaerens* added to spores in the laboratory. Under the specific conditions in the laboratory, spore germination did not proceed beyond the activation stage, and relatively little change occurred in the numbers of both *E. adhaerens* and *B. subtilis*. This was also true in soil, although some germination with destruction of spores and vegetative cells did occur if the soil had been nutritionally enriched by preincubation with incorporated ground alfalfa.

Large numbers of bacterial spores survive in soil as though they were immune to biological or physical changes in their environment. Nevertheless, *Ensifer adhaerens* 7A, a bacterial predator of certain other bacteria (3), was originally isolated from soil that had been incubated with *Bacillus subtilis* spores. Indigenous strain 7A cells apparently had proliferated in this soil in response to the spores. Direct microscopic study of soil preparations for this apparent *E. adhaerens* response presented a problem, however, because conventional endospore stains differentiate only poorly between soil bacteria and soil debris in soil smears. Nevertheless, we have now found that a modification of the Schaeffer-Fulton endospore stain does differentiate in soil smears. In addition, this stain appears to differentiate between the state of the spores as originally added to soil and a state that develops if there is a localized multiplication of certain indigenous soil bacteria.

The object of this study was to evaluate the use of the modified Schaeffer-Fulton stain for soil preparations. We also wished to examine an apparent change in the response of the stain to spores that had been exposed in soil to certain bacteria that developed in the soil, such as an *E. adhaerens*-like bacterium.

MATERIALS AND METHODS

Soils. All soils were stored at room temperature in sealed polyethylene bags. Soils A and B were Hagerstown silty clay loams (pH 6.4 and 5.5, respectively). The last crop grown on soil A was alfalfa. Soil B was collected from under the drip zone of a pine tree. This soil was not penetrated by roots. The soil was autoclaved, when necessary, by the procedure of Labeda et al. (9).

Cultures and maintenance. The bacteria studied were *B. subtilis* PSU 46A, *E. adhaerens* ATCC 33212 (strain A), and *E. adhaerens* ATCC 33499 (strain 7A). Stock cultures were maintained on slants of heart infusion agar made up at 1/10 strength. They were stored at 4°C.

Indigenous soil bacteria responding to the presence of *B. subtilis* spores were isolated by the soil-column slide technique (2). The control slides did not contain smears of the spores. The bacteria were incubated for 4 to 7 days at 27°C. Those that developed on the slides were streaked on the following media: live and autoclaved *B. subtilis* spore agar, 1/10 strength and full-strength heart infusion agar with and without 0.1% glucose, nutrient agar, brain heart infusion agar, and Noble and Bacto water agars (1.5%). Most of these media were obtained from Difco Laboratories, Detroit, Mich. The plates were incubated for 2 to 14 days at 27°C. Colonies were selected based on differences observed between plates from the spore soil column slides and those from the control soil column slides. The selection was aided by phase-contrast microscopy examinations of the cells. The isolates were maintained at 4°C on slants of 1/10 strength heart infusion agar. If growth was poor on this medium, then nutrient agar was used.

The live spore agar consisted of 5.0 ml of concentrated *B. subtilis* spore preparation per 500 ml of 1.5% Bacto-Agar in distilled water. The spores were added after the medium was autoclaved and tempered to 47°C. Autoclaved spore agar was similar, but the spores were added before autoclaving.

Cell and spore suspensions. *E. adhaerens* A and 7A were grown in nutrient broth as shaken cultures for 2 to 3 days at 27°C. The cells were then washed three times in sterile distilled water. The resulting concentrated cell suspension in distilled water was used immediately; it was not stored. Concentrated cell suspensions of the soil isolates were prepared in a similar manner, except that nutrient broth was substituted for 1/10 strength heart infusion broth in cases in which the organisms did not grow well in the latter broth.

For certain experiments, the *E. adhaerens* cells and *B. subtilis* spores were suspended in Burk buffer. This buffer contained (per liter) 0.64 g of K_2HPO_4, 0.16 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.05 g of $CaSO_4 \cdot 2H_2O$, and 1 ml each of $Na_2MoO_4 \cdot 2H_2O$ (0.25 g/100 ml) and ferric citrate (0.9 g/100 ml).

For preparing concentrated, washed suspensions of *B. subtilis* spores, an inoculum of vegetative cells was first produced by growing the cells (shaken culture) for 18 to 24 h at 27°C in nutrient broth containing 0.1% L-alanine and 0.5% glucose. These cells were nearly devoid of spores. The cells were washed three times in distilled water and resuspended in distilled water to give a 50-fold concentration. For spore

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TABLE 1. Occurrence of light-green-staining *B. subtilis* spores after incubation in aqueous solutions of L-alanine and glucose

Germinant	Concn (%)	Avg incubation time required (days)	% light-green-staining spores produced[a]
L-Alanine	0.01	14	85–90
L-Alanine plus glucose	0.01 + 0.2	11–12	95
Glucose	0.2	14	50–60
None		28	5–10

[a] Based on the modified Schaeffer-Fulton endospore stain.

production, 0.1 ml of the above cell suspension was spread on the surface of a plate of 1/10 strength heart infusion agar supplemented with 0.011% $MnSO_4 \cdot H_2O$. The plates were incubated for 2 weeks at 27°C. The resulting spores were harvested by suspension with a glass spreading rod from each plate in 10 ml of sterile distilled water. These suspensions were then combined and washed three times in sterile distilled water. The final pellet, representing 10 to 15 plates, was suspended in 5 ml of distilled water. It was stored at 4°C.

CFU for spores in these preparations or for spores from experiments were enumerated with 1/10 strength heart infusion broth solidified with 0.6% Gelrite (12). Agar was not used. The Gelrite per se had no effect on the counts, but it did control *B. subtilis* colony spreading. For solidification, the Gelrite required addition of 0.07% $MgCl_2 \cdot 6H_2O$. In addition, 0.01% L-alanine was added to increase the CFU count. Dilutions for the counts were made in sterile tap water. The counts were run with and without heat activation (80°C for 20 min) of the spores. For spore counts from soil and nonsoil preparations, the heat shocking was applied to the 10^{-1} dilution. A 10^{-1} dilution of soil was obtained by adding the entire 10 g of soil in the bottle to 90 ml of sterile tap water. The plates were incubated for 2 days at 27°C before the colonies were counted.

Stains. All stains were stored at room temperature. All dyes were reagent grade. The dye solutions were filtered through Whatman 2V filter paper (Whatman, Inc., Clifton, N.J.). The 5% aqueous malachite green was prepared by adding 100 g of malachite green (Fisher Scientific Co., Fair Lawn, N.J.) to 2.0 liters of distilled water. The mixture was stirred to dissolve the dye, allowed to sit for 1 h, and then filtered. The 7.6% aqueous malachite green was prepared in a similar manner.

The 5% crystal violet solution was prepared by mixing equal volumes of two solutions. One consisted of 10.0 g of powdered crystal violet (Allied Chemical Co., Morristown, N.J.) per 100 ml of 95% ethanol. The other consisted of 1.0 g of ammonium oxalate per 100 ml of distilled water.

The 0.24% safranin solution was prepared from a stock solution consisting of 2.5 g of safranin-0 (J. T. Baker Chemical Co., Phillipsburg, N.J.) dissolved in 100 ml of 95% ethanol. To achieve the final 0.24% concentration, 10 ml of the stock solution was added to 100 ml of distilled water.

Carbolfuchsin was prepared by combining equal volumes of two solutions. The first consisted of 9.0 g of basic fuchsin (J. T. Baker Chemical Co.) dissolved in 300 ml of 95% ethanol. The second consisted of 150.0 g of phenol (J. T. Baker Chemical Co.) dissolved in 2,850 ml of distilled water.

Staining procedures. The Schaeffer-Fulton endospore stain (5) was modified as follows. The 5% aqueous malachite green primary stain was retained; however, a 5% crystal violet solution was used as the counterstain. One loopful of cell or spore suspension was placed as a smear approximately 2.5 cm in diameter on a slide. These slides, or slides resulting from soil column experiments, were then air dried and heat fixed. The slides were placed over a steaming water bath, and a piece of paper towel was placed on each slide to help prevent drying of the stain. The towel was flooded with malachite green. Periodic additions of malachite green were needed during heating to prevent drying. The slides were heated for 5 min after the dye started to steam. They were then washed with water and stained for 2 min with the crystal violet counterstain, or with the more conventional safranin counterstain if it was to be tested. The stained slides were washed with water and blotted dry.

In the cold method for malachite green staining (4), the 7.6% aqueous malachite green solution was added to the smear with paper towel and allowed to remain for 10 min without application of heat. After a water wash, the slides were stained with crystal violet for 2 min, washed again with water, and blotted dry.

For the staining done with carbolfuchsin (4), the same protocol was used as in the modified Schaeffer-Fulton endospore stain, except that carbolfuchsin was substituted for the malachite green. Our method is a further modification of the Dorner endospore stain as modified by Snyder (see reference 4). The carbolfuchsin was washed from the slide by water instead of the alcohol used by Snyder. Also, the counterstain used was crystal violet instead of the recommended nigrosin.

RESULTS

Modified Schaeffer-Fulton stain. Spores germinating at 27°C in shaken nutrient broth, plus or minus 0.01% L-alanine and 0.2% glucose (and under other conditions described below), went through several staining phases when stained with a modified Schaeffer-Fulton spore stain. Crystal violet was used as the counterstain instead of safranin. The spores appeared first as dark green, then as light green, light purple and finally dark purple for the vegetative outgrowth. Light purple signified a loss of phase brightness in the spore, a condition that indicates spore germination. Spore germination was also indicated by small breaks in the exosporium followed by outgrowth of the vegetative cell. The spores lost their ability to stain with malachite green before undergoing the break in the spore coat: i.e., only spores staining purple were found with breaks in the spore coat. If the crystal violet counterstain was omitted, the light purple spores stained extremely light green instead of light purple. The use of safranin as the counterstain did not affect the light- and dark-green staining characteristics of the spores. However, safranin was not an acceptable counterstain because it did not allow for observation of soil bacteria when used on soil smears. The Dorner endospore stain, modified to use crystal violet as the counterstain, did not reveal any difference between spores staining dark green and those staining light green by the modified Schaeffer-Fulton stain. The cold method, in which malachite green was used with a crystal violet counterstain, also was not acceptable, because all spores stained light green.

B. subtilis spores at 2×10^8/ml were shaken at 27°C in distilled water solutions (50 ml) of the spore germinants containing 0.01% L-alanine, 0.2% glucose, or both. These low levels of germinants caused the spores to take on the light-green staining characteristic (Table 1). In addition to the light-green staining, however, a small percentage (<3) of the spores treated with the L-alanine–glucose mixture stained light purple. Also, several spores were found with a cracked

exosporium. For these treatments and the control, the ability of the spores to stain light green was reversible with continued incubation. More than 97% of the light green spores reverted to the dark-green staining characteristic. This reversion occurred most quickly with glucose alone (1 to 2 days after taking on the light-green staining characteristic), followed in decreasing order by L-alanine (2 to 3 days) and L-alanine–glucose (3 to 4 days).

B. subtilis spores, averaging 3×10^8/ml, were shaken for 8 weeks in 50 ml of distilled water containing 0.01% L-alanine and 0.2% glucose. During this incubation they stained dark green, then light green, and then dark green. Control spores were shaken in distilled water, and they stained dark green at all times. There was no decrease in spore viability during this incubation (tested every 2 weeks) whether or not the spores were tested by heating for 20 min at 80°C just before dilution for plating. To test for the relative heat resistance of the spores, 1 ml of spores was boiled in 9 ml of water for various periods of time just before plating. There was a progressive decrease in spore viability with time: i.e., from 3×10^8 at zero time to 7×10^3 at 10 min. However, the curves were virtually identical for spores that had been incubated in distilled water and those incubated in the L-alanine–glucose solution (L-alanine without glucose was not tested). In addition, these curves were the same as those for spores incubated with *E. adhaerens* A in distilled water (see below for *E. adhaerens* incubations) instead of germinants.

Spores having the light-green staining characteristic were produced by incubation with shaking in distilled water containing 0.01% L-alanine and 0.2% glucose for 2 weeks at 27°C. They were then washed and further incubated for 2 to 3 weeks stationary at 4 to 7°C in nutrient broth (with no additional alanine or glucose). The spores were able to germinate under the cold conditions, as indicated by the cracked exosporiums and vegetative cell outgrowth. Spores having the dark-green staining characteristics (not preincubated with alanine and glucose) did not germinate when incubated in the nutrient broth at 4 to 7°C.

A comparison was made between the germination rates in nutrient broth of control spores (preshaken for 2 weeks in distilled water and having the dark-green staining characteristic) and spores having the light-green staining characteristic (preshaken for 2 weeks in distilled water containing 0.01% L-alanine and 0.2% glucose). In both cases, the spores were washed three times before further treatment. The spores in the light-green state began to grow almost immediately (Fig. 1). By comparison, the spores in the dark-green state exhibited a longer lag period with a more pronounced decrease in optical density before the log-phase growth began.

Soil studies. *B. subtilis* spores and soil A (pH 6.4; not amended or sterilized) were incubated in soil column slides (2). The water content of the soil was maintained at about 65% of moisture-holding capacity (MHC) during incubation. At zero time on the slides, all of the spores stained dark green with the modified Schaeffer-Fulton stain. During a 1-week incubation at 27°C, areas developed on the slides in which the spores no longer stained dark green but instead stained light green. These areas varied in size from a few adjacent spores to areas encompassing two to three microscopic fields (100× objective). Purple-staining spores and spores with cracked spore coats were not detected. The light green areas were associated with the growth of two different indigenous soil bacteria (see below). The use of soil B (pH 5.5; not amended or sterilized) for the slides did not allow growth of these bacteria, or of most other indigenous soil

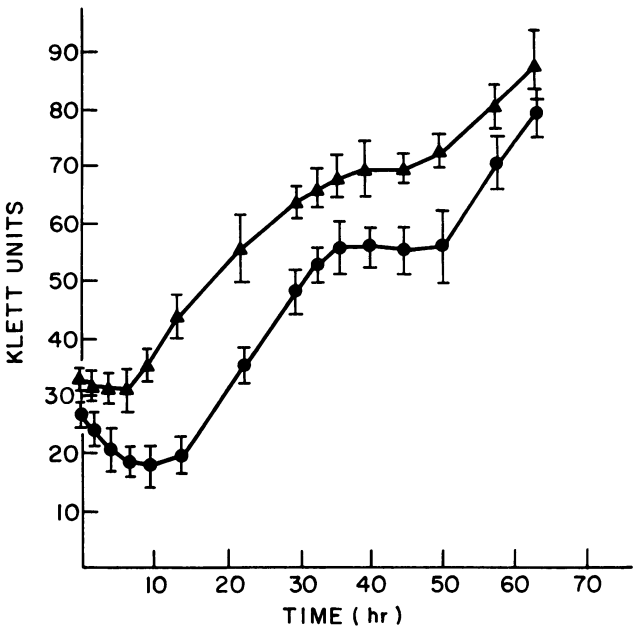

FIG. 1. Growth of *B. subtilis* spores. Symbols: ▲, spores pretreated with L-alanine plus glucose; ●, control (nontreated) spores. Incubations for growth were in shaken nutrient broth at 27°C. Bars represent extremes of variability in the data.

bacteria (and fungi). Under these conditions, virtually all of the spores retained their dark-green staining characteristic. Incubation of the spores for 2 weeks in soil column slides with autoclave-sterilized soils A and B also did not alter the dark-green staining characteristic of the spores.

The two indigenous soil bacteria that grew in the areas of soil A where the spores stained light green did not necessarily occur together, and they were not detected on slides to which spores had not been added. One of them occurred with greater frequency than the other. It was a short rod that at times could be seen attached to the spores in a picket fence arrangement as does *E. adhaerens* for its host cells (3). The other bacterium appeared to be an actinomycete that usually did not exhibit direct contact with the spores, even though the spores in the area took on the light-green staining characteristic. There was no apparent interaction of the activity of this bacterium with that of the *E. adhaerens*-like bacterium. The growth of these bacteria, and the limited growth of other indigenous soil bacteria, decreased after about 7 or 8 days of incubation. When this occurred, many of the spores that had taken on the light-green staining characteristic earlier during incubation reverted to the dark green state as though the phenomenon were reversible.

Soil column slides were incubated with *B. subtilis* spores and nonsterilized soil A that had been nutritionally preenriched by incubation for several months at room temperature with ground alfalfa. Again, areas of light-green-staining spores and the two aforementioned bacteria developed. A few residual dark-green-staining spores were present within these areas, however, which showed that the staining procedure was performed properly. Under these nutritionally richer soil conditions, the spores associated with the area of the *E. adhaerens*-like bacterium appeared to progress further along the pathway to germination. The spores in this area initially took on the light-green staining characteristic. By day 5 to 6 of incubation, however, the spores in the center of this area stained light purple and, within the outer

TABLE 2. Occurrence of light-green-staining spores after incubation of *B. subtilis* spores in distilled water with *E. adhaerens* cells

Ratio of *E. adhaerens* cells to *B. subtilis* spores	*E. adhaerens* strains	Incubation time required (days)	% light-green-staining spores produced[a]
2/1	A + 7A	14	90
2/1	A	14–18	80
2/1	7A	14–18	80
1/1	A	14–18	70
1/1	7A	14–18	70
0.5/1	A	21	60
0.5/1	7A	21	55
0.1/1	A	28	10
0.1/1	7A	28	10
0.0/1 (Control)	None	28	5–10

[a] Based on the modified Schaeffer-Fulton endospore stain.

light-green area, a few ghosted *Bacillus* vegetative cells were present. Along the outer edge of the light-green-staining area some spores with cracked spore coats and emerging vegetative cells were seen. After an additional 1 to 2 days of incubation, some of the original light green areas had changed to the point that they contained only light-purple-staining spores, plus ghosted, very light-purple-staining forms of the spores at the centers of the areas. On all of the above slides it was difficult to detect where reversion from the light-green to the dark-green staining characteristic had occurred for the spores. It was noted, however, that the numbers of areas with light-green-staining spores decreased with time, and there were areas of dark-green-staining spores that contained a few light-purple-staining spores.

The actinomycete noted earlier developed on these slides (alfalfa-preenriched) but did not cause any detectable staining changes for the spores. The apparent reason for this was that the *E. adhaerens*-like bacterium had attached to the actinomycete and held it in check in the manner described by Casida for *E. adhaerens* (2, 3). For all of the above instances involving changes in the staining characteristics of the spores, the areas that developed never involved more than 10% of the spores on the slides.

Additions to soil A (not sterilized) of 0.5% glucose solution, 0.05% L-alanine solution, a mixture of the two, or nutrient broth did not provide a clear picture of the fate of the spores. Some areas developed with conversion of the spores to the light-green staining characteristic, but many different soil bacteria also multiplied, making it difficult to attribute the staining change to any one bacterium.

B. subtilis spores were added to soil bottles (2) which contained 10 g of unamended, natural (nonsterile) soil A. Spore addition per bottle (zero-hour plating) was 1.2×10^9 to 1.6×10^9. The bottles were adjusted with water to 65% MHC and incubated with loosened caps for 12 weeks at 27°C. Additional water was added as needed to maintain the 65% of MHC, and periodic plate counts were performed. There was a reduction in CFU of approximately 40% over the incubation period. However, the true amount of reduction could be less than this because of the difficulty in freeing all the spores from soil after incubation.

E. adhaerens. Various ratios of *B. subtilis* spores and *E. adhaerens* cells (strain A or 7A) were incubated together by shaking in 50 ml of distilled water at 27°C for periods from 2 to 4 weeks. The *E. adhaerens* strains were tested separately and as a mixture in this manner. The *E. adhaerens* cells caused from 55 to 90% of the spores to take on the light-green staining characteristic (Table 2). The percentage of spores assuming this characteristic was in direct proportion to the ratio of *E. adhaerens* cells to spores. The spores were then incubated with the *E. adhaerens* cells for an additional 1 to 4 days after the spores had assumed the light-green staining characteristic. During this incubation, the spores reverted to the dark-green staining characteristic. These reverted spores, however, were unable to return to the light-green state when they were centrifuged and incubated with fresh *E. adhaerens* cells at any of the previous ratios. The reverted spores were still viable and had virtually no reduction (<5%) in CFU when plated.

B. subtilis spores shaken for 4 weeks in distilled water with *E. adhaerens* strain A cells (0.5 strain A cells per spore) decreased in CFU by 25%, as compared to a 5 to 10% decrease in the absence of strain A. During this time the CFU of strain A increased by 25% in the presence of the spores, but decreased by 5 to 10% in their absence.

When Burk buffer was used instead of distilled water as the suspension fluid, no amount (ratios as above) of *E. adhaerens* was able to convert the spores to the light green state. Incubation of strain A or 7A with the spores, at a ratio of about 0.75 to 1.0 in water containing 0.01% L-alanine, 0.2% glucose, or both in combination, produced light green spores as if the germinants were not present.

E. adhaerens growth responded strongly to calcium additions. It did not grow in the 0.01% L-alanine–0.2% glucose solution, but did do so if 0.1% $CaCl_2$, or a filtrate of *B. subtilis* spores which had been incubated in the 0.01% L-alanine–0.2% glucose solution until the spores had just started staining light green, was added. The slopes for both were 0.2. *E. adhaerens* incubated in 1/10 strength heart infusion broth had a slope of 0.9. Addition of either 0.1% $CaCl_2$ or the above spore culture filtrate gave slopes of 3.3.

E. adhaerens cells incubated in distilled H_2O with and without spores did not excrete L-alanine, or any other amino acids. The culture filtrates were tested both without concentration and after 10-fold concentration in a rotary vacuum evaporator. Detection was by paper chromatography with either isopropanol-acetic acid-H_2O (80:20:20 [vol/vol]) or ethanol-NH_4OH-H_2O (80:15:15 [vol/vol]). The limit of detection for L-alanine by chromatography was 10 μg/ml. In addition to the above, the culture filtrates did not cause development of light-green staining when they were incubated with *B. subtilis* spores.

E. adhaerens was not the only bacterium capable of inducing the light-green staining characteristic. A total of 42 bacteria was isolated from soil column slides that had been incubated with *B. subtilis* spores. Of these, four caused the production of 70 to 80% light-green-staining spores when they were tested as above with *B. subtilis* spores in distilled water (no germinants added). Also, as with *E. adhaerens*, the spores reverted to the dark-green staining characteristic with several days of additional incubation after assuming the light-green staining state. In addition, there was no reduction (<5%) in spore CFU. Of the other isolates tested, 21 were inactive because they did not survive the incubations in distilled water. Two of the four active isolates were gram-positive rods, one was a gram-negative pleomorphic rod, and the last, number 40, was a gram-negative coccoid rod. The cells of all four were larger than those of the short rods found with the light-green-staining spores on the soil column slides. Isolate number 40, however, was found to multiply by asymmetric budding in a manner similar to that described previously for *E. adhaerens* (3).

DISCUSSION

Our modified Schaeffer-Fulton stain allows the direct staining of bacterial endospores in soil smears, and the stained spores and related vegetative cells can be differentiated from other soil bacteria or inorganic or organic soil debris. This stain, however, also differentiates between inactive spores and spores undergoing the initial, reversible stages (13) of germination activation. Spores in these two phases stain dark green and light green, respectively. Spores further along the germination pathway lose phase brightness and heat resistance, and they stain light purple with this stain. These spores, theoretically at least, have completed germination. Outgrowth associated with germination stains dark purple. The use of continuously variable amplitude contrast microscopy (1) allows detection and visualization of nonstained spores in soil, but does not differentiate between dormant and activated spores.

The reversible, light-green staining characteristic was brought on by incubation of the spores in the presence of germinants, such as L-alanine and glucose, or by incubation with certain soil bacteria. Thus, the staining changes observed in soil when an indigenous *E. adhaerens*-like bacterium multiplied in response to the spores were duplicated when mixtures of pure spore cultures and *E. adhaerens* were incubated in distilled water, or when the spores were incubated with L-alanine and glucose (no other bacteria) in distilled water.

The percentage of spores staining light green and the incubation time required until light-green staining began was related to the relative ability of the germinant system to activate spores. The best activation system, L-alanine–glucose (11), was also the most successful in producing light-green-staining spores. When used in high concentrations, L-alanine–glucose mixtures induce spores to complete the germination process. In our study, however, the concentrations of L-alanine and glucose were kept at levels which insured that the spores would only become activated. When L-alanine and glucose were tested separately, L-alanine was the better producer of light-green-staining spores. Other workers have shown L-alanine to be a very effective spore germinant, especially with *B. subtilis* spores (6, 7). Other systems tested (glucose, EDTA, inosine and adenosine), but not necessarily reported, produced light-green-staining spores in direct proportion to their ability to activate *Bacillus* species spores (6, 11, 14, 15). Inosine, which is known to be ineffective as a germinant of *B. subtilis* spores (6), did not produce spores that would stain light green.

Incubation of the spores with *E. adhaerens* or with the germinants did not produce more than a minimal decrease in spore viability or increase in *E. adhaerens* CFU. This may be because whatever happened to the spore during its initial germination phase reversed itself with further incubation. Nevertheless, the initial, reversible phases of spore germination are known to release dipicolinic acid, calcium, and some amino acids into the environment (8, 10). Perhaps these are used by other bacteria, such as *E. adhaerens*, for promoting small amounts of growth or for maintaining viability of the cells. Use of the calcium, or possibly chelation of the calcium to cause spore activation, might be predicted from our results, however, because *E. adhaerens* did not promote light-green staining of the spores when incubated in Burk buffer. This is in agreement with the results of Keynan and Halvorson (8) who showed that the release of calcium was inhibited when the germination of spores was attempted in buffer. In addition, however, *E. adhaerens* was shown to have a strong growth requirement for calcium. Also, its response to culture filtrates of germinating spores was the same as its response to calcium. Thus it probably used any calcium that was released from the spores.

When additional exogenous nutrients were available, as in soil preenriched with alfalfa, there appeared to be some actual destruction of the spores. We did not attempt to duplicate these particular soil conditions with pure laboratory cultures. It is possible, however, that the extra nutrients of the enriched soil caused the spore already activated by *E. adhaerens* to proceed further along the germination pathway so that it became sensitive, as a germinated spore or vegetative cell, to attack by *E. adhaerens* or other soil bacteria or even by bacteriophage. However, an argument against the attack of *E. adhaerens* on the vegetative cell is that no attachment of *E. adhaerens* was seen. Also, other experiments (data not shown) indicate that *E. adhaerens* is not a predator of *B. subtilis* vegetative cells in pure cultures.

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