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Buoyant Density Heterogeneity in Spores of *Bacillus subtilis*: Biochemical and Physiological Basis

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The biochemical and physiological basis of density heterogeneity in Renografin of *Bacillus subtilis* W23 spores was determined by analysis of metals, macromolecules, and dipicolinic acid in the two density classes of the population. Germination rate and heat resistance were measured in both density classes. Atomic absorption spectrophotometry revealed that heavy spores (density = 1.335 g/ml) have 30% more calcium than light spores (density = 1.290 g/ml). Other metals found in greater amounts in heavy spores were manganese and potassium. However, light spores had more sodium than heavy spores. The amounts of carbohydrates, nucleic acids, and proteins were the same in both types of spores, but light spores contained more lipids, whereas heavy spores had 30% more dipicolinic acid than light spores. Calcium and lipid were excluded as causes of the heterogeneity in density in that alteration of their contents in spores did not detectably affect the density of these spores. Spores of two densities were genetically similar. Furthermore, light density spores arose earlier during sporulation than heavy spores as determined by releasing refractile forespores at various times during sporulation. We concluded that light spores represent an incomplete stage in development because they became heavy when reinoculated into spent sporulation medium. This must involve the additional accretion or synthesis of dipicolinic acid.

Buoyant density heterogeneity in populations of dormant bacterial spores has been previously described. Lewis, Snell, and Alderton (11) used a lead chelate to demonstrate three nonoverlapping bands of dormant *Bacillus megaterium* spores. The biochemical and physiological properties of these spores were not studied, however. Using Renografin, Tamir and Gilvarg (20) also demonstrated that dormant spores of *B. megaterium* were heterogenous with respect to buoyant density. Heterogeneity was not observed in a recent report on the separation of dormant from germinated spores (16) but, in this case, subfractionation of the broad band of dormant spores was not attempted. In their original description of density heterogeneity of spores on gradients of Renografin, Tamir and Gilvarg (20) suggested that the physiological basis for heterogeneity was the result of one of two possible processes. Light spores might be formed as a result of early release of spores before their maturation had been completed. Alternatively, light spores may be spores that were originally denser but have become lighter by germinating. This investigation presents evidence that light spores appear earlier in sporulation than heavy spores and become heavy if allowed to remain long enough in the medium. Thus, light spores may represent an immature stage of sporulation despite the fact that they are released from the sporangium.

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MATERIALS AND METHODS

Organisms. *Bacillus subtilis* W23 and a thymine, histidine auxotrophic mutant of *B. subtilis* W23 (BCW 503, a gift of James Copeland, Ohio State University, Columbus) were used in this study. The wild-type strain did not germinate well with common germinating agents so BCW 503 was used for germination studies.

Growth and sporulation conditions. Cultures were maintained as colonies on nutrient agar plates (Difco). Isolated colonies were inoculated into D medium (Difco nutrient broth, 8.0 g/liter; $MgSO_4 \cdot 7H_2O$, 3×10^{-2}M; KCl, 1.4×10^{-2}M; $MnCl_2$, 10^{-5}M; $FeSO_4$, 10^{-4}M; $CaCl_2$, 10^{-3}M) and shaken on a rotary shaker, at 30 C for 35 h, at which time free

spores were observed. Spores were washed five times with distilled water and stored at 4 C until used. Spores of both classes are stable against germination for at least one month when treated thus. Where indicated, spores were separated into density classes and lyophilized for storage. Spores of both classes are free from contaminating cell debris as visualized by phase-contrast and electron microscopy. The minimal medium used in the study using mutants was C medium (1) supplemented with $CaCl_2 \cdot 2H_2O$, 6.8×10^{-5} M; $MnSO_4 \cdot H_2O$, 3.6×10^{-5} M; $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 6.4×10^{-5} M, and L-alanine, 100 mM.

Separation of spores with different densities. Dormant spores were separated from germinated spores on continuous exponential gradients (1.400 to 1.140 g/ml) of Renografin (20). The peak fraction of dormant spores was collected by puncturing the bottom of the tube and collecting 10-drop fractions. The density of each fraction was determined from its refractive index by using a standard curve of refractive index versus pycnometric density for Renografin. Discontinuous gradients for the rapid purification of dormant spores of different densities were prepared by layering successively the following solutions of Renografin in SW27 centrifuge tubes: 1.340 g/ml, 1.330 g/ml, 1.240 g/ml, and 1.114 g/ml. Heavy spores (1.335 g/ml) band between the bottom two layers, light spores (1.290 g/ml) between the middle two layers, germinated spores band between the top two layers, and vegetative cells appear on the top layer. Conditions of centrifugation were: 27,000 rpm on an SW27 rotor, model L2-65B ultracentrifuge, at 20 C for 30 min. Pictures were taken of the centrifuge tubes and photographic negative images were traced with a Joyce-Loebl double beam recording microdensitometer (Mark IIIC).

Release of refractile forespores. To open sporangia with little damage to refractile forespores (RFS), a 5-ml sample of a sporulating culture was initially given two 15-s treatments with a Branson Sonifier at 90% maximum output. Lysozyme (0.1 ml of a 10 mg/ml solution) was added and allowed to act at room temperature for 30 s, followed immediately by another 15-s ultrasonic treatment. Phase-contrast microscopy showed that phase-bright refractile forespores had been released quantitatively from the sporangia.

Germination conditions. Spores were germinated without preheating in a complex medium (2) at room temperature in spectrophotometer cuvettes and the light scattered at 660 nm was recorded.

Heat resistance. Spore suspensions of each density type were diluted with water and placed in closed glass test tubes. These were immersed in a water bath for the indicated temperatures and times, cooled quickly, and plated in triplicate on nutrient agar plates containing 0.5% glucose. These were incubated at 30 C for 24 h and scored for numbers of survivors.

Nucleic acids. A 20-mg amount of spores of each density class was ground for 5 min with 40 mg of finely powdered sand in an agate mortar and pestle with polished surfaces. The ground spores and sand were reweighed and this weight was divided by three and taken as the weight of spores for calculations. The assumption was made that the loss of spores during grinding was proportional to the loss of sand. Enough 10% trichloroacetic acid was added to provide spore concentrations of 10 mg/ml for each assay. The slurry was heated at 90 C for 15 min and centrifuged at 6,000 rpm to rid the sample of interfering protein. Deoxyribonucleic acid (DNA) was assayed by the method of Burton (4) by using salmon sperm DNA (A grade, Calbiochem) as a standard. Ribonucleic acid (RNA) was assayed using orcinol by the method of Schneider (19) by using ribose (Calbiochem) as a standard. Calculations were made assuming that RNA is 40% ribose.

Carbohydrate, lipids, and protein. A 30-mg amount of lyophilized spores of each density class was ground with 60 mg of powdered sand as described for nucleic acids. Loss of spores and sand in the grinding process was compensated for as described above. Enough distilled water was added to provide spore concentrations of 10 mg/ml for each assay. Carbohydrates (non-amino sugars) were assayed by the phenol-H_2SO_4 method of Dubois et al. (7), by using glucose as standard. Lipids were extracted with chloroform-methanol (2:1) by the method of Bligh and Dyer (3). Lipids were estimated by the silver dichromate-H_2SO_4 method of Paul (14), by using stearic acid (octadecanoic) as a standard. Protein was estimated by the Biuret protein assay of Layne (10), by using bovine serum albumin (Schwarz-Mann) as a standard.

Dipicolinic acid. Dipicolinic acid (DPA) was measured by the method of Janssen et al. (9) by using DPA (Aldrich) as a standard.

Metal Analysis. Metals were analyzed with a Perkin-Elmer atomic absorption spectrophotometer 290B. The standards, $CaCl_2$, KCl, $Mg(CH_3COOH)_2$, and NaCl were purchased as 1,000 μg/ml (of cation) solutions from Haleco. Manganese was prepared from $MnCl_2 \cdot 4H_2O$ as a 1,000-μg/ml (of cation) stock solution.

Terminology. Heavy spores are dormant spores having an average buoyant density in Renografin of 1.335 g/ml. Light spores are dormant spores having an average buoyant density in Renografin of 1.290 g/ml. These values may apply only to *B. subtilis* and to this gradient solute. A density class is a term for either of these types of spores.

RESULTS

Number of density classes in the population. Previous reports of density heterogeneity in Renografin (16, 20) have failed to present evidence of the exact number of density classes in a population of dormant spores. Therefore, it was unknown whether the broad spore band observed in continuous gradients was formed by a density continuum, or from two or more overlapping classes of close but nonidentical density. To distinguish between these two possibilities, several fractions were taken

from both sides of the broad band (Fig. 1A). Each of these fractions was centrifuged on a separate continuous gradient. If the initial band had consisted of many overlapping density classes or a continuum of densities, each fraction would reband at approximately its own density. Conversely, if the initial band consisted of a small number of discrete density classes, only these few classes would be apparent upon recentrifugation. Figure 1B shows only two major classes of spores, although minor density differences within these major density classes appear to exist.

Discontinuous gradients. To effect a rapid separation of spores of these density classes, while providing a high degree of purity, a discontinuous gradient was constructed (Fig. 2A) as described in Materials and Methods. The

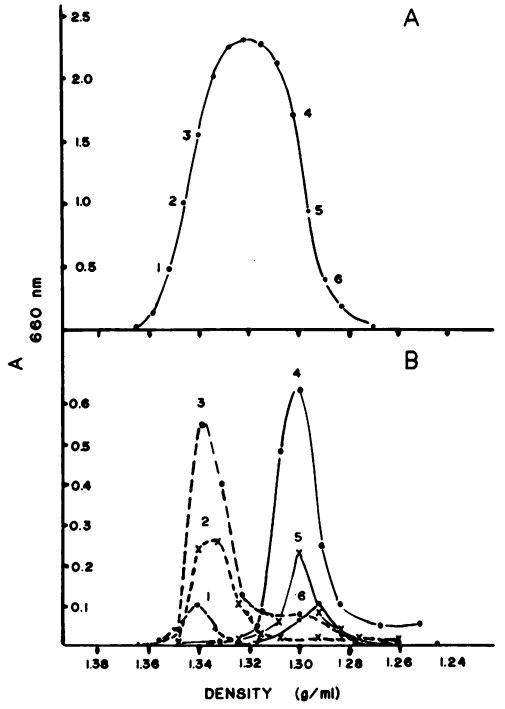

FIG. 1. *Continuous gradients of Renografin showing a broad band of B. subtilis W23 spores (A). Ten-drop fractions were collected and three sample fractions were taken from each side of the broad band. Each of these fractions was centrifuged on a separate continuous gradient (B). Two major bands of spores were resolved from these fractions, a heavy one at 1.335 g/ml and a light one at 1.290 g/ml. Fraction 3 is seen to consist of a small amount of the light class of spores, indicating that there are no major classes between these two.*

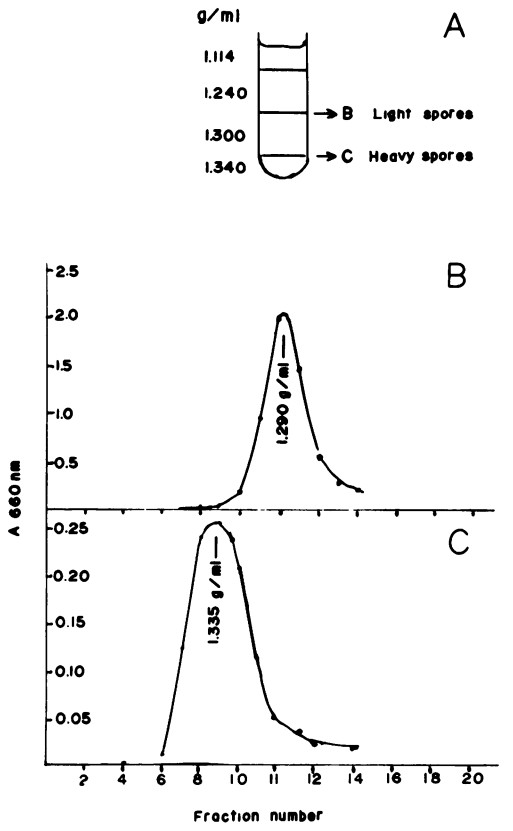

FIG. 2. *Discontinuous Renografin gradient used to separate heavy and light spores (A). Light (B) and heavy spores (C) were removed from the discontinuous gradient and centrifuged in continuous Renografin gradients to demonstrate homogeneity of these density classes after separation with the discontinuous gradient.*

ability of the discontinuous gradient to separate light from heavy spores is shown in Fig. 2. The two density classes were separated (Fig. 2A), and each density class was recentrifuged on a continuous gradient. Light (Fig. 2B) and heavy spores (Fig. 2C) each produced a single sharp homogenous peak with mean buoyant densities of 1.290 g/ml and 1.335 g/ml, respectively.

Heterogeneity is not an artifact of Renografin. Figure 2 also reveals that centrifugation does not cause one class of spores to give rise to the other. Light and heavy spores once separated (Fig. 2A) remain unchanged in their density upon recentrifugation in Renografin (Fig. 2B and C).

A discontinuous gradient prepared from an iodinated compound of different structure from Renografin, Conray (methyl glucamine iothala-

mate), using the same density layers as in the previous gradients of Renografin, yielded a mixed population of light, heavy, and phase-dark spores (Fig. 3) indicating that heterogeneity is not due to centrifugation through Renografin gradients.

Renografin is commercially available as a salt of methyl glucamine, and the commercial product also contains 0.32% sodium citrate and 0.04% disodium ethylenediamine tetraacetic acid (EDTA). The presence of citrate and EDTA was a cause of concern because they are chelating agents and might have reacted with bacterial spores by removing divalent cations. Sodium citrate and disodium EDTA were removed by precipitating and washing the iodinated component of Renografin with HCl. The precipitate was then mixed with equimolar methyl glucamine and adjusted to pH 7.0 with NaOH. Discontinuous gradients of acid-precipitated Renografin prepared of the same density layers as those described for the commercial preparation reveal heterogeneity as does the control (Fig. 3).

Heterogeneity is lost upon germination. It is observed in Fig. 2 and 3 that germinated or phase dark-spores have a much lower buoyant density than either light or heavy spores. From this it may be inferred that high density components are lost from spores during germination. The major components lost during germination are calcium, dipicolinic acid, hexosamine-peptide polymerase, and amino acids (15).

Purified light and heavy spores were germinated during the course of a 60-min incubation as described in Materials and Methods. These germinated spores were then centrifuged on separate continuous gradients (1.24 to 1.04 g/ml). The gradients were fractionated and the data were plotted in Fig. 4 as a function of density. The results show that heavy and light spores come to equilibrium at the same density position after germination, suggesting that the components responsible for density heterogeneity are lost during germination.

Heat activation has no effect on the density profile of a mixed population of *B. subtilis* spores. Thus, a heterogenous population heat-shocked at 60 C for 1 h and centrifuged on discontinuous gradients resulted in the usual profile of heavy and light spores.

Biochemical analysis. The major constituents of light and heavy spores were analyzed, and Table 1 shows the results. In terms of percent dry weight, there are no significant

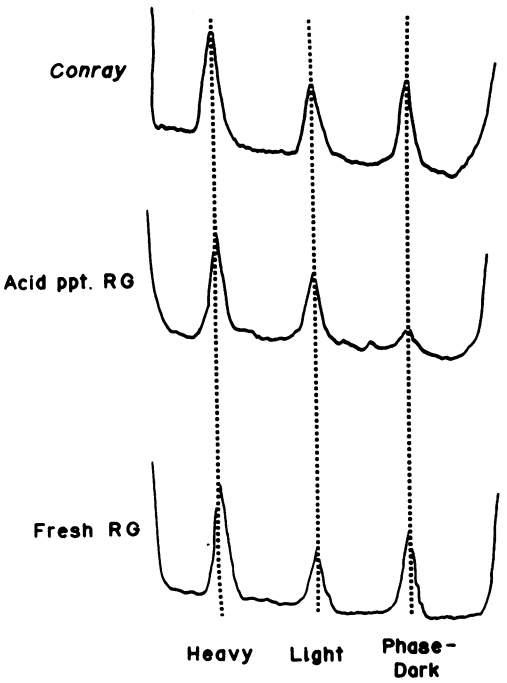

FIG. 3. *Microdensitometer tracings of photographic negatives of discontinuous gradient. Conray, acid-precipitated Renografin (acid ppt. RG), from which EDTA and sodium citrate have been removed, both show heterogenous density patterns for B. subtilis W23 spores as does Renografin (Fresh RG).*

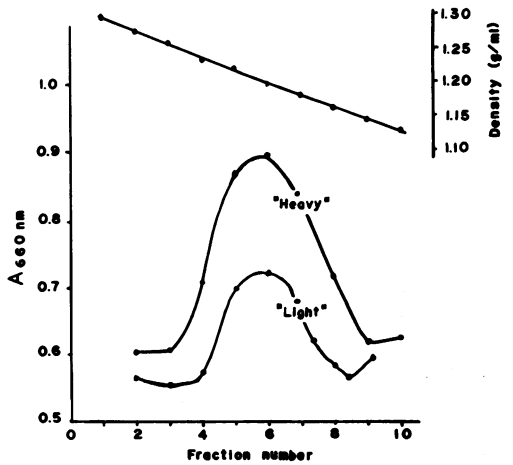

FIG. 4. *Continuous Renografin density gradients of germinated heavy and light spores with the gradient shown for a density reference. Germinated heavy and light spores have the same buoyant density (about 1.2 g/ml by this estimate).*

TABLE 1. *Analysis of macromolecules and DPA in light and heavy spores*

Compound	Dry wt (%)[a]	
	Light spores	Heavy spores
Protein	73 ± 7	80 ± 11
Carbohydrate	2.4 ± 0.2	2.6 ± 0.3
Lipid	2.0 ± 0.2	1.2 ± 0.4
RNA	4.5 ± 0.2	5.1 ± 0.6
DNA	1.0 ± 0.1	1.0 ± 0.3
DPA	7 ± 2	12 ± 2

[a] Mean of three determinations ± standard deviation. Total weight was determined gravimetrically with lyophilized spores.

differences in the contents of proteins, carbohydrates, or nucleic acids. Heavy and light spores, however, do vary in their lipids and DPA contents.

Since lipids are not lost by spores during germination, they probably are not a major cause of heterogeneity. To test the effect of changes of lipid content on the buoyant density of spores, light and heavy spores were separated and washed with chloroform methanol (2:1 vol/vol) under the same conditions used for extraction of lipids from ground spores. These treatments did not alter the density profile of light or heavy spores from that of untreated light or heavy spores. Light and heavy spores were also soaked in 1 mg of stearic acid per ml for 1 h at room temperature. Light spores are unaltered by this treatment and only a very small percentage of heavy spores are shifted to the light spore position (results not shown).

There is 30% more DPA in heavy spores than in light spores. This illustrates that DPA may be a major contributor to the buoyant density difference between the two types of spores.

Metal analysis. Five of the major metals found in spores were analyzed by atomic absorption spectrophotometry (Table 2). Heavy spores are found to have more calcium, manganese, and potassium than light spores. The sodium content of light spores is much in excess of that of heavy spores. The difference in calcium is of particular importance because it is the major metal in spores, is presumably chelated by DPA in vivo, and is implicated in the germination experiment (see above) as a possible cause of heterogeneity. Therefore, a series of experiments were performed to test the effect of extraction or addition of calcium on the buoyant density of light or heavy spores. A suspension of spores was adjusted to pH 4.0 with 0.1 N HNO_3 and incubated at room temperature for 1 h, a procedure which removes some, but not all of the calcium from spores (17, 18). Calcium may also be returned to spores thus depleted, by exposure to calcium acetate (50 mM, 60 C for 1 h) (17).

Table 3 shows that acid extract removes calcium from both light and heavy spores, as compared to the control. The control values in Table 3 are not meant to represent absolute values because of the method of determining weight. Nonetheless, results shown in this table are internally consistent because all treatments were done on spores from the control tubes. Note that acid-treated heavy spores have less calcium than control light spores. Calcium-acetate treatment causes a loss of calcium from light spores (see Ca^{2+}-treated, Table 3), but does not affect heavy spores. The loss of calcium from light spores is seen to be due to germina-

TABLE 2. *Metal analysis of light and heavy spores*

Metal	Dry wt (%)[a]	
	Light spores	Heavy spores
Calcium	1.9 ± 0.1	2.5 ± 0.3
Manganese	0.97 ± 0.07	1.18 ± 0.03
Magnesium	0.30 ± 0.04	0.32 ± 0.02
Potassium	0.043 ± 0.003	0.098 ± 0.005
Sodium	0.129 ± 0.004	0.0189 ± 0.0008

[a] Mean of three determinations ± standard deviation. Total weight was determined gravimetrically with lyophilized spores.

TABLE 3. *Analysis of calcium in light and heavy spores treated to add or remove calcium*

Condition	% Ca^{2+} by wt[a]
Control	
Light spores	1.5
Heavy spores	1.7
Acid-treated	
Light spores	0.2
Heavy spores	1.1
Ca^{2+}-treated	
Light spores	0.8
Heavy spores	1.7
Acid + Ca^{2+}-treated	
Light spores	1.2
Heavy spores	2.5

[a] Mean of three determinations. Total weight was determined optically from suspensions of spores by comparison of known values of suspensions of weighed lyophilized spores.

tion (Fig. 5). Calcium-acetate treatment of acid-treated spores demonstrated that calcium can be restored to acid-treated spores.

Figure 5 shows the effect of calcium extraction on the buoyant density of each type of spore. Neither extraction nor addition of calcium causes perceptible changes in buoyant density profiles. The only observable change is noted in Ca^{2+}-treated light spores. The decrease in the number of light spores indicates that this treatment caused some germination of light spores.

Germination. Light and heavy spores were germinated as described in Materials and Methods (Fig. 6). The lag period before germination and the rate of germination, as measured by percent decrease in turbidity (A660), is the same for both density classes. Total A660 drop for light and heavy spores was 40 and 45% of the initial readings, respectively.

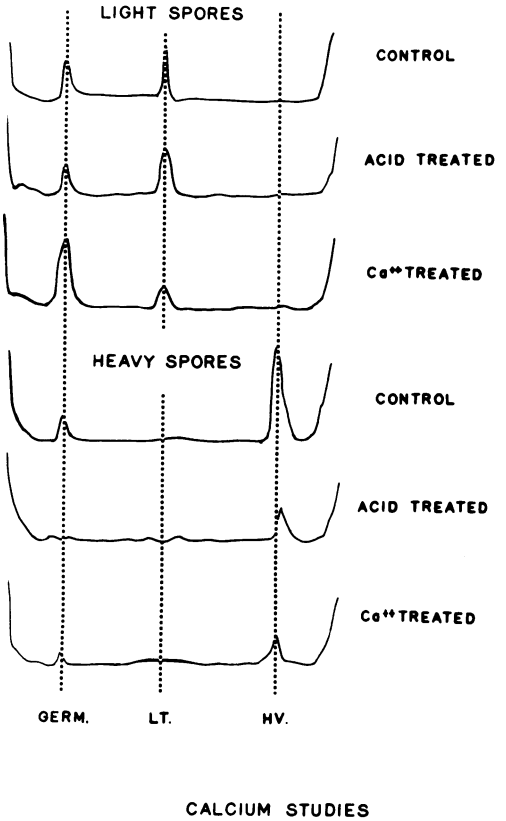

FIG. 5. *Microdensitometer tracings of photographic negatives of discontinuous Renografin gradients showing the effect of calcium extraction (acid treated) and addition (Ca^{2+} treated) on the buoyant density of light (top three) and heavy (bottom three) spores. Calcium extraction and addition is seen to have no preceptible effect on buoyant density with the exception of an increased germinated spore peak in Ca^{2+}-treated light spores. Germ., germinated spores; Lt., light spores; Hv., heavy spores.*

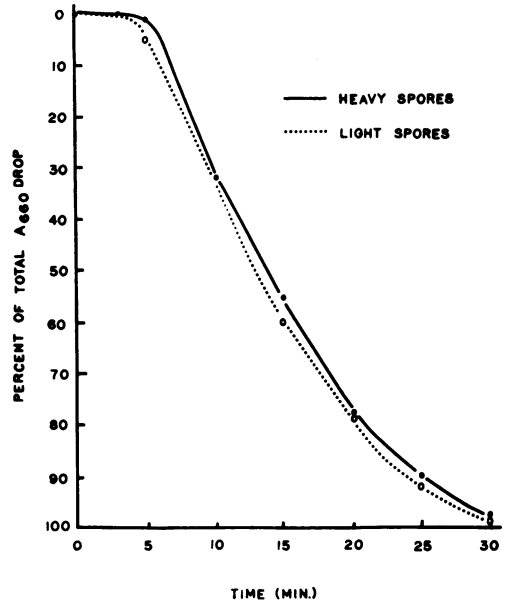

FIG. 6. *Germination of light and heavy spores as measured by total A660 decrease with respect to time. No significant difference is observed. Total absorbance decrease for light spores was 40% and for heavy spores was 45%.*

Heat resistance. Light and heavy spores show wide differences in heat resistance. The response of the two types of spores to temperatures below 80 C was about the same when they were heated for 30 min (data not shown). The decimal reduction time at 90 C ($D_{90°}$) of heavy spores was 17 min, whereas that of light spores was 8 min.

Genetic consideration. A major consideration in the study of population heterogeneity is genetic purity. Our first indication that genetic heterogeneity is not the cause of density heterogeneity in Renografin came from the finding that heterogeneity was apparent in clonal populations. Stock cultures were routinely streaked for purity and single colonies were used as inocula for the preparation of spores. We make the assumption that a colony represents a clone arising from a single cell. Phase-contrast microscopy reveals that *B. subtilis* W23 in these media is unicellular (not in chains) during later stages of growth. In every case (except as discussed below) spore populations have been

heterogenous with regard to density.

Another indication that density heterogeneity is not a genetic property is the finding that light spores give rise to heterogenous populations of spores as do heavy spores. Spores of each type were purified on discontinuous gradients, taking care to assure the purity of each class. Then these purified spores were streaked on nutrient agar plates and single colonies were isolated. Six isolates of each type were cultured for 36 h, harvested, washed, and centrifuged on discontinuous gradients. The spores for all six flasks cultured from each type of spore were heterogeneous, showing presence of both heavy and light spores in ratios similar to those of Fig. 3.

Furthermore, each class of spores retains the same auxotrophic requirements as the unfractionated population. Spores of a histidine and thymidine-requiring mutant of *B. subtilis* W23 (BCW 503) were separated into light and heavy density classes as described above, and these plus an unfractionated population were plated on complete medium (D medium) and minimal medium (C medium plus L-alanine). These results (not shown) also point out that density heterogeneity is not likely due to a contaminant.

Origin of heavy and light spores. The order of appearance of light and heavy spores was investigated (Fig. 7). At various times during sporulation RFS were released, as described in Materials and Methods, and their densities were determined. Figure 7A shows the population growth curve (A660) in relation to the increase in RFS during sporogenesis. The arrows indicate times at which samples were taken. Figure 7B through D shows the densitometric tracings of RFS banded in discontinuous gradients. Figure 7E shows the banding pattern of untreated free spores; the same pattern is produced when the population has been treated as if to release RFS. At 10 h the population is seen to consist predominantly of light spores with only a trace of heavy spores. When a sample was taken at 7 h and centrifuged after release of RFS no heavy spores and only a few light spores were seen (not shown). It is apparent from this that light spores arise before heavy spores.

Heterogeneity lost with time. Light and heavy spores could arise independently or in sequence. If the two types of spores arise in sequence, light spores giving rise to heavy spores, the population would consist entirely of heavy spores if allowed enough time to mature. A flask was inoculated with a log-phase culture of *B. subtilis* W23 and allowed to sporulate.

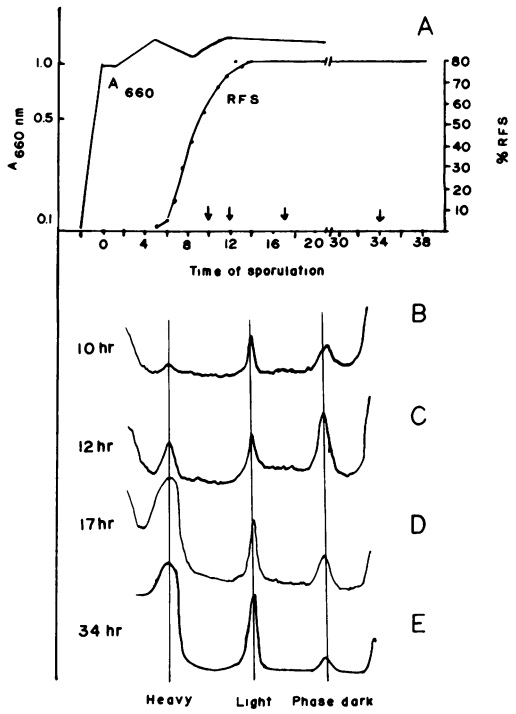

FIG. 7. *(A) Growth (A660) and sporulation (RFS) of B. subtilis W23. Arrows on the abscissa denote times at which samples were taken to release RFS. Microdensitometric tracings of photographic negatives of discontinuous Renografin gradients showing relative concentrations of heavy, light and phase-dark spores released from sporangia at 10 h (B), 12 h (C), 17 h (D), and 34 h (E) after the end of logarithmic growth.*

Spores were allowed to remain in the medium and samples were taken for centrifugation at 48, 60, and 84 h after inoculation. We found that light spores disappeared gradually from the population.

Light spores are incomplete heavy spores. Cells were cultured for 36 h, a time when the culture consists mainly of free spores. Free spores were then separated into light and heavy types, washed, and reinoculated into the same medium and incubated for 48 h longer. Figure 8A demonstrates the distribution of the population at 36 h (at the time of harvesting). Figures 8B, C, and D show the banding patterns of spore populations derived from light and heavy spores and a control (light and heavy together) after a total of 84 h of cultivation. It is evident that light spores will become heavy if allowed to remain in their same medium for 84 h. Heavy spores treated under the same conditions do not change their banding pattern. The culture of

light and heavy spores reinoculated together shows the same banding pattern as the 84-h culture inoculated with only heavy spores.

DISCUSSION

The number of major density classes comprising the density heterogeneity in Renografin has been determined to be two for *B. subtilis* W23 (Fig. 1). Evidence for this has not been published in earlier reports on heterogeneity in Renografin (16, 17). Using a lead chelate, Lewis, Snell, and Alderton (11) reported three nonoverlapping dormant spore classes in *B. megaterium* but only one band for spores of *B. subtilis* at 1.37 g/ml. It is possible that this gradient material would reveal heterogeneity in *B. subtilis* if this band were subfractionated.

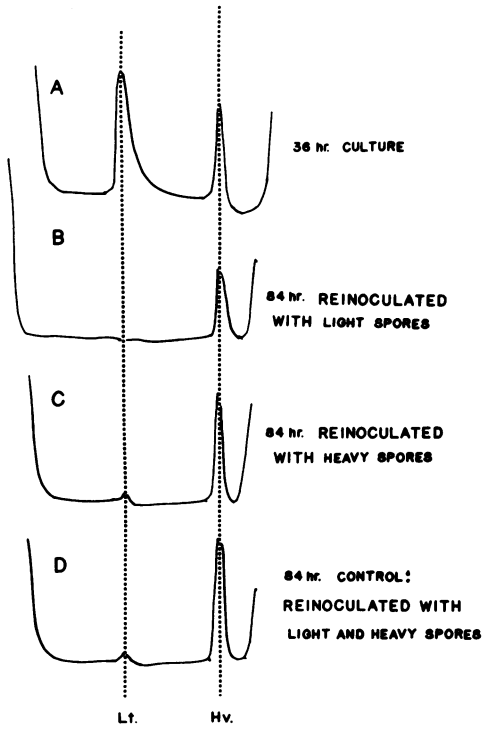

FIG. 8. *Microdensitometric tracings of photographic negatives of discontinuous Renografin gradients showing the reinoculation of heavy and light spores into spent medium. A heterogenous population of spores 36 h old was fractionated into heavy and light spores (A). Each spore type was reinoculated into the spent medium from which it was taken and allowed to continue incubation for a total of 84 h. The flask reinoculated with light spores (B) showed only heavy spores. The flasks reinoculated with heavy spores (C) and both light and heavy spores (D) showed a predominance of heavy spores.*

Renografin gradients appear not to contribute to density heterogeneity but it is not simple to demonstrate this. Separated heavy or light spores do not change to the other density upon recentrifugation (Fig. 2). Furthermore, other gradient materials, such as Conray, reveal heterogeneity as well (Fig. 3).

A significant finding in this work is that density heterogeneity is lost upon germination. From this result alone it can be predicted that the substance which determines density heterogeneity is one of those lost during germination, i.e., calcium, DPA, or hexosamine-peptide polymerase (15). A secondary prediction, relative to the suggestion that DPA is the chemical responsible for density heterogeneity, is that light and heavy spores should differ in heat resistance, since this property is known to be related to DPA content of the spore (12).

Biochemical analysis has substantiated both of the above predictions. No differences in the content of protein, DNA, RNA, and carbohydrates are found in each of the two types of spores (Table 1). Lipids were more predominant in light than heavy spores but experiments testing the effect of lipid extraction and addition on the buoyant density of spores did not reveal a clear relationship. Thus, lipids, found to be the basis of heterogeneity in spore mobility in an electrophoretic field and in ethylene oxide resistance (5), play little or no role in density heterogeneity.

DPA remains the most likely cause of density heterogeneity. This is consistent with the concomitant loss of DPA and heterogeneity during germination. These results agree with suggestions of Murrell (12) and Tamir and Gilvarg (20) that DPA or its chelated form with calcium is the likely determinant of density heterogeneity. Furthermore, Halvorson and Swanson (8) have demonstrated that DPA-less mutant spores have a lighter than normal buoyant density in Renografin.

Analysis of the major cations in spores revealed that heavy spores have larger amounts of calcium, manganese, and potassium than light ones, but have much less sodium (Table 2). However, reducing the calcium content of heavy spores below that of light ones did not cause a loss in detectable buoyant density (Fig. 5). Thus, we concluded that calcium, or other metals extracted by 0.1 N HNO_3, do not affect density heterogeneity significantly.

Light and heavy spores of *B. subtilus* W23 have been shown to behave similarly during

germination under the conditions used in these experiments (Fig. 6). This finding differs from a report on *B. megaterium* (20) which, however, does not present the germination data. It is possible that these discrepant results are due to strain differences. Both of the strains of *B. subtilis* W23 tested in this paper responded poorly to germination agents other than the complex germination medium used.

Spores of the two classes differ greatly in their response to heat. Light spores are much more heat sensitive than heavy ones. This was predictable because light spores have a relatively low DPA content and a high magnesium-calcium ratio compared to heavy spores. A positive relationship exists between these values and heat resistance (12, 13). Other workers (20; A. Evans, personal communication) have also reported differences in the heat resistance of heavy and light spores. The relationship between heat resistance and the specific gravity of bacterial spores has also been noted in earlier studies (6, 21).

Results discussed in this investigation indicate that density heterogeneity is not due to obvious genetic diversity in the populations. Three lines of evidence support this conclusion: first, spores cultured from a single colony (clonal populations) are heterogenous; second, spores which are cultured from purified light or heavy spores are heterogenous; and third, auxotrophic genetic markers are retained in both types of spores.

Results of experiments on the origin of density heterogeneity suggest that light spores are incomplete heavy spores. The process of formation of spores seems to involve a sequential change in density. Thus, light spores arise first and then give rise to heavy spores. This interpretation comes from the artificial release of RFS early in sporulation and the finding that spores of light density are predominant in the early stage (Fig. 7). Furthermore, the reinoculation of light or heavy spores into spent sporulation medium demonstrated that light spores may mature into heavy spores even after they are released from the sporangium (Fig. 8). This is a new interpretation of maturation because maturation seems to occur even after the spore is released from the sporangium. Since the major chemical difference between spores of the two densities seems to be DPA, it is reasonable to predict that the maturing process by which light spores become heavy ones involves the accretion or continued synthesis by the spore of DPA. There are only two density classes of spores rather than a continuum of densities in *B. subtilis* W23. Thus, it is possible that DPA deposition occurs in two stages, the first of which leads to light spores. In the second stage light spores would be converted to heavy spores.

In this investigation we have verified density homogeneity in bacterial spores and described the biochemical and physiological differences in heterogenous populations. These results suggest that future analysis of the biochemistry and physiology of bacterial spores should include steps to assure homogeneity of spore populations. We believe density heterogeneity offers unique possibilities for study of the heterogeneity of heat resistance in spore populations, not available in inter-species surveys, because these studies may be performed on genetically homogenous populations.

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