Protoplast Water Content of Bacterial Spores Determined by Buoyant Density Sedimentation†

JAMES A. LINDSAY,‡ TEOFILA C. BEAMAN, AND PHILIPP GERHARDT*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Received 6 March 1985/Accepted 23 May 1985

Protoplast wet densities (1.315 to 1.400 g/ml), determined by buoyant density sedimentation in Metrizamide gradients, were correlated inversely with the protoplast water contents (26.4 to 55.0 g of water/100 g of wet protoplast) of nine diverse types of pure lysozyme-sensitive dormant bacterial spores. The correlation equation provided a precise method for obtaining the protoplast water contents of other spore types with small impure samples and indicated that the average protoplast dry density was 1.460 g/ml.

The water content of protoplasts in situ within fully hydrated dormant spores has recently been quantified in lysozyme-sensitive spores that vary widely in heat resistance and morphology (2, 8). The extent of protoplast dehydration appears sufficient in itself to account for the low level of heat resistance in Bacillus megaterium spores but not for the greater resistance in other Bacillus species.

The investigation of dehydration as a main basis for bacterial spore resistance should be extended to extreme thermophiles, particularly Clostridium species, the spores of which are usually obtainable only in relatively small and impure amounts. The current permeability method for determining the water content of spore protoplasts requires decagrams of pure spores and multiple determinations for accuracy. However, the determination of apparent wet density by buoyant density sedimentation requires only milligrams of spores, separates different populations into density bands, and is very precise. If determined by use of a density medium such as Metrizamide (Nyegaard and Co., Oslo, Norway), which permeates a lysozyme-sensitive spore through the disrupted peripheral integument to the intact protoplast membrane (9), the resulting density value should reflect that of the protoplast and should be inversely correlated with the protoplast water content. In the work reported here, we established this correlation with nine types of pure lysozyme-sensitive dormant spores and thereby have provided a precise method for obtaining the protoplast water content of other spore types with small impure samples.

MATERIALS AND METHODS

Various types of clean lysozyme-sensitive dormant spores that varied widely in heat resistance were produced and prepared as described previously by Koshikawa et al. (5), Beaman et al. (2), and Nakashio and Gerhardt (8). B. macquariensis ATCC 23466, a sub-Antarctic psychrophilic species (7), was additionally obtained as strain B683 from the Division of Food Research, Commonwealth Scientific and Industrial Research Organization, North Ryde, New South Wales, Australia. It was grown on the sporulation medium (modified with 1.5% agar and without salts) of Warth (10) and was made lysozyme sensitive by treatment with 0.5% sodium dodecyl sulfate-0.1 M dithiothreitol in 0.1 M NaCl at pH 10. Clostridium perfringens 8-6, a mutant which produces lysozyme-sensitive spores with only a cortex-encased protoplast (3), was obtained as described previously (J. A. Lindsay, R. W. Sleigh, C. Chitgas, and J. D. Davenport, Eur. J. Biochem., in press).

The water content of the protoplasts in situ within the lysozyme-sensitive dormant spores was determined by differential permeability measurements with 3H-labeled water and 3C-labeled glucose by the method and with some of the results described previously (2, 8). The protoplast water content was additionally determined for spores of B. macquariensis and B. cereus (8). The latter spores were obtained anew from the original stock culture, and a slightly different protoplast water content (55.0%) and D100 value (2.87 min) were obtained than reported before (8).

Commercial gradient media which had chemical (dry) densities higher than the dry densities of the heaviest spore types were selected. Metrizamide is the trade name for 2-(3-acetamido-5'-N-methylacetamido-2,4,6-tri-iodobenzamido)-2-deoxy-β-glucose; it is nonionic and has a molecular weight of 789, a chemical density of 2.17 g/ml, high water activity and solubility, low osmolarity, and intermediate viscosity. Discontinuous gradients with density increments as small as 0.0025 g/ml were used. Confirming results were obtained with linear continuous gradients. Metrizamide solutions were prepared with double-distilled deionized sterile water on a weight basis; to obtain a solution of 1.575 g/ml (the maximum achievable), for example, 7.0 g of Metrizamide was slowly dissolved in 3 g of water at room temperature (caution: do not heat). The refractive indexes of the solutions were measured in a precision refractometer and converted to solution densities by use of a calibration curve. About 0.1 ml containing about 1 mg (dry weight) of spores (which had been equilibrated for 2 h in a Metrizamide solution with a density of 1.150 g/ml) was deposited on top of a 10 ml-gradient containing 10 1-ml layers of medium in a 13-ml polycarbonate tube. It was found unnecessary to remove gas from the spore suspension by evacuating before depositing on the gradient because the high centrifugal forces accomplished gas removal. The tube was centrifuged to equilibrium (usually 30 min) in a swinging bucket rotor at gravities suitable for each spore type (see the last column in Table 1 of reference 8; 6,000 × g was used for B. macquariensis spores). The apparent wet density of the spore type was determined by the position of the band at the interface above a medium layer, the true
density of which was predetermined. Maximum precision (<0.0025 g/ml) was obtained by using successively narrower gradients. Determinations were confirmed by duplication.

Nycodenz (Nyegaard and Co.) is the trade name for an iodinated compound similar to Metrizamide but improved by stability to heat, resistance to bacterial degradation, and absence of toxicity. Incremental discontinuous gradients with Nycodenz were prepared and used in the same way as with Metrizamide.

RESULTS AND DISCUSSION

Figure 1 shows the separation of different spore populations into density bands after buoyant density sedimentation (isodensity or isopycnic equilibrium sedimentation) of a mixture of lysozyme-sensitive \(\text{Lsz}^s\), lysozyme-resistant \(\text{Lsz}^r\), and germinated (Ger) spores of \textit{B. stearothermophilus} 7953 spores in an incremental discontinuous density gradient of Metrizamide. All three spore types were permeable to the relatively small (molecular weight, 789) Metrizamide molecule, but to different extents; consequently, each spore type sedimented into a separate density band. In the \text{Lsz}^r\) spore type, in which all structures were intact, Metrizamide permeated the exosporium and coat to the intact outer (pericortex) membrane; consequently, the apparent wet-density value (1.340 g/ml) reflects that of the dormant sporoplast. In the \text{Lsz}^s\) spore type, in which the coat-outer membrane complex was disrupted by treatment with sodium thioglycolate, Metrizamide permeated the exosporium, coat, outer membrane, cortex, and primordial cell wall to the intact inner (peritoplam) membrane; consequently, the apparent wet-density value (1.390 g/ml) reflects that of the dormant protoplast. In the Ger spore type (from either the \text{Lsz}^r\) or the \text{Lsz}^s\) type), in which the coat-outer membrane complex was disrupted, the cortex was digested, and the protoplast was demineralized and hydrated, Metrizamide permeated the integument to the intact outer membrane; consequently, the apparent wet-density value (1.240 g/ml) reflects that of the germinated protoplast. These explanations derive from spore permeability determinations with Metrizamide (9) and general permeability properties of bacterial spores for solutes of similar molecular size (1, 4).

Representative sedimentation results that were the same as those obtained with Metrizamide were obtained with density gradients of Nycodenz, a newer iodinated nonionic medium of similar molecular weight (821).

The true wet densities of entire \text{Lsz}^s\) spores and the water contents of their protoplasts are inversely correlated (8), and the apparent wet-density value obtained with a Metrizamide gradient reflects that of the protoplasts in \text{Lsz}^s\) spores, as explained above. Consequently, the protoplast wet densities obtained by this method should correlate inversely with the protoplast water contents obtained by the differential permeability method (2, 8) for diverse types of \text{Lsz}^s\) spores. Such a correlation was found to occur among nine types of pure \text{Lsz}^s\) dormant \textit{Bacillus} spores (Fig. 2). The correlation equation \(y = -0.0025x + 1.460\), where \(y\) is the protoplast wet density and \(x\) is the protoplast water content, provided a precise method for obtaining the protoplast water content of other spore types. Further advantages are that only milligram samples of spores are needed for buoyant density sedimentation and that they need not be pure, as the population of dormant \text{Lsz}^s\) spores sediments into a heavier density band separate from the lighter bands containing dormant \text{Lsz}^r\) spores, Ger spores, vegetative cells, and debris.

The linear correlation equation indicates that, when the protoplast water content is zero, the average protoplast density is 1.460 g/ml. This value represents the dry density of the protoplasts in situ within the dormant spores. A considerably higher value (1.7 g/ml) had been predicted by Lindsay et al. (6) from dry densities of protoplast constituents, mainly calcium dipicolinate, nucleic acid, protein, and carbohydrate. We have now recalculated this value to 1.462 g/ml by using more accurate values for the constituents.

The determination of the protoplast water content from the apparent wet density obtained by buoyant density sedimentation of \text{Lsz}^s\) spores in Metrizamide or Nycodenz should be useful for investigating the dehydration basis for heat resistance of species in which spores are not readily obtainable in large masses or in pure populations, particu-
larly *Clostridium* species. This future extension appears attainable, as diverse genetic and chemical methods are available for obtaining *Lsz* spores (8). This extension was exemplified with the coatless mutant and therefore *Lsz* spores of *C. perfringens* 8-6, for which an apparent wet-density value of 1.375 g/ml was obtained by buoyant density sedimentation in Metrizamide. This value agrees with that of 1.38 g/ml for the heaviest of three density bands obtained previously in this laboratory (9) and that of 1.375 g/ml obtained by Cassier and Ryter (3). The corresponding protoplast water content of *C. perfringens* 8-6 spores calculated from the correlation equation was 33.5%.

The apparent wet densities determined by buoyant density sedimentation in gradient solutions may not be exactly the same as true wet densities or water contents determined with gravimetric and volumetric measurements, although they can be correlated by the use of plots such as that in Fig. 2.

Metrizamide (or Nycodenz) molecules cannot penetrate into the solid polymeric matrix of the peripheral integument, and this would cause an underestimation of the true protoplast density. However, the probing molecules penetrate incompletely into the liquid water voids of ultrastructural solids in the peripheral integument because the probing molecules are larger in size than the water molecule, and this would cause an overestimation of the true protoplast density. The two limitations thus may neutralize each other.

**ACKNOWLEDGMENTS**

This work was supported by contract DAAG 29-83-K-0057 from the Biological Sciences Program of the U.S. Army Research Office. We thank Jody McCormack and Laurie Jankowiak for technical assistance.

**LITERATURE CITED**


**FIG. 2.** Protoplast wet density correlated by least-squares analysis with protoplast water contents of *Lsz* spores of *Bacillus* species identified by numbers as follows: 2, *B. stearothermophilus*; 4, *B. subtilis*; 8, *B. thuringiensis*; 10, 12, 13, and 14, *B. megaterium*; 20, *B. cereus*; 21, *B. macquariensis*. Identity numbers 2 to 14 correspond to the spore species and types used by Beaman et al. (2) and Nakashio and Gerhardt (8).