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Trehalose and Sucrose Protect Both Membranes and Proteins in Intact Bacteria during Drying

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The microorganisms *Escherichia coli* DH5α and *Bacillus thuringiensis* HD-1 show an increased tolerance to freeze-drying when dried in the presence of the disaccharides trehalose and sucrose. When the bacteria were dried with 100 mM trehalose, 70% of the *E. coli* and 57% of the *B. thuringiensis* organisms survived, compared with 56 and 44%, respectively, when they were dried with sucrose. Only 8% of the *E. coli* and 14% of the *B. thuringiensis* organisms survived drying without the sugars. Fourier transform infrared spectroscopy was used to investigate the role of membrane phase transitions in the survival of the organisms during drying and rehydration. Both *E. coli* and *B. thuringiensis* showed an increase of 30 to 40°C in the temperature of their phospholipid phase transition when dried without the sugars, while phase transition temperatures of those dried with the sugars remained near those of the hydrated cells. A Fourier transform infrared spectroscopy microscope made it possible to investigate the effects of drying on the protein structure in the intact cells. The amide II peak shifts from 1,543 cm^{-1} in the hydrated cells to about 1,533 cm^{-1} in the cells dried without sugar. There is no shift in the amide II peak when the cells are dried with trehalose or sucrose. We attribute the increased survival to the sugars' ability to lower the membrane phase transition temperature and to protect protein structure in the dry state. In addition to increasing the immediate survival of both species, the addition of trehalose protected the cells from the adverse effects of exposure to light and air while dry. *E. coli* dried with trehalose and exposed to light and air for 4 h had an increase in CFU of between 2,000 and 4,000 times the number obtained with *E. coli* dried without trehalose. *B. thuringiensis* showed an increase in CFU of 150% in samples dried with trehalose compared with samples dried without trehalose. The cells dried with sucrose did not show an increased tolerance to exposure following drying.

Freeze-drying is often used for preservation and storage of biological samples; however, it has some undesirable side effects, such as denaturation of sensitive proteins (7) and decreased viability for many cell types (29, 30). To prevent or reduce these adverse effects, protective substances such as skim milk, sucrose, glycerol, and dimethyl sulfoxide are commonly added to samples before freezing or freeze-drying (33). While the addition of solutes is known to increase the number of viable cells in a freeze-dried sample, viability remains below that of the initial culture (29), and the physical mechanism of their protective action remains to be established. Previous work has shown that nonreducing disaccharides such as sucrose and trehalose can protect liposomes, isolated biological membranes, and some intact cells from the adverse effects of freezing and drying (9, 10, 17, 28, 32). Liposomes dried and rehydrated without the addition of a disaccharide suffer imbibitional damage and leak their contents to the surrounding media, while those dried with a disaccharide retain their contents (13, 16). Vesicles of isolated lobster sarcoplasmic reticulum dried without a disaccharide suffer fusion and a total loss of Ca^{2+} transport activity. Vesicles from the same sarcoplasmic reticulum preparation dried with a disaccharide exhibit no adverse effects (11). Work with intact pollen from the cattail *Typha latifolia* has shown that sucrose plays a vital role in pollen's ability to tolerate drying and storage (12, 21–23). Pollen species having low levels of sucrose are more sensitive to desiccation than species having high levels (23).

Damage to biological systems resulting from freeze-drying can be attributed to two primary causes: changes in the physical state of membrane lipids and changes in the structure of sensitive proteins. Removal of hydrogen-bonded water from the headgroup region of phospholipid bilayers increases the headgroup packing (16) and forces the acyl chains together, increasing the probability of van der Waals interactions. As a result, the lipid may undergo a transition from liquid crystalline to gel phase (10). Upon rehydration, dry membranes, which are in gel phase at room temperature, undergo a transition from gel to liquid crystal phase. As the membranes pass through this phase transition there are regions with packing defects, making the membranes leaky (1). Adding a disaccharide such as trehalose before drying lowers the transition temperature (T_m) of the dry membranes by replacing the water between the lipid headgroups, preventing the phase transition and its accompanying leakage upon rehydration (10, 12).

In addition to lowering the T_m of membranes, trehalose and sucrose have been shown to preserve both structure and function of isolated proteins during drying (7–9). This ability to stabilize proteins during drying results from the disaccharides forming hydrogen bonds with the proteins when water is removed, thus preventing protein denaturation (4, 5).

On the basis of the results described above, it seems that the disaccharides trehalose and sucrose could be used to preserve intact cells during freeze-drying. If the disaccharide can be taken up by the cells so that it is present on both sides of the membrane and in contact with internal, cytosolic proteins, the cells should exhibit an increased tolerance to drying. We now

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show that drying in the presence of trehalose and sucrose increases the survival of bacterial samples and that this increase is likely to be due to the sugars' ability to lower the temperature of the membrane phase transition and to maintain protein structure in the dry state.

MATERIALS AND METHODS

Organisms and growth conditions. The microorganisms used in this study were *Escherichia coli* DH5α and *Bacillus thuringiensis* HD-1 (ATCC 39756). The organisms were grown in 100 ml of Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) at 30°C with shaking at 200 rpm to early stationary phase (18 h for *E. coli* and approximately 26 h for *B. thuringiensis*). Cell densities were adjusted to an optical density of 1.8 (at 600 nm), which corresponded to a population of about 3.10×10^9 CFU/ml. The cells were washed twice with 50 ml of cold, sterile 20 mM phosphate buffer (pH 7.5) and suspended in sterile distilled water or a 100 mM trehalose or sucrose solution. Colony counts were made on LB agar after 48 h at 30°C.

Survival. Aliquots of cells prepared as described above (200 μl each) were placed in Eppendorf tubes and frozen in liquid nitrogen. The frozen samples were dried on a freeze-dryer (Super Modulo, Edwards High Vacuum International, Crawley, Sussex, England) for 24 h to a final pressure of 10 to 20 μm Hg (1.3 to 2.7 Pa). Immediately after drying, samples were rehydrated with 200 μl of 22°C distilled H_2O, diluted in 20 mM phosphate buffer (pH 7.2), and plated in triplicate on LB agar. Three replicates were performed for each condition, and survival was calculated as a percentage of non-freeze-dried colony counts.

Spectroscopic investigations. (i) Determination of phase transitions. Lipid phase transitions were measured by using Fourier transform infrared (FTIR) spectroscopy following methods described previously (14). Briefly, the samples were sandwiched between two CaF_2 windows and loaded into a temperature-controlled holder. The holder was then loaded into a Perkin-Elmer series 1750 FTIR spectrometer controlled by a personal computer running Perkin-Elmer's IRDM software (version 3.3). The samples were rapidly cooled to −15°C and subsequently warmed as spectra were recorded. Twenty scans were averaged at each temperature point. Spectra were taken every 2 to 3°C, and recording scans over the entire temperature range took no longer than 2 h. Dry samples were removed from the lyophilizer and transferred under a vacuum to a glove box flushed with dry air (dew point, −100°C) before and during the loading process. The relative humidity within the box remained between 0 and 6% throughout the loading procedure. Silicone grease was used to seal the windows before they were removed from the dry box to prevent the possibility of partial rehydration of the sample during scanning. The windows were washed in warm chloroform between samples.

(ii) Determination of protein stability. Effects of drying on protein structure were determined by using a Perkin-Elmer FTIR spectrometry microscope attached to a model 1620 FTIR spectrometer. Samples were loaded between CaF_2 windows as described above. All scans were done at room temperature (approximately 22°C), and the microscope sample chamber was continuously flushed with dry air. Each spectrum was obtained by averaging 64 scans at a 4 cm^{-1} resolution. A background scan of clean CaF_2 windows was automatically subtracted, and the resulting spectra were smoothed with Perkin-Elmer's interactive smoothing routine.

Sugar accumulation. To test whether trehalose and sucrose are able to gain access to the inside of intact cells, *B. thuringiensis* was grown to stationary phase and washed as described above. The final pellet was resuspended in uniformly labeled 100 mM [^{14}C]trehalose (prepared as described in reference 35) at specific temperatures in the range of the hydrated phase transition (2.5 to 25°C). The cells were then filtered on a 0.22-μm-pore-size filter in a Millipore filter apparatus and washed with 2 ml of unlabeled 100 mM trehalose at 22°C. The filters were placed in scintillation vials with Ready-Saf scintillation cocktail (Beckman Instruments Inc., Fullerton, Calif.) and counted on a Beckman LS-5 scintillation counter. Cells incubated at room temperature for 2 min in the presence of the labeled trehalose served as controls.

Exposure experiments. (i) Freeze-drying. Aliquots (1 ml each) of cell suspensions were frozen in 10-ml glass ampules (Wheaton Scientific, Millville, N.J.) by dipping in a dry-ice–methanol mixture. The frozen cultures were kept at −80°C until dried with a freeze-drier (Virtis Co. Inc., Gardiner, N.Y.) at room temperature (22°C) to a final pressure of 1 to 10 μm Hg (0.13 to 1.3 Pa). Directly after the completion of the drying process the dried cultures were exposed to air for different periods in an environmental chamber (model 3980; Forma Scientific Inc., Marrietta, Ohio) at 20°C and 60% relative humidity. Light exposure consisted of exposing dried cultures, in their ampules, to light from a Lucalox LU-1000 BU lamp (General Electric, Cleveland, Ohio) at an intensity of 9×10^{-3} W/cm^2. Cultures not exposed to light were wrapped in aluminum foil prior to exposure to air. For each combination of exposure factors, three replicates were used.

(ii) Survival determination. At exposure intervals of 0, 0.5, 1, 2, 3, and 4 h, culture-containing ampules were reconstituted by adding 1 ml of 20°C distilled water and mixing on a vortex mixer. Solutions of the reconstituted cultures were diluted 10-fold in phosphate buffer (0.1 M, pH 7.2) and plated in triplicate on LB agar within 15 min. Plating was carried out with a spiral plater (model D; Spiral Systems Inc., Cincinnati, Ohio). The number of CFU was determined after 48 h of incubation at 30°C.

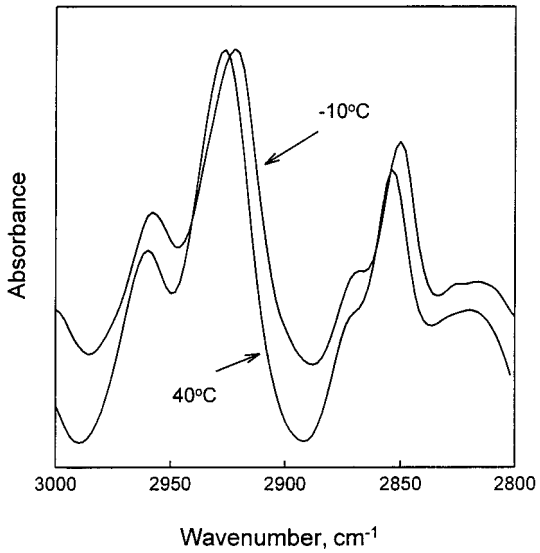

FIG. 1. Representative FTIR spectra of hydrated *E. coli* at two temperatures, showing the shift in vibrational frequency (wave number) of the CH_2 peaks which occurs with a change in temperature.

(iii) Data analysis and evaluation. Survival was calculated as the percentage of zero time colony counts for all timed exposures to air and light or dark, and a regression of length of exposure to percent survival, including both linear and quadratic terms, was carried out with SAS, version 6.03 (SAS Institute, Inc., Cary, N.C.). The rates of change of log CFU over time, as measured by the coefficient of time in the regression model for different strains, were statistically compared. *P* values of 0.05 or less were considered statistically significant.

RESULTS

Membrane phase transition temperatures. The T_m of the phase transition was determined by measuring the wave number of the symmetric CH_2 absorbance peak at wave numbers around 2,850 (Fig. 1) at various temperatures and then plotting the wave number against temperature. This method has been used successfully to measure the T_ms of artificial liposomes (16), intact pollen (12), yeast cells (28), and sperm (14). Drying cells of either species caused an increase in the T_m compared with that of the hydrated cells (Fig. 2 and 3). *E. coli* had a dry T_m of 50°C, 40°C higher than its hydrated T_m of 10°C (Fig. 2). Addition of a sugar to the cells before freeze-drying prevented this increase, maintaining the T_m of the dry samples near that of the hydrated ones. The results for *B. thuringiensis* were even more striking, with the sugars lowering the T_m of the dry cells from 42°C to around 5°C (Fig. 3). There appears to be little difference between trehalose and sucrose in terms of their ability to prevent the rise in T_m when the cells are dried.

Protein structure in freeze-dried cells. The effects of drying on proteins can be monitored by observing the frequency of the amide I and amide II peaks in the region at wave numbers between 1,500 and 1,700 (Fig. 4). Amide I is the carbonyl stretch, which is of use in assigning secondary structure, while amide II is composed primarily of N-H bending and stretching modes (37). The amide II band has been used before to monitor the effects of drying on proteins (5) and was used here to investigate the effects of freeze-drying on proteins in *E. coli* and *B. thuringiensis*.

In both organisms freeze-drying caused the amide II peak to shift down in frequency compared with the peak in the hy-

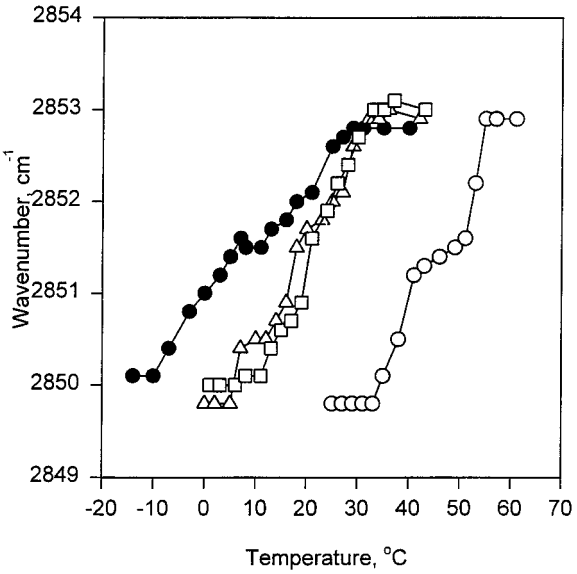

FIG. 2. Vibrational frequencies for the CH_2 symmetric stretch from hydrated *E. coli* (●), *E. coli* dried without sugar (○), and *E. coli* dried in the presence of trehalose (△) and sucrose (□) as a function of temperature. The temperature of the phase transition is at the center of each curve.

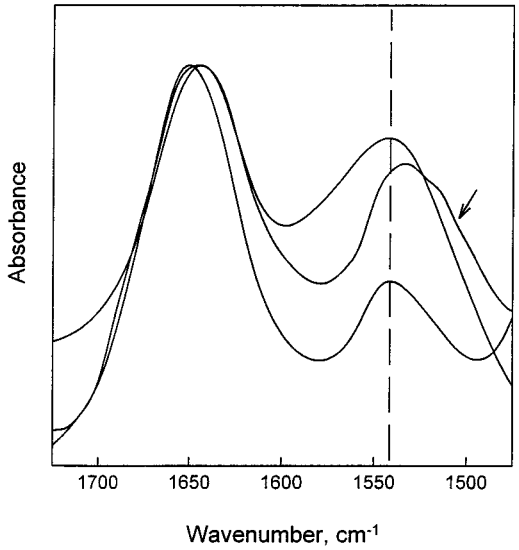

FIG. 4. Vibrational frequencies for the amide I and amide II regions from *E. coli* showing the shift of the amide II band when the cells are dried without trehalose or sucrose and that no shift occurs when the cells are dried with the sugars. The arrow indicates the spectrum for the sample dried without sugar. The vertical dashed line indicates the center of the amide II band in hydrated cells.

drated spectra. In *E. coli* the peak shifted from a wave number of 1,543 to 1,533 with drying, while in *B. thuringiensis* the amide II peak moved from a wave number of 1,546 to 1,533 (Fig. 4 and 5). This decrease in vibrational frequency represents a change in protein structure in the freeze-dried cells which may account for the decreased viability seen in these samples. Drying the cells with trehalose or sucrose prevented this change in protein structure.

Sugar access to the cytosol. For trehalose or sucrose to protect cytosolic proteins during drying they must have access to the inside of the cells. Figure 6 shows that when cells are forced through their phase transition, in this case by cooling, trehalose can flow down its concentration gradient into the cell. Cells resuspended in 100 mM trehalose at 20°C and above took up 0.43 μmol/mg (dry weight) of cells, while cells resuspended at temperatures below that of the start of the phase transition had a higher internal trehalose concentration (Fig. 6). Similar results indicate that sucrose also enters the cells as they start into their phase transition (27).

Survival of freeze-dried *E. coli* and *B. thuringiensis* strains. Cells dried with a sugar had substantially higher survival rates

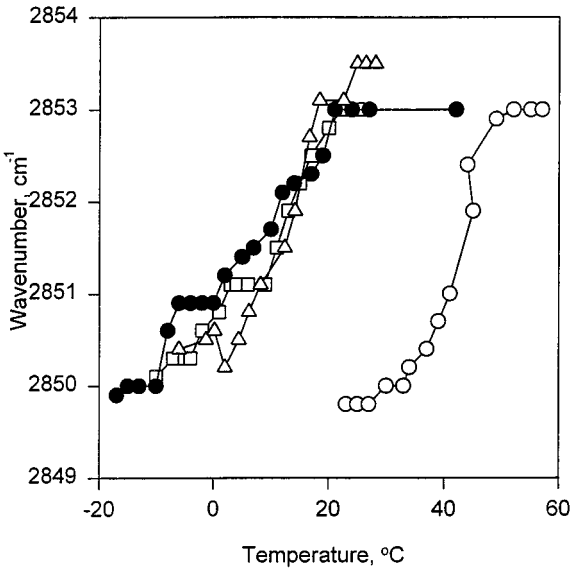

FIG. 3. Vibrational frequencies for the CH_2 symmetric stretch from hydrated *B. thuringiensis* (●), *B. thuringiensis* dried without sugar (○), and *B. thuringiensis* dried in the presence of trehalose (△) and sucrose (□) as a function of temperature.

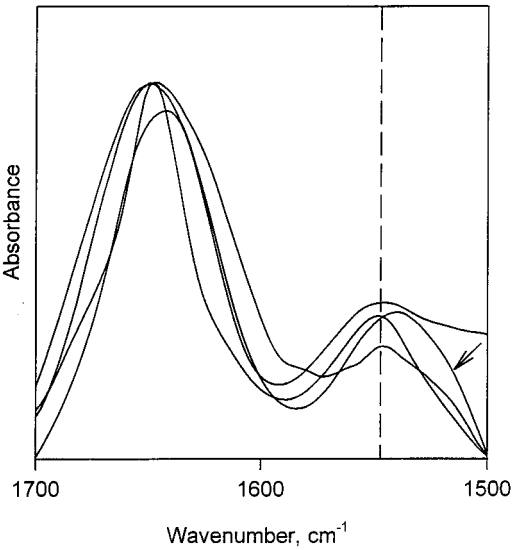

FIG. 5. Vibrational frequencies for the amide I and amide II regions from *B. thuringiensis* showing the shift of the amide II band when the cells are dried without trehalose or sucrose and that no shift occurs when the cells are dried with the sugars. The arrow indicates the spectrum of the sample dried without sugar. The vertical dashed line indicates the center of the amide II band in hydrated cells.

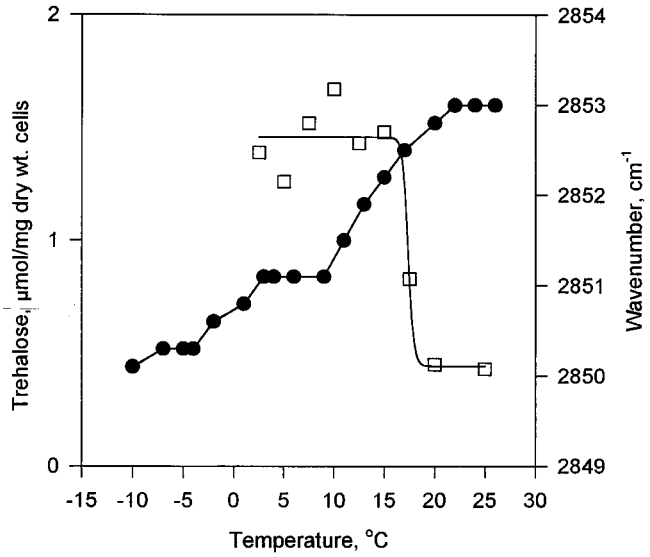

FIG. 6. Trehalose content (□) and vibrational frequencies of the symmetric CH_2 stretch (●) in hydrated *B. thuringiensis* as a function of temperature.

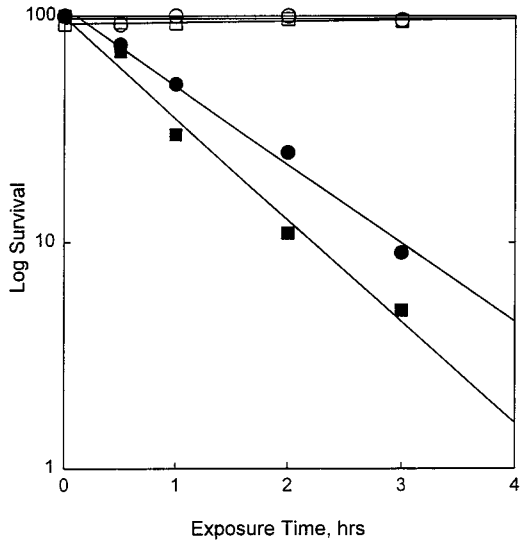

FIG. 8. Survival of *B. thuringiensis* dried with trehalose (○ and □) and without trehalose (● and ■) following exposure to light (● and ○) and dark (■ and □) as a function of time of exposure. All samples were exposed to air at 60% relative humidity during the light and dark treatments. The rate of change of log CFU over time is significantly different ($P \leq 0.05$) between the cells dried with trehalose and those dried without trehalose but not between the light and dark exposure when trehalose is present.

than cells dried in water alone (Fig. 7). *E. coli* dried with trehalose had 69.8% survival, compared with only 8.1% for *E. coli* dried without the sugar. Drying in the presence of sucrose increased survival to 56.2%. Similarly, *B. thuringiensis* dried with the sugars had higher survival rates than samples dried without the sugars: 56.7% when dried with trehalose, 43.9% when dried with sucrose, and 14.3% when dried in water.

Exposure of freeze-dried samples. Previous work has shown that *E. coli* dried with 100 mM trehalose is significantly more tolerant to exposure to light, humidity, and air than samples dried without the sugar (24). *B. thuringiensis* shows a similarly significant increase in survival following exposure when dried with trehalose (Fig. 8). The number of CFU in the trehalose-treated samples was around 150% the number in nontreated cultures. Practically no decrease of viability was observed in trehalose-treated cells during 3 h of exposure at 63% relative humidity and 21°C. While trehalose had a marked effect on the tolerance of freeze-dried *E. coli* and *B. thuringiensis* to exposure stresses, sucrose had no measurable effect (27).

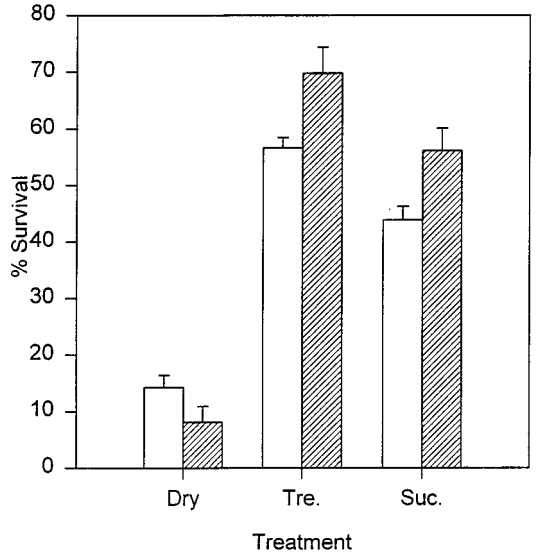

FIG. 7. Survival of *B. thuringiensis* (open bars) and *E. coli* (hatched bars) following freeze-drying with or without sugars. Cultures dried with the sugars were resuspended in 100 mM trehalose (Tre.) or 100 mM sucrose (Suc.) prior to freeze-drying. The values are averages of three determinations, and the error bars show standard errors.

DISCUSSION

Trehalose, phase transitions, and survival. While the importance of adding solutes to samples prior to freeze-drying is well-known (33), the mechanism of their action has remained unclear. This work proposes two possible requirements for these solutes (protecting membrane integrity and protecting protein structure during drying and rehydration) and demonstrates how the disaccharides trehalose and sucrose fulfill both these roles. To understand how the sugars could protect cell membranes it is necessary to understand what happens to membranes during drying and during rehydration. As water is removed from lipid bilayers the headgroups are brought closer together, resulting in an increase of van der Waals interactions between the acyl chains (16, 17). The increase in attraction between the acyl chains forces the dry bilayer into the gel phase at room temperature, which could, it has been proposed (10), lead to phase separation of membrane components. When the dry lipid is rehydrated it undergoes a phase transition from the gel back to the liquid crystalline phase, during which leakage may occur (10). Drying with trehalose or sucrose can prevent the damaging effects of drying and rehydration on membranes by maintaining the lipid in the liquid crystalline phase at room temperature, even when dry (10). It has been proposed that the sugars depress the phase transition in dry phospholipids by hydrogen bonding to the polar headgroup (9–11, 16), an idea known as the water replacement hypothesis (10). This suggestion has been called into question recently (25).

In addition to a transition from the liquid crystal to the gel phase, certain phospholipids can undergo a transition from

liquid crystal to hexagonal II phase as water is removed (15, 18). Hexagonal II phase is produced by an increased interaction between adjacent phospholipid headgroups as the separating water is removed. This type of transition is especially common in membranes high in phosphatidylethanolamine, such as the inner membrane of *E. coli*, and may play a role in the mortality seen in the cells dried without sugars. While a transition from the liquid crystal phase to gel phase differs from a transition to hexagonal II phase, the overall result for cells is the same. As the membranes pass from one phase to another membrane defects form, resulting in a loss of membrane integrity (15). There is no doubt that formation of nonbilayer phases is detrimental to membrane integrity (15). However, we doubt that hexagonal phase is involved in this particular instance, for the following reasons. Increased temperature favors formation of hexagonal phase (15), probably by increasing the volume occupied by the hydrocarbon chains (18). Thus, increased temperature would decrease survival if the cause of damage were formation of hexagonal phase. But survival is improved when the bacteria are hydrated at increased temperatures (27)—the opposite result from that predicted. We conclude that formation of hexagonal phase is not likely to be involved here.

When dried without a sugar, both *E. coli* and *B. thuringiensis* have a T_m above room temperature (Fig. 2 and 3), and rehydrating these cells with water at room temperature will cause them to undergo a thermotropic phase transition. Such transitions have recently been shown to account for the high mortality rate of dry baker's yeast, *Saccharomyces cerevisiae*, rehydrated below 38 to 40°C (28), and it may well play a part in the low viability seen in the bacteria dried without the sugars. Drying in the presence of trehalose or sucrose, however, lowers the T_m of the dry cells to below room temperature so that when rehydrated at room temperature they do not suffer a phase transition.

That trehalose and sucrose are capable of protecting both gram-negative and gram-positive bacteria is evident from the survival of each strain following freeze-drying with and without the sugar (Fig. 7). For sugars to protect a bilayer they must be on both sides of the membrane (16). Although both *E. coli* and *B. thuringiensis* transport trehalose and sucrose (27), the amount of time before drying makes it unlikely that they could transport enough to be effective in protecting their membranes. However, when the cells start to enter the phase transition the membrane becomes leaky and the sugar flows down its concentration gradient and into the cells (Fig. 6). Because of the short time before freezing and drying and the large amount of sugar entering the cell, the amount lost to metabolism is extremely small (27). Thus, these cells rapidly accumulate trehalose in the cytosol and so are protected during drying.

Sugars and protein structures. Although important, preventing a membrane phase transition would have little effect on the viability of freeze-dried cells if cytosolic or integral membrane proteins were irreversibly damaged during drying. By following the amide II band at wave numbers around 1,550 it is possible to monitor the general state of proteins (5, 34), and this is the method employed here to detect protein changes within the bacterial cells as a result of drying (Fig. 4 and 5). The scans collected represent an average for all the proteins in the cell and, while not providing information about specific proteins, can provide a good indication of the average condition of all proteins within the cell (34). In addition to the general information obtained from the amide II band it is possible to determine in-depth information about protein secondary structure from the amide I band at a wave number of 1,650 (2, 20, 31, 36). Amide I data were collected and analyzed for *B. thuringiensis* dried with and without trehalose and sucrose and will be reported in a separate publication (26).

The shift of the amide II band to lower wave numbers in the bacteria dried without sugar indicates a change in protein conformation in these cells (Fig. 4 and 5). When either trehalose or sucrose is present there is no change in the amide II band compared with that in the hydrated samples. Similar results have been reported for isolated lysozyme and other proteins when dried with and without trehalose (5) and indicate that the sugars maintain the dry proteins in their hydrated conformations. This ability to maintain proteins in a conformation similar to that of the hydrated protein, perhaps by binding to the hydrophilic domains of the proteins and preventing inter- and intraprotein hydrogen bonding during drying and rehydration (3, 6), further explains the ability of trehalose and sucrose to protect intact bacteria during freeze-drying.

Sugars and surviving exposure while dry. Trehalose has been shown to preserve viability in dry *E. coli* cultures even after 3 h of exposure to light and air with 60% relative humidity (24). Similarly, trehalose preserves dry *B. thuringiensis* cultures following exposures to light and air (Fig. 8). The ability to preserve dry cells for long periods of exposure may result from the sugar's ability to protect proteins in the dry state, but this idea is not supported by the finding that sucrose does not protect the dry cells. Both sugars protect *E. coli* and *B. thuringiensis* when the bacteria are rehydrated immediately after drying (Fig. 7), but only trehalose exhibits the ability to protect during exposure after drying. We are investigating two potential explanations for this intriguing difference between trehalose and sucrose. (i) Trehalose could be a more effective antioxidant than sucrose. We doubt that this is the case, but we are investigating this possibility nevertheless. (ii) Previous studies have established the importance of maintaining dry biological materials in the vitrified state during storage. If, for example, dry liposomes are heated above the glass transition temperature (T_g) for the stabilizing sugar, they fuse and leak their contents to the medium (13). Green and Angell (19) reported that trehalose has an anomalously high T_g, and we have recently obtained evidence that it is even higher than Green and Angell reported. Thus, it is possible that when the bacteria are exposed to moist air (which depresses T_g), the samples containing trehalose are maintained in the vitrified state while those with sucrose are not. We are investigating this possibility using liposomes as models.

Conclusions. The data presented here show that addition of trehalose or sucrose to samples of *E. coli* and *B. thuringiensis* before freeze-drying can increase the overall viability of the samples. This increased tolerance to drying appears to result from the sugars' ability to lower the temperature of the dry membrane phase transition and maintain general protein structure in the dry state. It seems only reasonable that any substance which was to increase the tolerance of organisms to drying would need to protect both the membranes and the proteins, and the disaccharides trehalose and sucrose meet both of these requirements.

ACKNOWLEDGMENT

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