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Small, Acid-Soluble Proteins Bound to DNA Protect *Bacillus subtilis* Spores from Being Killed by Freeze-Drying

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Wild-type spores of *Bacillus subtilis* were resistant to eight cycles of freeze-drying, whereas about 90% of spores lacking the two major DNA-binding proteins (small, acid-soluble proteins α and β) were killed by three to four cycles of freeze-dryings, with significant mutagenesis and DNA damage accompanying the killing. This role for α/β-type small, acid-soluble proteins in spore resistance to freeze-drying may be important in spore survival in the environment.

Dormant spores of *Bacillus* species are much more resistant than their growing counterparts to a variety of treatments, including heat, UV radiation, and chemicals such as hydrogen peroxide (6–8, 13). While many factors are involved in spore resistance to heat and hydrogen peroxide (6, 11, 13), one common factor is the saturation of spore DNA with a group of α/β-type small, acid-soluble proteins (α/β-type SASP) (11, 13). These proteins are made in the forespore late in sporulation in amounts sufficient to completely cover the spore chromosome but are degraded in the first minutes of spore germination (12, 13). *B. subtilis* spores lacking the two major α/β-type SASP (termed $\alpha^-\beta^-$ spores) are significantly more heat and hydrogen peroxide sensitive than are wild-type spores (5, 11). Studies both in vivo and in vitro have shown that binding of α/β-type SASP to DNA greatly retards heat-induced DNA depurination as well as DNA strand cleavage by hydrogen peroxide (5, 7, 12). In addition to their significant role in heat and hydrogen peroxide resistance of spores, α/β-type SASP are the major, if not sole, factor in spore UV resistance, exerting their effect by causing a change in the spore DNA's UV photochemistry (13). Given the central role that these proteins play in providing DNA protection against UV radiation, heat, and hydrogen peroxide (and thus spore resistance to these treatments), it would not be surprising if α/β-type SASP were important factors in spore resistance to other treatments. Indeed, in the course of sending spores from the United States to the United Kingdom, significant decreases in the viability of $\alpha^-\beta^-$ spores (but not wild-type spores) were observed when the spores were shipped after freeze-drying. Consequently, we undertook to study this phenomenon in more detail.

The "wild-type" *B. subtilis* strain used in this work was a derivative of strain 168 carrying plasmid pUB110, which provides kanamycin resistance. Vegetative cells of this strain were prepared by growth at 37°C to the late log phase (optical density at 600 nm [OD_{600}] of 1.0 in an LKB Ultrospec II spectrophotometer) in 2× YT medium (11) plus kanamycin (10 μg/ml). An aliquot of the culture (3 ml) was centrifuged in a microcentrifuge, washed once with sterile saline phosphate (SP) (25 mM potassium phosphate [pH 7.0], 0.1 M NaCl), and suspended in 1 ml of SP. Aliquots of three appropriate dilutions in SP were spread on L broth plates containing (per liter) tryptone, 10 g; yeast extract, 5 g; sodium chloride, 10 g; and agar, 15 g, with kanamycin (10 μg/ml), and the plates were incubated overnight at 37°C to determine the viable-cell count. The remainder of the suspension was centrifuged; the pellet was frozen in dry ice-ethanol and freeze-dried under vacuum, the dry residue was suspended in water, and the viable-cell count was determined as described above. Strikingly, the vegetative cells were killed significantly by this single freeze-drying cycle (Table 1). Essentially identical results were obtained in a repeat of this experiment. Presumably, if additions such as skim milk had been added to the cells before freezing, this would have prevented much if not all of this killing (2).

Spores of various *B. subtilis* strains were prepared, cleaned, and stored at 10°C in water as described previously (5, 11, 14). Spore suspensions (1 ml of 0.7 mg [dry weight]/ml) were stored at 10°C (no freeze-dryings) or centrifuged and the pellet was frozen. After freeze-drying, the pellet was resuspended in water for 10 to 12 h at 10°C to ensure complete dispersal of the spore pellet and recentrifuged; the pellet was freeze-dried again, and the supernatant fraction was saved for subsequent analysis of dipicolinic acid (9). After appropriate numbers of freeze-dryings, aliquots of spores were analyzed for viable-cell counts as described above. As has been observed previously (8), wild-type spores of *B. subtilis* were resistant to up to eight cycles of freeze-drying and rehydration (Table 1) as well as up to six freeze-thaw cycles (data not shown). However, in one experiment, the viability of $\alpha^-\beta^-$ spores decreased ~30-fold after six cycles of freeze-drying (Table 1), although six freeze-thaw cycles had no effect (data not shown). This loss in viability upon freeze-drying was seen with four different preparations of $\alpha^-\beta^-$ spores, with the viability loss for each freeze-drying cycle ranging from 1.5- to 4-fold (data not shown). Most of the latter variation occurred between results with different spore preparations; however, the precise reasons for this variability are not clear. No significant difference in the killing of $\alpha^-\beta^-$ spores was observed whether spores were freeze-dried under vacuum as a pellet or from suspension or dried from suspension at room temperature in a vacuum dessicator (data not shown). Storage of freeze-dried $\alpha^-\beta^-$ spores for up to 1 month at room temperature did not decrease their viability further (data not shown). Strikingly, $\alpha^-\beta^-$ spores carrying $pSspC^{wt}$, which directs the synthesis of high spore levels of $SspC^{wt}$, normally a minor *B. subtilis* α/β-type SASP, were resistant to freeze-

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TABLE 1. Survival of cells and spores after freeze-drying and rehydration[a]

No. of freeze-drying cycles	Survival[b] (%)				
	Wild-type cells	Wild-type spores	$\alpha^-\beta^-$ spores	$\alpha^-\beta^-$ $SspC^{wt}$ spores	$\alpha^-\beta^-$ $SspC^{Ala}$ spores
0	100	100	100	100	100
1	2	—[c]	—	—	—
2	—	—	26	80	30
4	—	—	10	75	15
6	—	115 (<10)	3 (<10)	73 (<10)	5 (<10)

[a] Cells and spores were prepared and survival after freeze-drying was determined as described in the text. The 95% confidence limits for the viability measurements were less than ±20% of the values given. The strains analyzed were wild-type strain PS533(pUB110) (Km^r), which is derived from a laboratory strain of 168; $\alpha^-\beta^-$ strain PS578 (Δ*sspA* Δ*sspB*[pUB110] Km^r), which has deletions in the genes coding for the two major α/β-type SASP (4, 13); $\alpha^-\beta^-$ $SspC^{wt}$-producing strain PS1450 (Δ*sspA* Δ*sspB*[p$SspC^{wt}$] Km^r), which is analogous to PS578 but expresses high levels of $SspC^{wt}$ in spores (14); and $\alpha^-\beta^-$ $SspC^{Ala}$-producing strain PS1465 (Δ*sspA* Δ*sspB*[p$SspC^{Ala}$] Km^r), which is analogous to PS578 but expresses high levels of $SspC^{Ala}$ in spores (14).

[b] Values in parentheses are the percentage of the total spore dipicolinic acid released in all six freeze-dryings combined.

[c] —, not done.

drying, while a mutant form of $SspC^{wt}$, termed $SspC^{Ala}$, was not effective in protecting spores against being killed by freeze-drying (Table 1). Previous work has shown that high $SspC^{wt}$ levels restore most or all of the heat, UV, and hydrogen peroxide resistance to $\alpha^-\beta^-$ spores (11, 13, 14). In contrast, $SspC^{Ala}$ does not bind to DNA and is ineffective in restoring spore heat, hydrogen peroxide, and UV resistance (11, 14).

An obvious question concerning the killing of $\alpha^-\beta^-$ spores by freeze-drying is the mechanism of this process. Since the freeze-dried spores that we tested released no significant amounts of dipicolinic acid (Table 1), this suggested that the killing caused by freeze-drying was not due to breakdown of spore permeability barriers. However, transfer of survivors of $\alpha^-\beta^-$ spores from the rich medium plates used to determine survival to either sporulation or minimal medium agar plates, as described previously (5), and incubation of the latter for 24 to 48 h indicated that a significant percentage of the survivors had asporogenous or auxotrophic mutations (Table 2). Similar results have been obtained previously upon analysis of survivors of heat and hydrogen peroxide killing of $\alpha^-\beta^-$ spores (5, 11). In contrast, heat or hydrogen peroxide killing of wild-type spores does not generate mutations in the survivors (5, 11).

TABLE 2. Survival and mutagenesis of $\alpha^-\beta^-$ spores after freeze-drying and rehydration[a]

No. of freeze-drying cycles	Survival (%)	Mutations (%)		
		Auxotrophy	Asporogeny	Auxotrophy and asporogeny
0	100	<0.5	<0.5	<0.5
3	7	10.8	5.7	2.8

[a] Spores of strain PS578 ($\alpha^-\beta^-$) were either incubated at 10°C in water or subjected to three cycles of freeze-drying and rehydration. Survivors were quantitated, and the percentage of survivors having mutations causing auxotrophy or asporogeny was determined as described in the text. Two hundred ten colonies of the culture incubated at 10°C and 248 survivors of the freeze-drying were tested for mutations. Note that spore preparations different from that shown in Table 1 were used for this experiment. This difference presumably accounts for the difference in killing as a function of number of freeze-drying cycles. Induction of mutations upon freeze-drying of $\alpha^-\beta^-$ spores was also observed with a different preparation of $\alpha^-\beta^-$ spores.

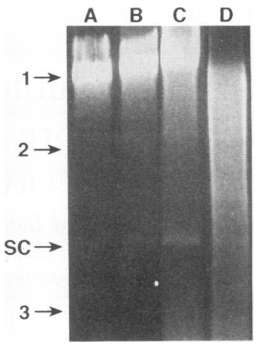

FIG. 1. Agarose gel electrophoretic analysis of DNA from untreated and freeze-dried wild-type and $\alpha^-\beta^-$ spores. Spores of strain PS533 (wild type) or PS578 ($\alpha^-\beta^-$) (from the same batch used for the experiment giving the results shown in Table 2) were either stored at 10°C in water (no freeze-dryings) or subjected to four cycles of freeze-drying, and DNA was isolated as described in the text. Approximately 7 mg (dry weight) of spores was used for each condition. The wild-type spores showed no detectable loss in viability on freeze-drying; the $\alpha^-\beta^-$ spores lost ~99% of their viability. Aliquots containing either 3 μg (wild-type spores) or 7 μg ($\alpha^-\beta^-$ spores) of DNA were run on a 1% agarose gel, and the gel was stained with ethidium bromide. Lanes: A, wild-type spores without freeze-drying; B, wild-type spores after freeze-drying; C, $\alpha^-\beta^-$ spores without freeze-drying; D, $\alpha^-\beta^-$ spores after freeze-drying. The arrows labeled 1, 2, and 3 mark the migration positions of DNA markers of 23, 6.7, and 2.1 kb, respectively. The arrow labeled SC marks the migration position of supercoiled pUB110.

The fact that freeze-drying generated mutations in $\alpha^-\beta^-$ spores strongly suggested that freeze-drying was causing DNA damage. Indeed, when DNA was extracted and purified (5, 11) from wild-type and $\alpha^-\beta^-$ spores after four freeze-drying cycles and electrophoresed on agarose gels, the wild-type DNA appeared to be identical before and after the freeze-drying—in particular in that the intensity of the faint band of supercoiled plasmid pUB110 did not change (Fig. 1, lanes A and B). In contrast, the DNA from the freeze-dried $\alpha^-\beta^-$ spores had a significant number of single-strand breaks, as seen both by the overall size reduction in the chromosomal DNA fragments in the DNA from the freeze-dried spores and by the virtual loss of the supercoiled DNA band (Fig. 1, lanes C and D).

The killing of bacterial cells by freeze-thawing and freeze-drying is a common observation (3, 4), and there have also been reports of mutagenesis associated with freeze-drying of cells (1, 4, 10, 15). However, the mechanisms by which freeze-drying kills cells and how freeze-drying might cause DNA damage leading to mutagenesis are not clear. Whatever the mechanism of these effects, it is clear that binding of α/β-type SASP to spore DNA is a significant factor in spore resistance to freeze-drying, just as these proteins are major factors in spore resistance to UV radiation, heat, and hydrogen peroxide (5, 11, 13). Since α/β-type SASP effect spore resistance to UV radiation, heat, and hydrogen peroxide by preventing DNA damage from these treatments, it seems most likely that these proteins also effect spore resistance to freeze-drying by preventing DNA damage. The generation of both DNA damage and mutations in $\alpha^-\beta^-$ spores by freeze-drying is certainly consistent with this idea. Clearly, the presence of high levels of the unique DNA-binding proteins, the α/β-type SASP, in spores of *Bacillus* as well as *Clostridium* species plays a major role in the extreme resistance of such spores to a variety of harsh conditions and thus to their long-term survival both in the laboratory and in natural environments.

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