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Deoxyribonucleic Acid Strand Breaks During Freeze-Drying and Their Repair in *Escherichia coli*

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Freeze-drying of *Escherichia coli* cells caused strand breaks of deoxyribonucleic acid (DNA) in both radiation-sensitive and -resistant strains. However, in the radiation-resistant strain *E. coli* B/r the damaged DNA was repaired after rehydration, whereas in the radiation-sensitive strain *E. coli* B_{s-1} the damaged DNA was not repaired and the DNA was degraded. Repeated freeze-drying did not break the damaged DNA into smaller pieces.

In previous work on the effect of repeated freeze-drying on the survival of radiation-resistant and -sensitive strains of *Escherichia coli*, we found that survivals of the radiation-resistant strain *E. coli* B/r after freeze-drying one, two, and three times were 53, 20, and 0.95%, respectively, whereas those of the radiation-sensitive strain *E. coli* B_{s-1} were 0.32, 0.0018, and 0.00013%, respectively (21). These results suggest that freeze-drying somehow affects deoxyribonucleic acid (DNA) so that the radiation-sensitive mutant, which cannot repair damaged DNA, cannot survive after freeze-drying.

It has been reported that radiations, such as X-ray and ultraviolet-ray (UV) irradiation, damage DNA and that the damaged DNA is repaired by repair enzymes in the cell (4, 5, 9, 14). It has also been reported that the modes of damage of DNA by X-ray irradiation and UV irradiation are different (8, 13, 22). *E. coli* B/r is uvr^+ and can repair pyrimidine dimers formed by UV irradiation and DNA base damage induced by chemical agents, such as 4-nitroquinoline-1-oxide (6, 7). This strain is also exr^+: that is, it can repair strand breaks caused by X-ray or gamma-ray irradiation (8). On the other hand, *E. coli* B_{s-1} is $uvrB^-$ and cannot repair base damage (1). It is also exr^- and cannot repair strand breaks of DNA, and strand breaks are followed by DNA degradation. Using these characters of *E. coli* B/r and B_{s-1}, we studied DNA damage induced by freeze-drying and its repair in *E. coli*. Preliminary accounts of this paper were reported elsewhere (19, 20).

Cells of *E. coli* B/r were subjected to either UV irradiation or freeze-drying, suspended in a 0.9% (wt/vol) NaCl solution, and incubated at 37°C for 90 min. The effect of liquid holding on the survival of treated cells is shown in Table 1. Liquid holding increased the survival of *E. coli* B/r after UV irradiation, but decreased the survival after freeze-drying. UV-induced damage can be enzymatically repaired during liquid holding, whereas X-ray-induced damage cannot (2). Thus, it seems likely that DNA damage due to freeze-drying may be similar to that induced by X-ray irradiation. This assumption is consistent with the fact that *E. coli* B_{s-1} has the character of exr^- and is sensitive to X-ray irradiation.

Figure 1 shows the effect of freeze-drying on the degradation of DNA. When *E. coli* B/r and B_{s-1} were not subjected to freeze-drying, no degradation of their DNA was observed (Fig. 1A). After freeze-drying once, the DNA of *E. coli* B_{s-1} was degraded significantly, whereas that of *E. coli* B/r was not (Fig. 1B). After freeze-drying three times, even the DNA of *E. coli* B/r was degraded slightly (Fig. 1C), but the extent of its degradation was significantly lower than that of the DNA of *E. coli* B_{s-1}.

The damage to DNA of *E. coli* B/r and B_{s-1} was investigated by alkaline sucrose density gradient centrifugation. Figure 2A shows that single-strand breaks appear in the DNA of *E. coli* B_{s-1} as a result of freeze-drying. After incubation of the freeze-dried cells in medium containing excess unlabeled thymidine, a significant decrease was found in the amounts of their DNA. This observation is consistent with data

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TABLE 1. *Liquid holding recovery of E. coli B/r after UV irradiation and freeze-drying*[a]

Treatment	Survival of cells/ml		Survival ratio after liquid holding
	Before liquid holding	After liquid holding	
UV irradiation (J/m²)			
0	3.1×10^8	3.5×10^8	1.1
50	1.8×10^7	2.6×10^7	1.4
100	6.3×10^4	1.8×10^5	2.9
Freeze-drying (no. of times)			
0	2.8×10^{10}	2.8×10^{10}	1.00
1	5.8×10^9	2.3×10^9	0.40
2	3.9×10^8	2.8×10^8	0.72
3	2.8×10^7	2.7×10^7	0.97

[a] The cells were grown overnight at 37°C with shaking in medium containing 5.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 1 g of NH_4Cl, 10 g of polypeptone, 5 g of yeast extract, 1 g of Casamino Acids (Difco), and 10 g of glucose per liter of distilled water (pH 7.2). The cultured cells were washed twice with 0.9% NaCl solution and then suspended in a 0.9% NaCl solution to give an absorbance of 0.3 at 540 nm. UV irradiation of the suspension was done as described previously (24). The cell suspension was poured into a petri dish to a depth of 1 mm and irradiated with a germicidal lamp (Mitsubishi GLK-10, 10 W) with gentle shaking at room temperature. The intensity of UV light was determined with a UV photometer (Toshiba Electric Co., GI-I), which was standardized against a Latarjet UV photometer. The dose rate used was 20 J/m² per s. Freeze-drying of cells was carried out as described previously (21). Cultured cells were harvested by centrifugation and washed twice with a 0.9% NaCl solution. They were then suspended in a cold solution (0 to 4°C) of 10% skim milk and 1% sodium glutamate. A 0.2-ml sample of the suspension in an ampoule was frozen in an acetone bath (−40°C) and then dried for 16 h with a temperature of not more than 4°C at a gas pressure of 10^{-3} to 10^{-4} mm of Hg, using a Daia freeze-dryer. When freeze-drying was repeated, the samples were rehydrated by adding 0.2 ml of sterile distilled water and then rapidly frozen again. The UV-irradiated and freeze-dried cells were suspended in a 0.9% NaCl solution to give an absorbance at 540 nm of 0.3 and incubated at 37°C for 90 min. Cells were plated after appropriate dilutions. Each value is an average of duplicate determinations, and counting errors between two determinations were less than 30%.

shown in Fig. 1B. Strand breaks of the DNA of *E. coli* B/r were also observed after freeze-drying (Fig. 2B). However, after incubation in medium containing excess unlabeled thymidine, the size of the DNA molecules of *E. coli* B/r was found to be the same as that of control cells. These data indicate that the DNAs of *E. coli* B_{s-1} and B/r were both damaged by freeze-drying, but when the freeze-dried cells were incubated after freeze-drying, the damaged DNA of *E. coli* B_{s-1} was degraded, whereas that of *E. coli* B/r, which has the *exr*⁺ character, was repaired. Recently, Takano and his co-workers reported DNA damage during freezing of *Salmonella typhimurium* (17, 18). It is probable that strand breaks of cellular DNA of bacterial cells occur during freeze-drying and damaged DNA is repaired during rehydration when the bacteria have a repair enzyme(s). The effects of repeated freeze-drying on DNA damage of *E. coli* B_{s-1} are shown in Fig. 2C. The size of the DNA was almost the same after freeze-drying one and three times. Similar results were obtained with *E. coli* B/r. Since the degradation of DNA was observed after repeated freeze-drying (Fig. 1B and C), these data suggest that freeze-drying may have another effect(s) on DNA molecules besides causing strand breaks of DNA. For instance, it is possible that freeze-drying may affect some mechanism for protecting DNA against degradation.

About 58% of the cells of *E. coli* B/r survived

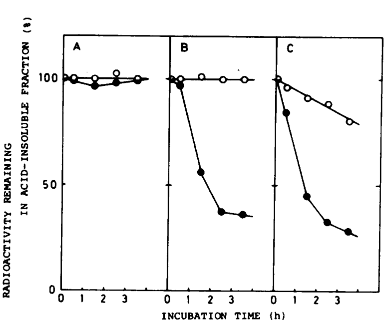

FIG. 1. *Degradation of DNA of E. coli B/r and B_{s-1} after freeze-drying. After overnight culture, cells were diluted 100-fold with EM9 medium (containing 11 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 1 g of NH_4Cl, 0.011 g of $CaCl_2$, 0.13 g of $MgSO_4$, 4 g of glucose, and 5 g of Casamino Acids per liter of distilled water, pH 7.2) supplemented with 10 μCi of [6-³H]thymidine (Radiochemical Centre, Amersham, England) per 3 μg and 200 μg of deoxyadenosine per ml. The cell suspension was incubated overnight with shaking at 37°C, and the cells were then washed three times with EM9 medium and suspended in 2 ml of a solution of 10% skim milk and 1% sodium glutamate. The suspension was freeze-dried, resuspended in 2 ml of a 0.9% NaCl solution, and washed once with a 0.9% NaCl solution. The cells (about 10^{10}) were then suspended in 5 ml of EM9 medium containing 300 μg of thymidine per ml and incubated with shaking at 37°C. Samples of 1 ml were taken at the indicated times and mixed with an equal volume of 10% trichloroacetic acid. The cold trichloroacetic acid-insoluble material was collected on a Whatman glass fiber disk (GF/C), dried, and counted in a Beckman liquid scintillation spectrometer. The radioactivities of cold trichloroacetic acid-insoluble material in the cells after incubation for various intervals are expressed as percentages of those in nonincubated cells. Symbols: ○, E. coli B/r; ●, E. coli B_{s-1}. (A) Cells not freeze-dried; (B) cells freeze-dried once; (C) cells freeze-dried three times.*

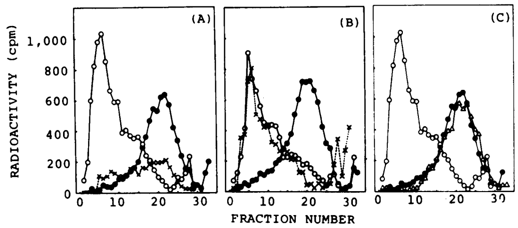

FIG. 2. *Analysis of DNA of freeze-dried E. coli B_{s-1} and B/r by alkaline sucrose density gradient centrifugation. Sedimentation analysis of DNA molecules by alkaline sucrose density gradient centrifugation was carried out essentially as described by McGrath and Williams (8). Cells were cultured overnight, labeled with [6-^{3}H]thymidine as described in the legend of Fig. 1, and suspended in 1 ml of solution containing 10% skim milk and 1% sodium glutamate. A 0.2-ml sample of the suspension was subjected to freeze-drying. After freeze-drying, the cells were washed once with a 0.9% NaCl solution and suspended in 0.2 ml of SSC solution, which contained 0.15 M NaCl, 0.015 M trisodium citrate, and 0.01 M ethylenediaminetetraacetate (pH 8.0). In some experiments, where indicated, cells that were freeze-dried once and washed once with a 0.9% NaCl solution were suspended in 2 ml of EM9 medium containing 300 μg of thymidine per ml and incubated at 37°C for 90 min. Cells were then collected and washed once with a 0.9% NaCl solution and suspended in 0.2 ml of SSC solution. Control cells were treated in the same way, but not subjected to freeze-drying. For alkaline sucrose density gradient centrifugation, a linear sucrose density gradient (5 to 20% sucrose solution containing 0.2 N NaOH and 0.001 M ethylenediaminetetraacetate, in a total volume of 4.4 ml) was prepared. The first 0.1 ml of 0.5 N NaOH and 0.015 ml of 5% sodium lauryl sulfate were layered on the gradient, and then 0.04 ml of cell suspension was applied on top. The gradient was held at room temperature for 15 min and then centrifuged at 30,000 rpm for 90 min in an SW40 rotor. Fractions of 2 drops were collected, placed on paper disks, and washed first with 5% cold tricholoroacetic acid and then with a mixture of ethanol and ether (1:1, vol/vol). The radioactivity on the paper disk was counted in a Beckman liquid scintillation spectrometer. (A) E. coli B_{s-1}: ○, cells not freeze-dried; ●, cells freeze-dried once; ×, cells freeze-dried once and then incubated in EM9 medium. (B) E. coli B/r: ○, cells not freeze-dried; ●, cells freeze-dried once; ×, cells freeze-dried once and then incubated in EM9 medium. (C) Effect of repeated freeze-drying on DNA of E. coli B_{s-1}: ○, cells not freeze-dried; ●, cells freeze-dried once; △, cells freeze-dried three times.*

after freeze-drying once (21). However, no degradation of their DNA was observed (Fig. 1B), and most of their damaged DNA could be repaired (Fig. 2B). This indicates that damage of cells due to freeze-drying cannot be due only to strand breaks of DNA. As reported by others (10, 11, 15, 16), this treatment may also damage other biological functions of the cells, such as cell membrane and some enzyme systems. Nevertheless, the difference in the survivals of *E. coli* B/r and B_{s-1} after freeze-drying may be explained as being due to DNA damage and its repair. Moreover, this work suggests that the DNA damaged during freeze-drying can be repaired by a repair enzyme, especially an *exr* gene-dependent enzyme. These findings seem consistent with the results of others, who showed that freeze-drying of cells increased the mutation rate (3, 12) and that drying induced temperate phages (23).

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