BIKEN JOURNAL Vol. 18, 267–269, 1975

PRELIMINARY REPORT

LETHAL EFFECT OF FREEZE-DRYING ON RADIATION-SENSITIVE MUTANTS OF *ESCHERICHIA COLI*

YOSHINORI TANAKA[1], TAKEO OHNISHI[2], YOSHIFUMI TAKEDA[1,2], and TOSHIO MIWATANI[1,2]

Laboratory for Culture Collections[1] and Department of Bacteriology and Serology[2], Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

(Received June 18, 1975)

Injury of bacterial cells by freeze-drying has been reported by several workers. It has been assumed that this treatment damages the cell membrane so causing metabolic disorders which kill the bacteria (Postgate and Hunter, 1963; Sinskey and Silverman, 1970; Speck and Cowman, 1969). This assumption is supported by the findings that the injured bacteria undergo repair when incubated in an appropriate medium (Baird-Parker and Davenport, 1965; Wasserman and Hopkins, 1957) and that energy is required for this repair (Ray, Jezeski and Busta, 1965).

On the other hand, the occurrence of mutations during freeze-drying has been observed in *Serratia marcescens* and yeast (Servin-Massieu and Cruz-Camarillo, 1969; Hieda and Ito, 1974), suggesting that this treatment damages cellular DNA. Recently, evidence of DNA-damage during slow freeze-thawing of *Salmonella typhimurium* was also reported (Takano, 1974; Takano, Sinskey and Baraldi, 1974).

To test the possibility of DNA-damage during freeze-drying, we studied the effect of freeze-drying on the survival of strains of *Escherichia coli* sensitive and resistant to radiation.

The strains used in this study were *E. coli* B/r, B_{S-1}, H/r-30R and NG30. B/r and B_{S-1} were derived from *E. coli* B (Hershey strain) and originally described by Hill (1958). B/r is radiation-resistant and B_{S-1} is radiation-sensitive. B_{S-1} (*uvr* B^-, *exr*$^-$) is unable to repair UV or X-ray damage of its DNA (Mattern, Zwenk and Roersch, 1966). *E. coli* H/r-30R and NG30 were described by Kato and Kondo (1967). H/r-30R is a revertant of H/r-30 which is an arginine-requiring substrain of a radiation-resistant variant (B/r type) of Harm's B *phr*$^-$ strain (Harm and Hillebrandt, 1962). NG30 is a radiation-sensitive mutant and is classified phenotypically as *rec* A^- (Kato and Kondo, 1967).

Loopfuls of stock cultures were inoculated into modified EM9 medium (10 ml) containing 0.058 g Na_2HPO_4, 0.03 g KH_2PO_4, 0.05 g NaCl, 0.01 g NH_4Cl, 0.1 g polypeptone, 0.05 g yeast extract, 0.01 g casamino acids and 0.1 g glucose and cultured by shaking overnight at 37 C. Cells were harvested by centrifugation and washed twice with saline. They were suspended in a cold solution (0–4 C) containing 10% skim milk and either 1% sodium glutamate or 1% glucose.

Aliquote of 0.2 ml of the suspension (about 10^9–10^{10} cells per ml) in 3 ml ampoules were

subjected to freeze-drying using a Daia freeze-dryer. Each sample was frozen in an acetone bath (—40 C). The frozen samples were then dried for 16 hr at a gas pressure of 10^{-3}–10^{-4} mmHg. When freeze-drying was repeated, the samples were rehydrated by adding 0.2 ml of sterile distilled water and immediately frozen again. Under these conditions, no cell division of the samples was observed.

After rehydration, 10-fold serial dilutions of the samples were made in modified EM9 medium. The samples were then spread on modified EM9 medium with 1.5% agar and incubated overnight at 37 C. Then viable cells were counted.

As shown in Fig. 1A, when B/r and B_{s-1} suspended in 10% skim milk and 1% sodium glutamate had been subjected to freeze-drying once, their survival rates were 53% and 0.32%, respectively, indicating that most cells of the radiation-sensitive mutant, B_{s-1}, had been killed. After freeze-drying two and three times, the survival rates of B/r decreased to 20% and 0.95%, respectively, while those of B_{s-1} decreased tremendously to 0.0018% and 0.00013%, respectively. The survival rates of *E. coli* B (Hershey strain) after freeze-drying were almost the same as those of *E. coli* B/r. Similar results were obtained when these cells were suspended in a solution containing 10% skim milk and 1% glucose for freeze-drying (Fig. 1B).

As shown in Fig. 2, similar results were obtained in experiments using another radiation-sensitive mutant, NG30 and its parent strain, H/r-30R.

It is known that the radiation-sensitive mutant, B_{s-1} cannot repair UV or X-ray damage of its DNA (Mattern et al., 1966). Thus, it seems probable that freeze-drying somehow affects cellular DNA so that radiation-sensitive mutants which cannot repair damaged DNA cannot survive after freeze-drying. The high survival of B/r may be explained by its ability to repair the damaged DNA. However, decrease in survival of B/r

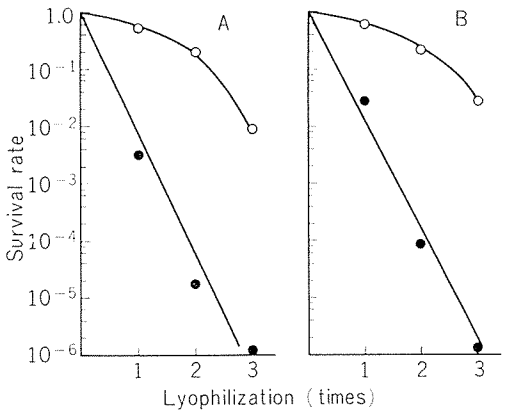

FIGURE 1. *Effects of freeze-drying on survival of E. coli B/r and B_{s-1}. E. coli B/r and B_{s-1} were cultivated and suspended in cold solution containing 10% skim milk and 1% sodium glutamate (A) or 10% skim milk and 1% glucose (B) and freeze-drying was carried out as described in the text. The viable counts before freeze-drying were: (A) B/r (○), 9.5×10^{9}/ml; B_{s-1} (●), 7.6×10^{9}/ml; (B) B/r (○), 9.4×10^{9}/ml; B_{s-1} (●), 7.6×10^{9}/ml. Viable counts after each freeze-drying are expressed as survival rates relative to these values.*

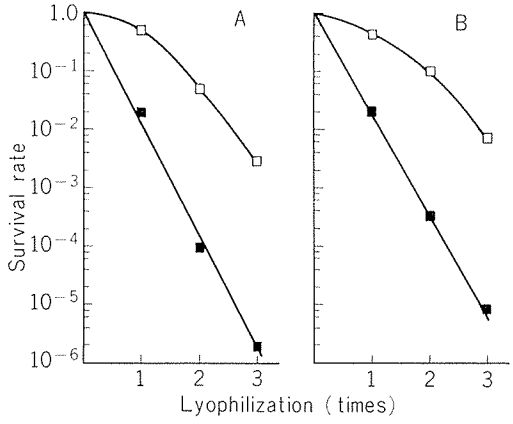

FIGURE 2. *Effects of freeze-drying on survival of E. coli H/r-30 R and NG 30. Experimental conditions were as for Fig. 1. (A) The suspension solution contained 10% skim milk and 1% sodium glutamate. The viable counts before freeze-drying were: H/r-30 R (□), 1.53×10^{10}/ml; NG30 (■), 6.68×10^{9}/ml. (B) The suspension solution contained 10% skim milk and 1% glucose. The viable counts before freeze-drying were: H/r-30R (□), 1.31×10^{10}/ml; NG30 (■), 5.38×10^{9}/ml.*

to 53% after freeze-drying once may show that damage of cells by freeze-drying is due not only to DNA damage but also to membrane damage and metabolic injury.

On the other hand, NG30 is radiation-sensitive although it contains repair enzymes. This strain is known to be of the *rec A* type. Cellular DNA of this strain is degraded by UV or X-ray irradiation. It is supposed that freeze-drying causes similar damages of cellular DNA of NG30 to those caused by radiation. A detail study on the effect of freeze-drying on cellular DNA is now in progress in our laboratory.

ACKNOWLEDGMENTS

This work was supported in part by a Grant in Aid for Developmental Scientific Research from the Ministry of Education, Science and Culture. We thank Dr. K. Nozu for critical reading of the manuscript and Miss M. Arita for technical assistance.

REFERENCES

Baird-Parker, A. C., and E. Davenport. 1965. The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat treatment and after the storage of frozen or dried cells. J. Appl. Bacteriol. 28: 390–402.

Harm, W., and B. Hillebrandt. 1962. A non-photoreactivable mutant of *E. coli* B. Photochem. Photobiol. 1: 271–272.

Hieda, K., and T. Ito. 1974. Induction of genetic change by drying in yeast, p. 71–78. *In* T. Nei [ed.] Proc. Symp. on Freeze-Drying of Biological Materials, International Institute of Refrigeration, Paris.

Hill, R. F. 1958. A radiation-sensitive mutant of *Escherichia coli*. Biochim. Biophys. Acta 30: 636–637.

Kato, T., and S. Kondo. 1967. Two types of X-ray-sensitive mutants of *Escherichia coli B*: Their phenotypic characters compared with UV-sensitive mutants. Mutat. Res. 4: 253–263.

Mattern, I. E., H. Zwenk, and A. Roersch. 1966. The genetic constitution of the radiation-sensitive mutant *Escherichia coli* B_{s-1}. Mutat. Res. 3: 374–380.

Postgate, J. R., and J. R. Hunter. 1963. Metabolic injury in frozen bacteria. J. Appl. Bacteriol. 3: 405–414.

Ray, B., J. J. Jezeski, and F. F. Busta. 1965. Repair of injury in freeze-dried *Salmonella anatum*. Appl. Microbiol. 22: 401–407.

Servin-Massieu, M., and R. Cruz-Camarillo. 1969. Variants of *Serratia marcescens* induced by freeze-drying. Appl. Microbiol. 18: 689–691.

Sinskey, T. J., and G. J. Silverman. 1970. Characterization of injury incurred by *Escherichia coli* upon freeze-drying. J. Bacteriol. 101: 429–437.

Speck, M. L., and R. A. Cowman. 1969. Metabolic injury to bacteria resulting from freezing, p. 39–51. *In* T. Nei [ed.] Freezing and Drying of Microorganisms, University of Tokyo Press, Tokyo and University Park Press, Baltimore and Manchester.

Takano, M. 1974. Effect of freeze-thawing rate on minimum medium recovery and DNA damages of *Salmonella typhimurium* LT-2. The First International Congress of the International Association of Microbiological Societies, Tokyo.

Takano, M., A. J. Sinskey, and D. Baraldi. 1974. DNA damage in *Salmonella typhimurium* LT-2 by a combination of freezing and nutrition, p. 61–70. *In* T. Nei [ed.] Proc. Symp. on Freeze-Drying of Biological Materials, International Institute of Refrigeration, Paris.

Wasserman, A. E., and W. J. Hopkins. 1957. Studies in the recovery of viable cells of freeze-dried *Serratia marcescens*. Appl. Microbiol. 5: 295–300.