J. Hyg., Camb. (1974), **72**, 467
Printed in Great Britain

Preservation of corynebacteriophages by freeze-drying

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(*Received* 24 *October* 1973)

SUMMARY

A method of freeze-drying is described by which the activity of a series of corynebacteriophages was maintained over a test period of 30 months, whereas some of these strains became inactive if stored as liquid filtered lysates at 4° C., or frozen and held at −25° C.

INTRODUCTION

One of us (H.R.C.) has been engaged in an extended series of studies on bacteriophages carried by a number of pathogenic species of corynebacteria isolated from various lesions in man and animals. Observations on their morphological characters have recently been reported (Nagington & Carne, 1971).

The collection comprised some seventy phages, and for purposes of comparative study it was desired to be able to store multiple samples of individual phages for considerable periods without loss of activity. Preliminary observations indicated that some of these corynebacteriophages lost their activity relatively rapidly when held at 4° C. as liquid bacteria-free filtrates of lysed bacterial cultures. Considerable loss of titre also occurred when such filtrates were distributed in 0·25 ml. amounts in sealed glass ampoules and stored at −25° C. However, no loss of activity occurred if such ampoules were first rapidly frozen by immersion and then subsequently stored in liquid nitrogen at −196° C. This method proved excellent for limited numbers of samples, but because the large number of phages involved threatened to overtax the available liquid nitrogen refrigeration space, the possibility that freeze-drying might provide a satisfactory solution was then examined.

Davies & Kelly (1969) investigated the sensitivity of one of our phages to freezing and drying under different conditions and the method we have employed is based upon their findings.

MATERIALS AND METHODS

Bacteriophages

Fourteen phages were tested. These were derived from four different species of *Corynebacterium* isolated from infections in seven mammalian species as shown in Table 1.

Preparation of bacteriophage suspensions

Overnight cultures of sensitive indicator strains on tryptic digest agar slopes were washed off with 3 ml. of tryptic digest broth, shaken with glass beads in a high-speed mechanical shaker (H. Mickle, Hampton, Middlesex, England) for

Table 1. *Bacteriophages tested; lysogenic bacteria from which they were derived, and lesions from which the latter had been isolated*

Designation of phage	Lysogenic bacterium of origin	Mammalian species and type of lesion from which isolated
ov 1	*Corynebacterium ovis*	Sheep; caseous lymphadenitis
ov 2	*C. ovis*	Sheep; caseous lymphadenitis
ov 3	*C. ovis*	Sheep; caseous lymphadenitis
cap 1	*C. ovis*	Goat; caseous lymphadenitis
hq 1	*Corynebacterium* closely resembling *C. ovis*	Man; suppurative lymphadenitis
hq 2	*Corynebacterium* closely resembling *C. ovis*	Man; suppurative lymphadenitis
UH 3	*C. ulcerans*	Man; sore throat
uh 3	*C. ulcerans*	Man; sore throat
uh 5	*C. ulcerans*	Man; sore throat
uh 6	*C. ulcerans*	Monkey: sore throat
UB 1	*C. ulcerans*	Buffalo; ulcerative dermatitis
ub 2	*C. ulcerans*	Cow; mastitis
equi 4498	*C. equi*	Foal; suppurative pneumonia
pyog. 29	*C. pyogenes*	Cow; arthritis

2 min. to break up clumps. Sufficient of this suspension was then added to 100 ml. bottles of warmed (37° C.) tryptic digest broth to give a just perceptible opalescence. Bottles were then incubated for $1\frac{1}{2}$ hr. at 37° C. in a horizontal shaker giving 80 traverses per minute. Bacteria-free, high-titred phage suspensions were then added and the cultures shaken at 37° C. for a further 5 hr. After centrifugation at 10,000 rev./min. for 20 min. the supernatant was passed through Millipore filters (22 μm.) in all but two instances in which small volumes were passed through Membrane Filter No. 17 Oxoid.

The supernatant was dispersed in 0·25 ml. volumes into sterile 0·5 ml. freeze-drying ampoules (Johnson and Jorgensen, London), sealed, and rapidly frozen by immersion in liquid nitrogen, and then held in a liquid nitrogen refrigerator to await results of tests for freedom from bacteria and determination of the phage titre.

Filtrates were tested for freedom from bacteria by sowing liberally on appropriate culture media, and phage titres were determined by the Adams overlayer method (Adams, 1959).

Preparation of experimental samples

Appropriate numbers of ampoules of frozen, high-titre, bacteria-free lysate were rapidly thawed by agitation in water at 37° C. To the liquid phage suspension was then added an equal volume of a mixture of equal parts of 20% peptone, 10% sucrose and 2% sodium glutamate (sterilized by filtration). The phage-additive mixture was dispensed in 0·1 ml. amounts into sterile 0·5 ml. freeze-drying tubes (Johnson and Jorgensen, London) plugged with cotton wool, and the tubes were rapidly frozen by standing in a metal rack in a vacuum bowl containing liquid nitrogen. The frozen tubes were then transferred to the liquid nitrogen refrigerator until a convenient time to freeze-dry.

Table 2. *P.F.U./ml. of phages before freeze-drying, shortly after, and* 30 *months later after storage at* $-25°$ *C.*

Phage	At time of freeze-drying	3–15 days after freeze-drying	30 months after freeze-drying
ov 2	10^8	10^7	4×10^7
ov 3	10^{10}	(Not done)	10^{10}
cap 1	10^9	10^8	10^9
hq 1	3×10^9	10^{10}	10^8
hq 2	3×10^9	10^{10}	10^9
UH 3	10^8	10^7	10^8
uh 3	3×10^{10}	10^9	10^9
uh 5	2×10^{10}	10^{10}	10^9
uh 6	10^7	10^7	10^7
UB 1	10^{10}	4×10^9	10^{10}
ub 2	5×10^9	10^9	10^{10}
equi 4498	10^{10}	10^9	10^9

Freeze-drying

The apparatus used was that described by Greaves & Davies (1965). This comprised a two-stage thermoelectric refrigerator mounted on a thick brass plate which was water-cooled on the opposite side. A recessed 'O' ring in this plate gave a vacuum seal for the 'bell jar' top of the desiccator. The desiccator chamber was connected to a Cenco 'Megavac' pump via a vacuum valve and phosphorus pentoxide trap. An aluminium block, drilled to take 25 freeze-drying tubes, was placed empty in the centre of the dryer plate; the metal heat screen was placed in position and the bell jar placed over; refrigerator and pump were started and the temperature adjusted to $-35°$ C.

The apparatus was allowed to run for 30 min. to cool the aluminium block. The frozen tubes of phage suspension were then transferred from liquid nitrogen into the cavities of the block, and the apparatus started up again. After 30 hr. the temperature was raised to $-25°$ C. to increase the rate of drying and held at this for another 24 hr. The temperature was then allowed to come up to room temperature and held at this overnight. Tubes were then constricted, put on a manifold for secondary drying over P_2O_5 and after 4–6 hr. were sealed *in vacuo* and stored at $-25°$ C.

Dried samples were tested for phage titre by overlayer assays at 3 days and 30 months after freeze-drying. For this purpose the dried deposit in tubes was dissolved in 0·45 ml. of sterile 0·85% NaCl solution to give a 1/10 dilution, and from this further dilutions of 10^{-2} to 10^{-10} were prepared in tryptic digest broth for overlayer assays.

RESULTS

Table 2 gives the p.f.u./ml. of each phage, (*a*) just before freeze-drying, (*b*) 3 days after, and (*c*) 30 months after.

It will be seen that no significant loss of titre occurred as a result of the freeze-drying procedure used, and it remained essentially unchanged throughout a period of 30 months. The minor variations in titre are within the limits of experimental

variation with such phages which have a marked propensity to clump, probably because of the high lipid content of their bacterial hosts.

The authors are greatly indebted to the following for phages and/or strains of Corynebacteria – Dr M. H. Ayoub, Veterinary Research Laboratories, Dokki, Cairo, Egypt; Miss Ivonne Batty, Public Health Laboratory, Toowoomba, Queensland, Australia; Professor S. D. Henriksen, Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo, Norway; Dr D. H. Howard, Department of Medical Microbiology, School of Medicine, Los Angeles, California, U.S.A.; Dr D. J. Jayne-Williams, National Institute for Research in Dairying, Shinfield, Reading, Berkshire; Dr W. H. H. Jebb, Public Health Laboratory, Radcliffe Infirmary, Oxford; Dr Eugenia Meitert, Cantacuzino Institute, Bucarest, Romania; the late Dr G. Ramon, Institut Pasteur, Paris, France; Dr Phyllis M. Rountree, Faifax Institute of Pathology, Royal Prince Alfred Hospital, Sydney, Australia.

This work was supported by a grant from the Medical Research Council.

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