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Preservation of *Staphylococcus aureus* with unstable antibiotic resistance by drying

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SUMMARY

Cultures of *Staphylococcus aureus* showing unstable resistance to kanamycin, streptomycin, lincomycin and penicillin have been preserved by drying. Particular attention has been paid to organisms showing linked resistance to pairs of these antibiotics. Recoveries of viable cells from the desiccates was high and the proportion of resistant and sensitive cells was maintained both during storage and during heating. The preservation of the organisms on ceramic beads stored in air over silica gel promises to be an effective and convenient method for maintaining staphylococcal desiccates during studies of unstable antibiotic resistance.

INTRODUCTION

It is now well established that resistance to some antibiotics in *Staphylococcus aureus* is often controlled by extrachromosomal determinants (plasmids). Such resistance is often lost spontaneously and rapidly during growth and when maintained on ordinary culture media. Also in some studies, linkage has been demonstrated between different resistance factors and also between these factors and other metabolic markers. In investigations in this field it is an advantage to have readily available populations reliably characterized with regard to their content of resistant and sensitive cells. Although desiccation is a common means of preserving bacteria and could be expected to fulfil this requirement, there appears to be no published evidence to support this contention. This paper supplies some data on the subject and some also upon the effect of heat on such desiccates. The strains investigated were those previously shown to have linked and unstable antibiotic resistance to various combinations of kanamycin, lincomycin, streptomycin and penicillin (Grubb & Annear, 1972).

METHODS

Suspensions for drying

The organisms (Tables 1–3) were grown on nutrient agar and harvested after 20 hr. at 37° C. Heavy suspensions (10^{10} orgs/ml.) were prepared in sodium glutamate the concentration of which varied with the drying method. Colonies grown from platings of the suspensions were screened for antibiotic resistance. The suspen-

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sions were in the mean time frozen and stored at $-20°$ C. to await drying. If the screening indicated satisfactory proportions of resistant and sensitive cells the suspension was considered suitable for drying. Suspensions showing between 10 and 90 % of resistant organisms were regarded as ideal for the purpose. The frozen suspensions were usually stored for no more than 24 hr. before being thawed and dried. Preliminary experiments were invariably necessary to determine the degree of instability associated with the organisms concerned and occasionally several attempts were necessary before a suitable suspension was obtained. Growth at 43·5° C. was used in one experiment (Table 2) to obtain penicillin-sensitive variants in a strain which also yielded linked kanamycin- and lincomycin-sensitive variants (Annear & Grubb, 1972*b*).

Screening for antibiotic resistance

For most of the work screening colonies for antibiotic resistance was done by multipoint replication (Annear & Grubb, 1972*a*). In one experiment where the resistant cells were only a small fraction ($< 1/1000$) of the total, their incidence was determined by surface counts on antibiotic plates. Screening of desiccates was always done on colonies which developed directly from freshly rehydrated cells.

Viable counts

In some experiments viable counts on blood agar plates were made to assess losses due to drying and storage. In other experiments only rough assessments of viability were made, by noting the density of growth on plates inoculated with heavy suspensions of either undried cells or dried cells after rehydration.

Drying methods

General. Several methods of drying were used and each involved drying suspensions from the liquid state *in vacuo* on a manifold over P_2O_5 (Annear, 1962). The suspensions were dried on various surfaces from which they could be readily recovered by gentle agitation in broth.

Method A. The organisms were suspended in 5 % sodium glutamate and single drops were dried on cellulose fibres held in small tubes (Annear, 1962). The desiccates were sealed *in vacuo* and stored at room temperature.

Methods B and C. These methods are based to some extent upon previous work (Polding, 1943; Lange & Boyd, 1968; Grivell & Jackson, 1969). Bacterial suspensions were dried on ceramic insulating beads and stored either in air or *in vacuo*. The methods are illustrated in Fig. 1. Ampoules containing approximately 50 beads (2×1 mm) were prepared and inoculated with three drops of bacteria suspended in 5 % glutamate. The inoculum was distributed over the beads by gentle shaking. The cotton-wool plug was pushed firmly into the constriction and the ampoule was dried on the manifold for 24 hr. During the first 15 min. of drying the ampoule was tapped occasionally to prevent beads sticking to each other and to the glass surfaces. The beads were then stored in two ways. For method B they were stored in small screw-capped containers in air with one or two pieces of silica gel previously sterilized by dry heat. The vessels were sealed as an added security against leakage

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Table 1. *Preservation in dried cultures of unstable and linked resistance to kanamycin and lincomycin in* Staphylococcus aureus (*M* 4)

Drying method	Storage	% resistant: Before drying	% resistant: After drying: Immediately	% resistant: After drying: After storage
A	4 years at 25° C.	40	36	46
B	3 years at 4° C.	28	20	18
C	3 years at 25° C.	80	76	84

All cultures showed confluent growth on direct plating.

Table 2. *Preservation in dried cultures of penicillin resistance and linked kanamycin and lincomycin resistance in* Staphylococcus aureus (*M* 4) (*drying method D*)

Stage of examination	Viable cell recovery (%)	% resistant: Penicillin	% resistant: Kanamycin and Lincomycin
Before drying	—	14	0·00021
After drying	90	20	0·00025
After storage for 4 years at 25° C.	85	16	0·00032

and were stored at 4° C. In earlier experiments, $\frac{1}{4}$ oz. glass bottles were used, but currently polypropylene tubes (Nunc No. 1078) are employed. These tubes were developed for storage of biological materials in liquid nitrogen and have proved extremely satisfactory for the purpose described here.

For method C a few beads were transferred to small tubes which were then constricted. The beads were dried for a further period of 1–2 hr. on the manifold, sealed *in vacuo* and stored at room temperature.

Method D. In this method, single drops of bacterial suspension in 10 % glutamate were dried on tufts of quartz fibres (Annear, 1964). The ampoules were sealed *in vacuo* and stored at room temperature.

Method E. Three-drop volumes of suspensions in 20 % glutamate were dried as foams (Annear, 1970). Small tubes of moist P_2O_5 (Annear, 1971) were included in the ampoules before they were sealed. Sealing was carried out in air and the desiccates were stored at room temperature. Some sealed desiccates were subjected to heating at 100° C. for 24 hr.

RESULTS AND DISCUSSION

In all experiments (Tables 1–3) high recoveries of viable organisms were obtained after drying and after storage at 25° and 4° C. Also, the screenings showed that these treatments did not modify the fractions resistant to the antibiotics concerned. The

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Table 3. *Preservation in dried cultures of unstable and linked antibiotic resistance in* Staphylococcus aureus (*drying method E*)

Unstable resistance	Kanamycin and lincomycin	Kanamycin and streptomycin	Kanamycin and penicillin
Before drying			
Antibiotic resistant (%)	30	52	44
After drying			
Immediately			
Viable cell recovery (%)	85	60	90
Antibiotic-resistant (%)	40	48	40
24 hr. at 100° C.			
Viable cell recovery (%)	0·0015	0·0022	0·0034
Antibiotic-resistant (%)	28	56	40
2 years at 25° C.			
Viable cell recovery (%)	50	50	85
Antibiotic-resistant (%)	32	62	52

heating of desiccates at 100° C. in air in the presence of P_2O_5 although reducing the number of viable cells had little effect on the proportion of antibiotic-resistant organisms. Such a treatment is of prognostic value as it may accelerate some of the events likely to occur during long-term storage. Of particular interest are the results in the experiment (Table 2) in which the suspension dried had been derived from growth at high temperature and the two groups of variants had a widely separated incidence. Again the ratio of resistant to sensitive cells for each group was maintained after drying and storage.

The results presented here are representative of a much larger body of unpublished data. As well as results from organisms with linked antibiotic resistance, data from other strains in which the resistance was unlinked were also obtained. The antibiotics concerned were those mentioned in this paper and also tetracycline.

With some experiments, faulty sealing has led to moisture absorption by the desiccate as indicated by colour change of the silica gel. In such desiccates, although the viable cell content was much reduced the fraction of resistant cells invariably remained unchanged.

In comparing the various methods of drying investigated here, both the fundamental and applied aspects of the work should be considered. For example, the sealing of desiccates in the presence of P_2O_5 provides data from desiccates stored under defined conditions but is not a procedure with practical appeal. Where drying of cultures for no particular immediate purpose is undertaken, method A has remained the routine. It has proved highly reliable since its adoption some 10 years ago (Annear, 1962). There has, however, been an increasing trend towards the use of methods B and C which may be conveniently carried out together. The storage of beads in air (method B) has great practical appeal and has undoubtedly stimulated and accelerated studies of antibiotic instability in this laboratory. The ready availability of a large number of standard inocula in one small readily accessible vessel is of obvious value. However, until more quantitative evidence is available, it is considered wise to maintain some of the beads *in vacuo* (method C).

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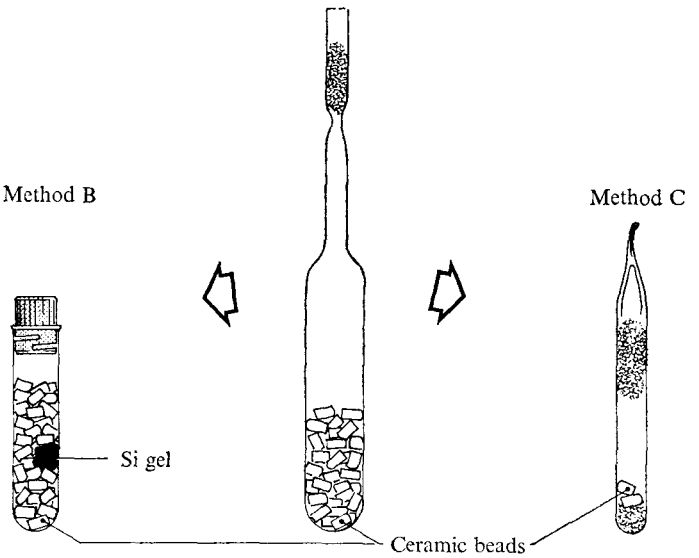

Fig. 1. Preparation of desiccates on ceramic beads.

As yet, no reliable evidence has been obtained in this laboratory for drying organisms other than staphylococci by method B, although several workers already cited have enthusiastically recommended the method for a number of other organisms.

The temperature of 4° C. has been arbitrarily adopted for storage as likely to give better survival rates than room temperature. The silica gel in the sealed desiccate has been included not for the purpose of achieving or maintaining dryness but as an indicator of effective sealing. The final equilibrium humidity level is not known or controlled with any precision but is obviously reproducible within reasonable limits.

While it is usual to preserve cultures being used in genetic projects as a precaution against loss, contamination or change, their preservation at various stages of such experimentation and as an integral part of it does not appear to be widely practised. The advantage of such a practice is that it enables experiments to be interrupted or to be reinvestigated at definite points. It also relieves the pressure for immediate investigation where rapid changes are occurring and enables the investigator to plan his work more conveniently and under less duress. More specifically, it has been found that one of the advantages of 'characterized' desiccates in studies of unstable antibiotic resistance has been their ready availability for testing for additional linkages to those already recognized.

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