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A standardized monitor for the control of ethylene oxide sterilization cycles

By A. H. DADD, CELIA M. STEWART and MARGARET M. TOWN
Bacteriological Laboratories, Imperial College of Science and Technology, London, SW7 2BB, U.K.

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SUMMARY

The resistance of spores of *B. subtilis* var. *niger* produced in liquid synthetic medium and exposed to ethylene oxide on a nylon surface, has been shown to be almost identical to that for spores produced on a traditional solidified complex medium with exposure to the sterilant on aluminium foil. The use of short lengths of nylon tube as carriers allowed easy production and handling, with self-protection of the spore-bearing surface. Addition of a dye provided visual evidence of inoculation without affecting resistance to ethylene oxide. Such a monitor is suitable for use as a standardized biological challenge in routine ethylene oxide sterilization cycles.

INTRODUCTION

The routine control of ethylene oxide sterilization cycles usually includes the insertion of biological monitors into each sterilization load. The monitor takes the form of a carrier inoculated with a known number of spores of a resistant bacterium, and the one most frequently used is *Bacillus subtilis* var. *niger* N.C.T.C. 10073, as recommended by Beeby & Whitehouse (1965). Although not the most resistant spore known (Dadd & Daley, 1980), it is favoured as it is easy to sporulate, the spores store without substantial loss of viability and it produces an orange pigment which distinguishes it from incidental contamination during culture for survivors.

The resistance of biological monitors to inactivation by ethylene oxide is markedly influenced by the conditions existing during sporulation, the way in which the monitors are prepared and stored and the technique used to determine their resistance (Caputo & Rohn, 1982; Dadd, McCormick & Daley, 1983). It is therefore important that they are standardized both on spore count and on resistance to inactivation by ethylene oxide.

A biological monitor for use either in a hospital or in industry should, additionally, be packed in such a way that its spore-bearing surface is protected from abrasion, which could lead to the loss of spores and possibly dissemination of spores into the environment. This paper describes the preparation of such a monitor from spores produced on a synthetic medium and its evaluation against a traditional aluminium foil monitor.

MATERIALS AND METHODS

Media

Synthetic sporulation medium (SSM). This contained (g/l) K_2HPO_4, 0·5; KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·2; $MnSO_4.4H_2O$, 0·01; $ZnSO_4.7H_2O$, 0·01; $CaCl_2$, 0·005; $FeSO_4.7H_2O$, 0·001; L-glutamic acid, monopotassium salt, 9·265; D-glucose (Analar), 0·9008. The medium was adjusted to pH 7·0 by the addition of 1 M-NaOH before sterilization at 121 °C for 20 min.

Sporulation medium (SM 1). This contained (g/l) Oxoid nutrient broth No. 2, 3·125; $MnSO_4.4H_2O$, 0·03; K_2HPO_4, 0·25; Agar (Oxoid No. 3), 12·0. It was sterilized at 121 °C for 20 min.

Glucose tryptone agar (GTA). This contained (g/l) Difco Bacto-tryptone, 10·0; D-glucose (Analar), 5·0; Agar (Oxoid No. 3), 12·0. It was sterilized at 121 °C for 20 min.

Preparation of spore suspensions (*Bacillus subtilis* var. *niger* N.C.T.C. 10073 was used throughout).

Suspensions from synthetic medium SSM. Two litre conical flasks containing 125 ml SSM were inoculated with approximately 1×10^8 bacteria taken from a 7–8 h nutrient agar (Oxoid No. 2) culture of the organism incubated at 37 °C. The flasks were incubated on a reciprocating shaker at 35 °C for 4 days or 30 °C for 5 days when $\geqslant 95\%$ sporulation, counted microscopically, had occurred. The spores were harvested and washed ten times in sterile distilled water at 4 °C. Observation of the initial harvest showed the presence of an orange layer on top of the beige pellet of spores. This consisted of empty sporangia, debris and vegetative cells and was removed with the supernatant. The suspension was stored for 7 days at 4 °C to lyse any remaining vegetative cells and then washed once more with sterile distilled water. Finally, the spore count was determined on GTA and the suspension adjusted to contain 5×10^7 viable spores/ml.

Suspensions from complex medium SM 1. Medical flats containing 60–65 ml SM 1 were inoculated from 7–8 h nutrient agar cultures at 37 °C and incubated with loose caps at 37 °C for 7 days when $\geqslant 95\%$ sporulation had occurred. The spores were removed from the agar surface by gentle agitation with sterile distilled water at 4 °C and the spore suspension was washed and adjusted in a similar manner to that described for spores produced on SSM.

Preparation of monitors

Nylon tube monitors. Nylon tubing (Grilamid, nylon 12, Portex Ltd, Hythe, Kent), 6·0 mm diameter, cut into approximately 10·0 mm lengths, was used as the carrier for the spores. Before use, they were cleaned by soaking overnight in 0·8% aqueous Teepol (Shell Ltd) and rinsed thoroughly in tap and finally distilled water. They were sterilized by dry heat.

The tubes were arranged on a Perspex rack (Fig. 1) and each one inoculated, using a glass fixed-volume syringe pipette (Scientific Glass Engineering, London), with 20 μl of a suspension containing 5×10^7 spores/ml, to give an inoculum of 1×10^6 spores/monitor. The drops of suspension were dried in an air current generated within a short tunnel. The temperature of the air flow was not allowed

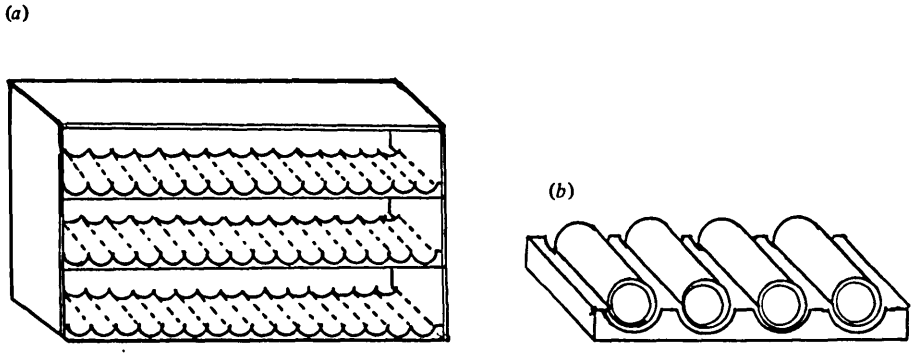

Fig. 1. Drying rack for inoculated nylon tube monitors: (*a*) general view, (*b*) detail of tube holder.

to exceed 25 °C and the relative humidity was maintained in excess of 25 %, conditions previously shown to be without effect on the resistance of the spores to ethylene oxide (Town, M., personal communication). The monitors were removed from the air flow immediately the drops were visibly dry.

Aluminium foil monitors. Aluminium foil, 0·1 mm thick (J. G. Smith, Clerkenwell, London) was cut into 25 × 8 mm strips and cleaned by the method of Beeby & Whitehouse (1965). After sterilization by dry heat, each strip was inoculated and dried as described for nylon tube monitors.

Exposure of spores to ethylene oxide

The apparatus and exposure technique followed that previously described by Dadd & Daley (1980) except that prior to the introduction of ethylene oxide the vacuum in the desiccator was standardized at $6{\cdot}66 \times 10^4$ Pa, achieved after 1 min evacuation.

Recovery and estimation of survivors

Recovery of the spores from the monitors after exposure to ethylene oxide was achieved by ultrasonication (Gurney & Quesnel, 1981). Each monitor was placed in 10 ml sterile distilled water in a 25 ml McCartney bottle and placed in a Dawe Sonicleaner (Baird & Tatlock Ltd, London) for 10 min. Appropriate dilutions were made and 1 ml aliquots plated on glucose tryptone agar incubated at 37 °C for 2 days. The colonies were counted using an Artek colony counter (Dynatech Laboratories, Billingshurst, Sussex), the mean determined and the results plotted on a logarithmic scale as percentage survivors against duration of exposure. From these curves, the D-value (the time required to reduce the population by one log cycle) was read off. All D-values were reproduced in at least five replicate experiments.

RESULTS

Resistance of spores produced on synthetic medium SSM

Spores produced on the liquid synthetic medium SSM exhibited a similar resistance to spores produced on solidified complex medium SM 1, when exposed

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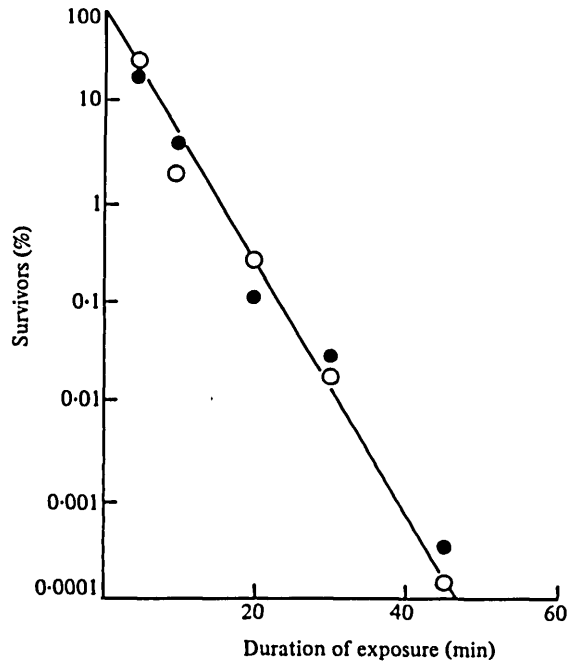

Fig. 2. Survivor curves for spores of *Bacillus subtilis* var. *niger* produced on SSM and exposed to 475 mg/l ethylene oxide at 29 °C on nylon tube (●) and aluminium foil (○).

to ethylene oxide on aluminium foil as carrier. The survivor curve for both types of spore was similar to the curve shown for aluminium foil monitors in Fig. 2, and the D-value was within the range 9 ± 2 min in five replicate spore suspensions.

Spores produced on SSM were stored in suspension for up to 3 months at 4 °C and their viable count and resistance to ethylene oxide determined at suitable intervals. In all samples, the survivor curves were similar to that for freshly produced spores with D-values within the range previously quoted. There was no evidence of loss of viability of the spores in suspension.

Resistance of nylon tube monitors

Nylon tube monitors and aluminium foil monitors prepared from spores produced on SSM, when exposed to ethylene oxide, exhibited survivor curves indicating similar resistance characteristics (Fig. 2). Both were logarithmic throughout, and the D-values in replicate batches of monitors from separate spore suspensions were within the range previously quoted.

Addition of a dye to spore suspensions

Inoculated nylon tube monitors are almost indistinguishable from uninoculated ones, and it was considered desirable that evidence of inoculation should be provided. Three dyes, methyl-violet, basic fuchsin and safranin, were added to aliquots of spore suspension to give a final concentration of 0·01 % (w/v) and monitors prepared from these suspensions as previously described. Survivor curves indicated that for suspensions containing basic fuchsin or safranin, the resistance

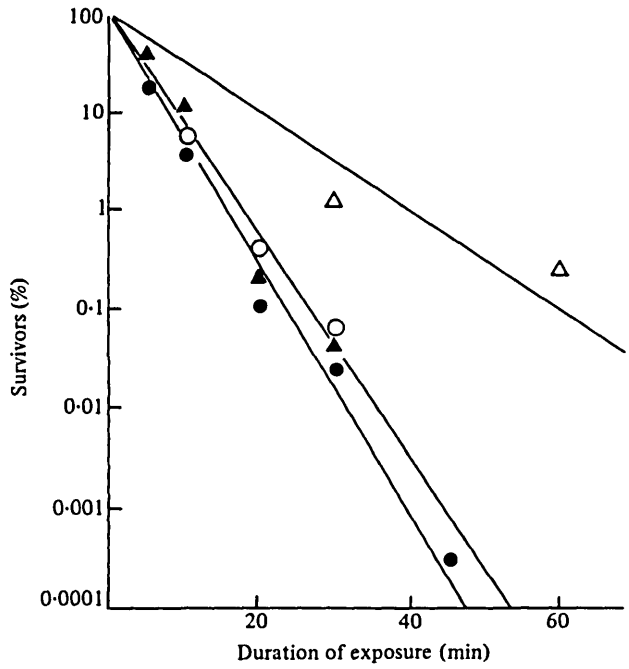

Fig. 3. Survivor curves for spores of *Bacillus subtilis* var. *niger* produced on SSM and exposed to 475 mg/l ethylene oxide at 29 °C on nylon tubes after addition of dye (0·01 % w/v final concentration) to spore suspension. △, Methyl-violet; ▲, basic fuchsin; ○, safranin; ●, no dye.

Table 1. *Recovery of spores from nylon monitors*

Time of exposure to ETO (min)	Viable spore count* following recovery by: Shaking with Ballotini for 10 min	Sonication for (min) 5	10	15
0	$1{\cdot}13 \times 10^6$	$1{\cdot}23 \times 10^6$	$1{\cdot}13 \times 10^6$	$1{\cdot}22 \times 10^6$
15	$9{\cdot}95 \times 10^4$	$9{\cdot}55 \times 10^4$	$9{\cdot}10 \times 10^4$	$6{\cdot}50 \times 10^4$
30	$1{\cdot}73 \times 10^3$	$1{\cdot}50 \times 10^3$	$2{\cdot}10 \times 10^3$	$2{\cdot}85 \times 10^3$
45	$1{\cdot}50 \times 10^1$	$0{\cdot}75 \times 10^1$	$4{\cdot}00 \times 10^1$	$0{\cdot}75 \times 10^1$

* Mean of 3 replicates. Spores exposed to 475 mg/l ETO at 29 °C.

characteristics were similar to those for untreated suspensions, but methyl-violet increased the resistance of the spores (Fig. 3). The graph shows a small difference between untreated spores and those to which basic fuchsin or safranin had been added (shown as a single line), but the D-value for the latter is within the range previously quoted for untreated spores and there is no evidence of tailing to the survivor curve.

Recovery of spores from nylon tube monitors

A comparison of the recovery of spores from nylon tube monitors by ultrasonication or shaking with grade 18 Ballotini, before and after exposure to ethylene oxide (Table 1), revealed a similar number of spores recovered by both techniques.

Although time of shaking with Ballotini is known to have a marked influence on the number of viable spores recovered, particularly after exposure to ethylene oxide (Dadd, McCormick & Daley, 1983), time of ultrasonication was relatively unimportant. Indeed, when ultrasonication was continued for 60 min, there was no significant reduction in the viable count of ethylene oxide-exposed spores. It was concluded that, although both methods are satisfactory, ultrasonication is preferred because it is less critical and requires less preparation time.

DISCUSSION

Many factors are known to influence the resistance of bacterial spores to inactivation by ethylene oxide, and of these the composition of the sporulation medium is one (Dadd, McCormick & Daley, 1983). Further, complex media are known to exhibit batch-to-batch variation, giving rise to changes in the resistance of spores to heat and chemical treatments (Hodges, Melling & Parker, 1980). Therefore, in the preparation of spores for the production of monitors to control ethylene oxide sterilization cycles, it is desirable that a standard medium be used and, to reduce batch-to-batch variation, this should be chemically defined.

Many synthetic sporulation media have been described (Bayliss, Waites & King, 1981; Donellan, Nags & Levinson 1964; Hodges, Melling & Parker, 1980; Rowley & Newcomb, 1964; Waites & Bayliss, 1980) and the influence of nutritional and physical conditions on the rate and amount of sporulation discussed (Buono, Testa & Lundgren, 1966). However, any medium to be used for routine spore production should, in addition to giving a high yield of spores of consistent resistance, be of simple composition and easy to prepare. The medium described was developed to provide high sporulation (95%), with spores of consistent resistance which could be stored for a period in excess of 3 months without measurable change. None of the published media fully satisfied all these requirements.

Packaging the spore monitors to avoid loss of spores from the surface and their dissemination into the environment is now accepted as essential. Flat foil monitors require to be contained in such a way that the spore-bearing surface is protected from abrasion and then packed in an envelope or pouch which is freely permeable to air and ethylene oxide. Rolling foil monitors following deposition of the spores on the surface, although providing the required protection to the spore-bearing surface, results in a variable loss of spores from the monitor, but the use of a preformed tube, with the spores deposited on the internal surface, overcomes this problem and provides a self-protecting monitor. Nylon tube, which is easily cut into short lengths and cleaned, has been shown to be suitable and to give resistance characteristics similar to monitors using aluminium foil as carrier. In addition, they are easy to handle and their small size allows them to be placed inside many medical devices, where this is considered desirable. One disadvantage with nylon tube monitors is that inoculated ones are almost indistinguishable from uninoculated ones. However, it has been shown that it is possible to add a dye to the spore suspension to make the inoculation visible without affecting the viability or resistance of the spores on the monitor. Basic fuchsin or safranin at a concentration of 0·01% (w/v) was shown to be reliable and effective.

Drying an aliquot of spore suspension on the surface of the carrier has been shown

to be a critical step in the preparation of a monitor of defined resistance. Temperature and humidity during drying must be controlled (Dadd, Town & McCormick, personal communication) such that the temperature does not exceed 25 °C and the relative humidity is in excess of 25 %. Drying the nylon monitors under these conditions took several hours but the time could be reduced to 30–40 min by the use of an air current. Resistance was not affected.

Since the resistance of a monitor is influenced by environmental conditions at all stages of preparation, it is desirable to be able to check the resistance of the completed monitor. Such a procedure was originally described by Dadd & Daley (1980) and, with modifications, was the one used in this work. However, recovery of spores from monitors is always a critical step in assessing resistance. Shaking with Ballotini, the method previously recommended and the one widely used, has to be carefully controlled so that the time of shaking is minimal to enable the maximum number of viable spores to be recovered (Dadd, McCormick & Daley, 1983). As an alternative, ultrasonication was found to be effective and the time of treatment less critical. The numbers recovered by ultrasonication were similar to, or above, those recovered by shaking with Ballotini.

In this paper, a new form of biological monitor has been described and shown to have a similar resistance to that of a traditional aluminium foil monitor prepared and tested under similar conditions. The nylon tube has the advantages that it is easy and cheap to produce and is self-protecting, allowing direct packaging without further containment. All monitors respond to environmental changes following preparation, but such changes are similar in both aluminium foil and nylon tube monitors. If desired, this may be prevented by packaging in a moisture-proof pack and storage at a temperature below 25 °C.

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