J. Pharm. Pharmacol. 1980, 32: 126–130 Received May 10, 1979

A comparison of chemically defined and complex media for the production of *Bacillus subtilis* spores having reproducible resistance and germination characteristics

N. A. HODGES*, J. MELLING† AND S. J. PARKER

School of Pharmacy, Brighton Polytechnic, Brighton, U.K. and †Centre for Applied Microbiology and Research, Porton Down, Wilts, U.K.

Spores of *Bacillus subtilis* were produced on five batches of Antibiotic Assay Medium No. 1 obtained from different suppliers, five batches obtained from a single supplier and five batches of a chemically defined liquid medium. The magnitude of the variation within each of the three groups was compared in terms of heat resistance, glutaraldehyde resistance, germination rate and sensitivity of germinated spores to neomycin. For all these parameters the degree of reproducibility achieved by using a chemically defined liquid medium was substantially better than that using batches of complex medium from a single supplier. Even larger variations in these parameters resulted when different suppliers were used. The value of defined media for the production of spore inocula to be used in sterilization control and similar procedures is discussed.

For many years spores of *Bacillus* species have found regular use as inocula for a variety of purposes in the fields of food, cosmetic and pharmaceutical microbiology. Spores have advantages over vegetative cell inocula in that they are relatively easy to produce, clean and count, whilst showing minimal changes in viability and other properties during storage. Applications of spore inocula include both the monitoring of heat sterilization procedures (Kelsey 1961) and the control of tests for sterility (European Pharmacopoeia 1971). They are used in the AOAC test for the evaluation of sporicidal disinfectants (Horwitz 1970) and Moore (1978) has recently recommended that consideration be given to the use of sporulating cells as inocula in 'challenge tests' to assess the efficacy of an antimicrobial preservative in cosmetics and pharmaceuticals. Perhaps their most extensive use as inocula, however, is in agar-diffusion antibiotic assays (Kavanagh 1972). In this context *Bacillus* spores are recommended in assays for 17 out of the 22 antibiotics described in the relevant appendix of the current British Pharmacopoeia (1973).

Clearly it is desirable that media used to produce spores for these purposes should provide spore crops showing minimal batchwise variation in such properties as percentage viability, rate and extent of germination and resistance to heat, chemicals and other adverse environments. To this end media of defined chemical composition has been used in research applications for many years. More recently similar liquid media have been described which are further defined in terms of the nature of the specific nutrient, which, when exhausted, limits vegetative growth and induces sporulation (Brown & Hodges 1974; Frieson & Anderson 1974). Cells produced from such media are, therefore, also defined in a physiological sense and their properties have been shown to depend upon the nature of the limiting nutrient (Hodges & Brown 1975; Lee & Brown 1975). Nevertheless, complex media are still routinely used in the production of spore inocula, although it has long been recognized that marked variations in certain spore properties may be observed in successive crops (El-Bisi & Ordal 1956). There appear to be no reports in the literature, however, in which the main intention was to examine the batch to batch variations in one or more complex media and quantify these variations in terms of the properties of the cells grown on those batches rather than in terms of chemical analyses. Because it is the former criterion which is frequently of interest, this present work was undertaken in order to compare reproducibility in terms of heat resistance, glutaraldehyde resistance, germination rate and antibiotic sensitivity of germinated spores between crops derived from batches of a selected complex medium obtained from five different manufacturers, batches of that medium from the same manufacturer and batches from a nutrient depleted defined medium.

* Correspondence.

MATERIALS AND METHODS

Culture methods and preparation of spore suspensions

The complex medium used was Antibiotic Assay Medium No. 1 supplemented with 0·0001% $MnSO_4$: it was selected as a medium specifically recommended for spore production. Plates were inoculated with a dense log phase nutrient broth culture of *B. subtilis* N.C.T.C. 7861 and incubated at 35 °C for 7 days. Spores were removed from the agar surface with water, centrifuged, washed repeatedly and stored at 4 °C as aqueous suspensions. Five crops of spores were simultaneously prepared in this manner using medium obtained from different manufacturers. Five further crops were similarly prepared using different batches of medium from a single manufacturer. Finally, another 5 crops were produced using a liquid medium prepared exclusively from Analar reagents of the following composition (M): $Na_2HPO_4.12H_2O$, 0·040; KH_2PO_4, 0·027; NH_4Cl, 0·01; $MgSO_4.7H_2O$, 5×10^{-5}; $CaCl_2.6H_2O$, 1×10^{-5}; $MnSO_4.4H_2O$, 5×10^{-6}; $FeSO_4.7H_2O$, 2×10^{-6}; glucose, $3{\cdot}5 \times 10^{-3}$; pH 7·0. Glucose was growth-limiting in this medium and was present at a concentration sufficient to support growth to an optical density of 1·5 at 430 nm. Flasks, 1 litre, containing 250 ml medium were placed on a reciprocating shaker operating at 95 × 5·5 cm throws min^{-1} and each flask individually inoculated with 10 ml of dense log phase culture grown in identical medium. After 24 h at 35 °C approximately 95% sporulation was observed. Lysozyme and trypsin were added to give final concentrations of 20 I.U. ml^{-1} and 0·02 mg ml^{-1} respectively. After 1 h further incubation at 35 °C the cultures were individually harvested and cleaned as before.

Heat resistance

Spores were suspended at a concentration of approximately 1×10^7 ml^{-1} by addition of 0·025 ml concentrated suspension to 20 ml preheated pH 4·0 acetate buffer in a 250 ml volumetric flask. The flask was immersed in a water bath thermostatically controlled at 85 ± 0·1 °C and the suspension agitated using a 25 mm Teflon-coated magnetic stirring bar driven by a motor beneath the bath. Samples were removed at suitable time intervals, diluted in nutrient broth and 0·2 ml volumes spread on the surface of nutrient agar plates. From the colony counts thus obtained after 48 h incubation at 35° C values were plotted for log percentage survivors with time.

Glutaraldehyde resistance

Spores were suspended at a concentration of approximately 1×10^7 ml^{-1} in glutaraldehyde 2% v/v adjusted to pH 7·9 using sodium bicarbonate 0·3% w/v in 100 ml conical flasks at 25 °C. 0·5 ml samples were removed at suitable time intervals and immediately placed in sodium metabisulphite 1% w/v for 30 min to inactivate the glutaraldehyde (Rubbo et al 1967). Control experiments showed this inactivation procedure to be satisfactory. Further dilutions were performed in nutrient broth and 0·2 ml volumes spread on the surface of nutrient agar plates. After incubation for 48 h at 35 °C colony counts were performed and survivor plots constructed.

Spore germination

0·2 ml concentrated spore suspension were added to 20 ml preheated germination solution in a 100 ml conical flask placed on a reciprocating shaker at 35 °C. The composition of the solution was identical to the full medium with the glucose concentration increased to 0·01 M and L-alanine 0·1 M added. The optical density at 430 nm was recorded for successive samples and germination plots constructed as percentage of the initial absorbance with time. Preliminary experiments had shown a fall in absorbance to be linearly related to germination as determined by phase contrast microscopy.

Effect of neomycin on germinated spores

An 0·2 ml sample of concentrated spore suspension was added to 20 ml germination solution prewarmed to 35 °C in a 100 ml conical flask on a shaker. After 90 min incubation >99% of the spores had germinated (phase contrast) and neomycin sulphate (Sigma ≮U.S.P. potency) was added to give a concentration of 50 μg ml^{-1}. Samples were removed, diluted rapidly in broth, plated and incubated as before. Control experiments demonstrated both that the extent of dilution was sufficient to render the neomycin carry-over negligible and that neomycin had no effect on dormant spores.

RESULTS

Figs 1a, b, c illustrate the heat resistance of spores produced on complex medium from different manufacturers, the same manufacture and on defined medium respectively. This order of presentation applies also to the results illustrating glutaraldehyde resistance (Fig. 2), germination rate (Fig. 3) and neomycin sensitivity of germinated spores (Fig. 4).

Comparisons between the three groups of spore crops were complicated by the fact that many of the

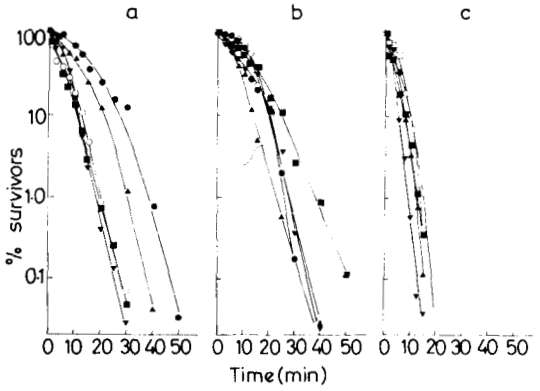

FIG. 1. Heat resistance of replicate batches of *B. subtilis* spores from three different sources.

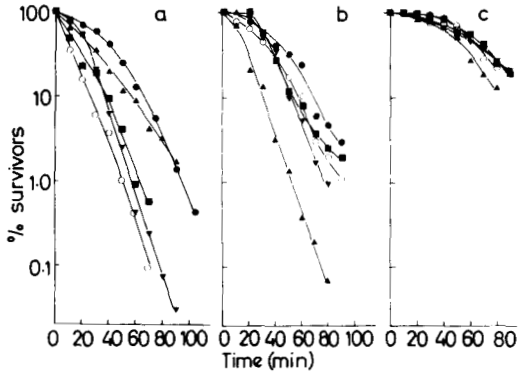

FIG. 2. Glutaraldehyde resistance of replicate batches of *B. subtilis* spores from three different sources.

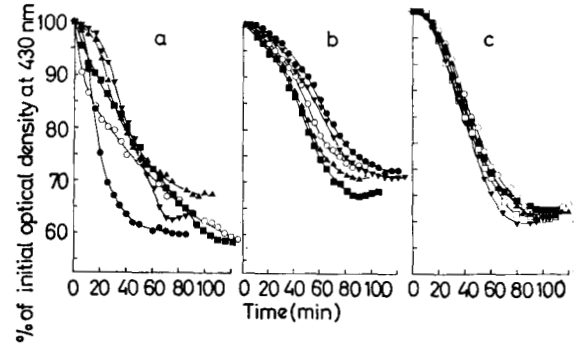

FIG. 3. Rate of germination of replicate batches of *B. subtilis* spores from three different sources.

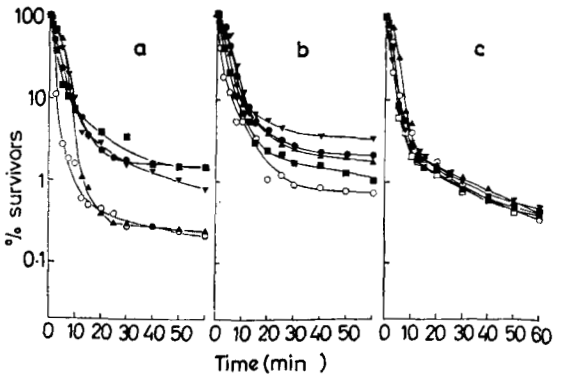

FIG. 4. Neomycin sensitivity of replicate batches of germinated spores of *B. subtilis* from three different sources.

survivor plots for both heat and glutaraldehyde resistance did not exhibit first order kinetics, but rather an initial shoulder followed by a region of linearity or slight 'tailing'. This, together with the low heat resistance and high glutaraldehyde resistance exhibited by the defined medium spores made simple visual comparisons of the 'spread' of results rather misleading. An approximate comparison may be made of the variations within each group of spore crops by using the times required to achieve a particular survivor level. If this procedure is applied to the heat resistance data in Fig. 1a and the times required to achieve a level of 0·1% survivors for the most resistant and the most sensitive batch are expressed as a ratio, a value of 1·84 results. The corresponding values for Fig. 1b, c are 1·61 and 1·56 respectively. This indicates the variability in results for spores from defined medium to be less than that in the other two cases, although this improved reproducibility may not be as great as that suggested by a visual comparison of the 'spread' of the plots on the graph. Values calculated in this way may, however, vary slightly relative to each other depending upon the survivor level chosen and the magnitude of the initial shoulder on the plots in question, consequently a full statistical analysis was considered inappropriate.

If the same procedure is applied to the data in Fig. 2, a level of 20% survivors must be used because of the high resistance of the defined medium spores. In this case the greater reproducibility of batches from defined medium is much more marked as indicated by a ratio of 1·27 compared with 2·27 for spores from different batches of complex medium from the same supplier and 3·00 for spores from complex medium from different suppliers.

Comparisons between the three groups of spores may be made more easily in terms of rate and extent of germination, because the magnitude of these is similar in each case (Fig. 3). It is evident that spores from defined medium are, once again, the most reproducible from batch to batch with the difference between the faster and slowest germinating crops being at no time greater than 5·5% of the initial optical density. The corresponding value for spores from complex medium from different suppliers was 17 and 9% for the other group.

The sensitivity of germinated spores to neomycin was selected as a parameter by which to assess their reproducibility because completed germination represents the last stage in which the cell is still recognizable as a spore rather than a vegetative cell. Reproducibility may, in this case, therefore, be regarded as indicating the extent to which the influence of sporulation conditions is retained throughout the life cycle.

The fact that neomycin is ineffective on dormant spores explains the observed shape of the survivor plots (Fig. 4) with the point at which the curve levels out indicating the approximate proportion of dormant spores remaining in the suspension. The reproducibility of neomycin sensitivity *per se* in germinated spores should not therefore be assessed on the spread of the plots at the end of the exposure period, this merely reflects the extent of germination, but on the slope of that region of the survivor plot covering the first 90% of the population. Using this criterion it is again evident that the use of a defined medium results in spore crops having better reproducibility than those from complex medium.

DISCUSSION

It is evident from the data in Figs 1–4 that improved reproducibility in the four parameters examined may result from the use of a defined liquid medium and that variations are greater when batches of a complex medium are obtained from several suppliers rather than a single supplier. Whilst the potential variation in chemical composition between batches of a complex medium can be predicted by consideration of the analyses published in manufacturers literature and elsewhere (Bridson & Brecker 1970) the consequences or magnitude of the resulting variations in selected cell properties cannot be so readily anticipated. Frequently, quality control of culture media is undertaken in clinical laboratories, particularly in the United States where it became mandatory in 1967. Nagel & Kunz (1973) reported the results of a period of such control in which a total of 900 lots of 46 different media from 3 suppliers were examined in terms of their ability to support characteristic growth of test strains, to permit haemolysis by streptococci and to suppress swarming by *Proteus* species. Using these qualitative criteria less than 2% of the samples were found to be unsatisfactory and the authors concluded that extensive routine testing of complex media in clinical laboratories was unnecessary. Using the criterion of rates of kill for *B. subtilis* and *Clostridium sporogenes* spores in alkaline glutaraldehyde however, Stark et al (1975) noted a variation in resistance between crops produced on different batches of a soil extract nutrient broth. This prompted the authors to suggest the use of a chemically defined medium for spore production, which, they anticipated, would improve the reproducibility of the AOAC sporicidal disinfection test.

Batchwise variations in media and consequently in the physiological properties of the cultures grown in them may be of limited significance in diagnostic bacteriology, but they assume greater importance when the cells are to be used as inocula when maximal reproducibility is desirable. The importance attached to this reproducibility is illustrrated by the recommendation of Rosenheim (1973) that spores of thermophilic bacilli should be regarded as being of only secondary value in the testing of heat sterilization procedures because the manufacture of spore preparations of consistent and appropriate resistance was difficult to achieve.

For a number of years there have been arguments favouring the more widespread use of defined media (Herbert 1961). The advantages they offer in terms of reproducibility and stability during storage have been recognized by their recent use for vegetative cell inocula in tests evaluating hospital disinfectants (Kelsey & Maurer 1974) and preservative efficacy in eye-drops and contact lens solutions (Davies 1978). Perhaps greater consideration should now be given to the routine use of defined media for testing procedures which require spore inocula.

REFERENCES

Bridson, E. Y., Brecker, A. (1970) in: Norris, J. R. Ribbons, D. W. (eds) Methods in Microbiology 3A Academic Press, London

British Pharmacopoeia (1973) London: Her Majesty's Stationery Office

Brown, M. R. W., Hodges, N. A. (1974) J. Pharm. Pharmacol. 26: 217–227

Davies, D. J. G. (1978) J. Appl. Bacteriol. 44: S19–28

El-Bisi, H. M., Ordal, Z. J. (1956) J. Bacteriol. 71: 1–9

European Pharmacopeia (1971) Sainte-Ruffine: Mainsonneuve S.A.

Frieson, W. T., Anderson, R. A. (1974) Can. J. Pharm. Sci. 9: 50–53

Herbert, D. (1961) Symp. Soc. Gen. Microbiol. 11: 391–416

Hodges, N. A., Brown, M. R. W. (1975) in: Gerhardt, P., Costilow, R. N., Webb, R. L. (eds), Spores VI 550–555, Washington: American Society for Microbiology

Horwitz, W. (1970) Official Methods of Analysis Association of Official Agriculture Chemists, 11th ed. Washington, D.C. p. 64

Kavanagh, F. (1972) Anal. Microbiol. volume II Academic Press. London

Kelsey, J. C. (1961) J. Clin. Pathol. 14: 313–319

Kelsey, J. C., Maurer, I. M. (1974) Pharm. J. 213: 528–530

Lee, Y. H., Brown, M. R. W. (1975) J. Pharm. Pharmacol. 27: 22P

Moore, K. E. (1978) J. Appl. Bacteriol. 44: S43–55

Nagel, J. G., Kunz, L. J. (1973) Appl. Microbiol. 26: 31–37

Rubbo, S. D., Gardner, J. F., Webb, R. L. (1967) J. Appl. Bacteriol. 30: 78–87

Rosenheim, The Lord (1973) Report on the prevention of microbial contamination of medicinal products H.M.S.O.: London

Stark, R. L., Ferguson, P., Garza, P., Miner, N. A. (1975) Dev. Ind. Microbiol. 16: 31–36