The destruction of spores of *Bacillus subtilis* by the combined effects of hydrogen peroxide and ultraviolet light

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Ultraviolet light irradiation of bacterial spores in the presence of hydrogen peroxide has been shown to produce synergistic kills when compared with ultraviolet light (u.v.) and hydrogen peroxide used sequentially. This use in combination has been patented for the commercial sterilization of packaging before filling with UHT-processed products. Previous results have shown that lamps producing u.v. light with a maximum output at about 254 nm were extremely effective. Results obtained using a Synchrotron radiation source to produce a narrow band of irradiation now shows that the greatest kill of spores of *Bacillus subtilis* in the presence of hydrogen peroxide is obtained with radiation at ~270 nm. Such results suggest that the action of the u.v. light is not directly on the spore DNA but may be related to the production of free hydroxyl radicals from hydrogen peroxide.

Dormant bacterial spores are relatively resistant to u.v. light (Germaine *et al.* 1973) and to hydrogen peroxide (Toledo *et al.* 1973) but we have previously shown that simultaneous treatment with far-u.v. and hydrogen peroxide results in a kill of up to 2000-fold greater than that produced by irradiation either alone or followed by treatment with hydrogen peroxide (Bayliss & Waites 1979). This combined treatment has been patented as a method of sterilization, particularly for use with packaging for aseptic filling of UHT-processed foods (Peel & Waites 1979). In this study we irradiated spores in the presence of hydrogen peroxide with synchrotron u.v. at wavelengths from 240 to 300 nm in order to determine the optimum wavelength for killing.

Materials and Methods

SPORE PREPARATION

Spores of *Bacillus subtilis* NCDO 2129 were produced by growth at 37°C on *Bacillus* spore agar as described by Franklin *et al.* (1970) with Oxoid Lab. Lemco added at 0·1% (w/v). After ~3 d, the culture, containing ~95% free spores, was harvested and washed five times with sterile deionized glass-distilled water before storage at −18°C.

ULTRAVIOLET IRRADIATION

Spores were suspended at a final concentration of $\sim 1 \times 10^7$/ml in hydrogen peroxide (1% w/v Analar grade) and placed in a silica spectrophotometer cuvette with a magnetic follower. Sufficient volume was used to allow the suspension to cover the magnetic follower to a depth of ~3 mm. The cuvette was then placed in the focused beam of a Synchrotron Radiation Source and the suspension stirred by a magnet. Irradiation was with light of wavelengths 240, 250, 260, 270, 280 or 300 nm (band width ±1 nm) for 30 min before dilution and plating on *Bacillus* spore agar and incubation for 2 d at 30°C in order to allow viable spores to form colonies. A photometer was used to measure the energy available

at each wavelength and allowed such differences to be used in the calculation of the kill as a normalized death coefficient, $\sigma_\lambda . \sigma_\lambda$ (per Joules) defined by the relation

$$N_t = N_o e^{-tI\sigma_\lambda}$$

where N_t is the number of surviving spores at time t, N_o the corresponding number at time $t = 0$ s, and I the intensity of radiation received (Joules/s). The irradiation time, t, was held constant at 1800 s.

Results and Discussion

The viable counts are plotted as the normalized death coefficient against wavelength of u.v. radiation in Fig. 1 and show an increased kill at ~270 nm. Similar results were obtained using a second preparation of *B. subtilis* spores.

These observations suggest that the action of the u.v. light is not directly on spore DNA since the maximum kill was not obtained close to a wavelength of 260 nm. It is possible that u.v. light may have a direct action on dipicolinic acid (DPA), whose absorption peak is close to 270 nm. However, the most likely explanation is that free hydroxyl radicals produced from the hydrogen peroxide by u.v. irradiation are only active against spores when formed close to or even within the spores themselves. Ultraviolet absorption by hydrogen peroxide is greater at shorter wavelengths so that at wavelengths less than ~270 nm, so much u.v. may be absorbed by hydrogen peroxide outside the spore that few free hydroxyl radicals are formed within the spore, hence reducing the kill at these wavelengths. This is supported by evidence from previous work (Bayliss & Waites 1976; Waites *et al.* 1979) where the presence of cupric ions markedly increased the lethal effect of hydrogen peroxide on spore suspensions of *Clostridium bifermentans*, suggesting that the levels of such ions within spores might determine their resistance to hydrogen peroxide. However, cupric ions did not increase the destruction of spores of other species by hydrogen peroxide. Examination of ultrathin sections of unstained spores by electron microscopy showed that spores of *C. bifermentans* bound cupric ions to their centres while those of other species only bound them to their peripheries.

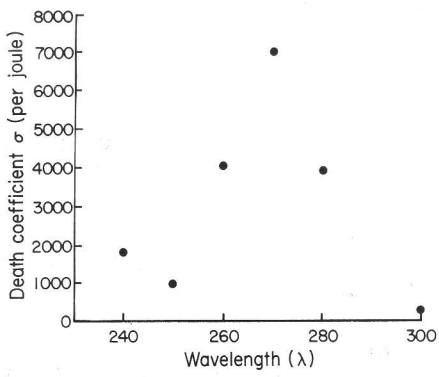

Fig. 1. Plot of the normalized death coefficient σ_λ (normalized for differences in intensity as a function of wavelength) versus wavelength, λ of synchrotron u.v. radiation for *Bacillus subtilis* spores.

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References

BAYLISS, C.E. & WAITES, W.M. 1976 The effect of hydrogen peroxide on spores of *Clostridium bifermentans*. *Journal of General Microbiology* **96**, 401–407.

BAYLISS, C.E. & WAITES, W.M. 1979 The synergistic killing of spores of *Bacillus subtilis* by hydrogen peroxide and ultraviolet radiation *FEMS Microbiology Letters* **5**, 331–333.

FRANKLIN, J.G., UNDERWOOD, H.M., PARKIN, A.G. & BURTON, H. 1970 Comparison of milks processed by the direct and indirect methods of ultra-high sterilization. II. The sporicidal efficiency of an experimental plant for direct and indirect processing. *Journal of Dairy Research* **37**, 219–226.

GERMAINE, G.R., COGGIOLA, E. & MURRELL, W.G. 1973 Development of ultraviolet resistance in sporulating *Bacillus cereus* *Journal of Bacteriology* **116**, 823–831.

PEEL, J.L. & WAITES, W.M. 1979 Improvement in methods of sterilisation. *UK Patent Application* 791091.

TOLEDO, R.T., ESCHER, F.E. & AYRES, J.C. 1973 Sporicidal properties of hydrogen peroxide against food spoilage organisms. *Applied Microbiology* **26**, 592–597.

WAITES, W.M., BAYLISS, C.E., KING, N.R. & DAVIES, A.M.C. 1979 The effect of transition metal ions on the resistance of bacterial spores of hydrogen peroxide and to heat. *Journal of General Microbiology* **112**, 225–233.