Cytochrome Pigments in Spores of *Bacillus cereus* T

G. BAHNWEG AND H. A. DOUTHIT*

Institut für Mikrobiologie, Universität Göttingen, Göttingen, West Germany, and Department of Botany, University of Michigan, Ann Arbor, Michigan 48104*

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Absorption spectra of dormant spores of *Bacillus cereus* T suspended in glycerol showed peaks characteristic of cytochrome pigments.

The terminal respiratory enzymes of *Bacillus* species have been the subject of several previous studies. Although the results of different investigations do not always coincide, presumably due to the different methods employed, the small amount of cytochrome pigments in spores is accepted generally. Keilin and Hartree (4) determined the cytochrome content of spores of *B. subtilis* spectrophotometrically, and found it to be only 6% of the amount present in vegetative cells. Nakada et al. (5) reported that extracts from dormant spores and even from germinated spores of *B. cereus* contained no cytochromes at all, whereas cytochromes a and b were present in vegetative cells. Doi and Halvorson (2) studied respiratory enzymes from mechanically disrupted cells of *Bacillus cereus* T. They found cytochromes b, c, and a in vegetative cells, but none in spores unless they were reduced with dithionite. Under these conditions a weak absorption band of cytochrome b was seen. These results and studies on the effect of various inhibitors on the respiratory activity in extracts led to the conclusion that terminal electron transport in spores is carried out via a soluble flavoprotein system, which is replaced by a particulate (membrane-bound) cytochrome system during germination and outgrowth (2).

Thus, the commonly held assumption that bacterial spores are devoid of respiratory pigments, or at least possess them in severely reduced quantity, has been based mainly on spectral data. But this conclusion is contradicted by cyanide sensitivity of respiration of particles from spores (2), which suggested the presence of cytochromes, and the isolation from spores of *B. subtilis* of particles that show cytochrome spectra (6). The present study was undertaken to ascertain whether optical problems were responsible for the failure to detect these pigments spectrophotometrically.

A property long used in experiments with bacterial spores is their high refractility. Since the amount of scatter by refraction is a direct function of the difference between the refractive indices of two media through which light passes, the apparent brightness of spores in the phase-contrast microscope is due to the large difference in the refractive indices of water and spore material. If cytochrome pigments are present in the spore, it is conceivable that they would not receive much of the incident light, which would be directed away by refraction.

Figure 1 shows data from an experiment designed to determine the refractive index of spores. In this experiment, dormant spores, prepared as previously described (3), were suspended (1 mg/ml, final concentration) in a

![Fig. 1. Absorbance of spores versus refractive index of suspending medium. Spores (250 mg) were suspended in 5 ml of 2-propanol and added to mixtures of 2-propanol and benzyl benzoate to 1 mg/ml final concentration. Refractive index was measured in an Abbe refractometer at 20°C, and absorbance was measured at 660 nm in a Gilford spectrophotometer.](image-url)
series of mixtures of 2-propanol and benzylbenzoate, and the refractive index measured in an Abbe refractometer at 20 C. The use of absorbance at 660 nm was based on the assumption that negligible absorption by cellular components would occur at this wavelength. As the refractive index of the mixture increased, light scattering decreased to a minimum at about \( \eta \) of 1.53, after which it increased again. We assume this minimal value to be the refractive index of these spores, and, as is demonstrated in Fig. 2, they are nearly transparent when viewed in the phase-contrast microscope under these conditions. When the organic solvents leading to minimal light scattering are removed by washing and the spores are suspended in water, they are still refractive and still germinate normally. Thus they are not badly damaged by these solvents.

Spores of \( B. \text{cereus} \) T suspended in a mixture (10:1.038; vol/vol) of benzylbenzoate and 2-propanol (\( \eta \) equals 1.545) were examined spectrophotometrically using an Amino-Chance dual wavelength split-beam recording spectrophotometer. A spectrum obtained in this way displayed peaks which could only be attributed to absorption by reduced cytochromes. However, due to the low concentration of these pigments in such a preparation, clearly defined peaks were difficult to obtain. They were significantly improved by measuring the spectrum at a refractive index between that of water and that of minimal light scatter (1). Glycerol (\( \eta \) equals 1.473) served this purpose remarkably well (Fig. 3), and its high viscosity also prevented appreciable settling of suspended spores during absorption measurements. The absorption spectrum shown in Fig. 4 was obtained by suspending 75 mg of dry spores in 3 ml of glycerol. This suspension was used in the sample cuvette and measured against the reference cuvette which contained glycerol and washed cell walls of vegetative cells of \( B. \text{cereus} \) T, adjusted so that its scatter was the same as that of the spore sample at 660 nm. Cell walls were prepared by incubating vegetative cells overnight in 25% sulfuric acid at 25 C and then washing them thoroughly with water.

The absorption spectrum in Fig. 4 shows \( \alpha \) and \( \beta \) bands of cytochrome \( b \) and one band of cytochrome \( a \), although we have as yet no confirmation of these designations. Similar spectra were obtained from vegetative as well as from sporulating cells. The lower wavelength range (400 to 500 nm) is omitted from the spectrum in Fig. 4 because the “Soret” region of the spectrum is obscured by a highly absorbing red pigment present in spores, but not in vegetative cells.

It was not expected that absorption spectra (as opposed to difference spectra) could be recorded directly, since this implies that the cytochromes in spores are in the reduced state. Neither oxidizing (sodium iodate) nor reducing (sodium dithionite) agents had detectable effect on the spectra of whole dormant spores, so difference spectra could not be recorded. These experiments and more drastic ones (3 h of boiling in 3% hydrogen peroxide) did not alter the spectra, indicating that the cytochromes

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**Fig. 2. Spores in 2-propanol-benzyl benzoate (1.038:10). Calculated refractive index of this mixture, \( \eta \) equals 1.545 (phase-contrast).**
were not due to surface contamination, and that they may reside in a portion of the spore inaccessible to such compounds.

These experiments also help to explain why cytochrome spectra have been difficult to obtain in the past. Only when the difference in refractive index between spore and surrounding medium is such that an appreciable fraction of incident light enters the former is it possible to obtain sufficient light absorption by these pigments.

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