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Reversion of Phase-Dark Germinated Spores of *Clostridium perfringens* Type A to Refractility

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The increase in optical density of suspensions of germinated spores of *Clostridium perfringens* NCTC 8238 and *C. perfringens* NCTC 8798, aerobically incubated in an outgrowth medium, was not attributable to growth; the phase-dark germinated spores had become phase-bright refractile bodies, similar in appearance to resting spores. Optical density increase and refractile body formation under aerobic conditions did not occur in buffer without the organic constituents of the outgrowth medium. At high pH levels, a lower temperature was required for optical density increase and for refractile body formation than at lower pH levels.

Microcycle sporogenesis (1, 3, 6) and other types of cytodifferentiation, as described by Vinter and co-workers (5, 7), involve the production of sporelike refractile bodies from germinated spores in the absence of an intervening stage of growth and multiplication. The present paper is, we believe, the first report of this phenomenon with germinated spores of an anaerobic species. We emphasize that these are preliminary results which we believe are important and unusual enough to be called to the attention of spore scientists; their interpretation and significance remain to be assessed. We shall document rather simple conditions for the reversion of phase-dark germinated spores to refractile bodies, and we hope that others will join us in pursuing further research on this fascinating phenomenon.

As part of a study of nutritional and other requirements for outgrowth, spores of two strains of *Clostridium perfringens* type A (NCTC 8238 and 8798) were produced as described previously (2). The spores were germinated in 10 mM $\text{KH}_2\text{-K}_2\text{HPO}_4$ buffer, pH 6.5, at 35°C for 1 h, by which time suspensions had lost 40 to 50% of their initial optical density (OD) of ca. 1.0, and ca. 95% of the spores had lost refractility, heat resistance, and dipicolinic acid and had become dark under phase-contrast optics (Fig. 1). Similar results were obtained with both strains, although only data obtained with strain NCTC 8238 are presented here. The germinated spores (Fig. 1B) were incubated in an outgrowth medium at a final ratio of germinated spore suspension to outgrowth medium of 1:4. The initial OD of the germinated spore suspension

was 0.125 to 0.150. The medium contained (final concentration): 1.0% salt-free casein hydrolysate (Hy-Case SF, Humko-Sheffield), 0.25% yeast extract (Difco), 25 mM glucose, 25 mM NH_4NO_3 , 1.0 mM K_2SO_4 , and 50 mM $\text{KH}_2\text{-K}_2\text{HPO}_4$ buffer. Incubation was at pH levels from 5.4 to 7.45 at temperatures ranging from 30 to 60°C. Parallel anaerobic and aerobic experiments were run, the latter serving as controls. Outgrowth was followed by change in OD as measured with a Spectronic 21 (Bausch & Lomb) spectrophotometer at 560 nm.

Results of the anaerobic experiments will be reported elsewhere. Briefly, outgrowth of germinated spores of *C. perfringens* was optimal at pH 6.75 and, at the optimal pH, was maximal at 45°C. The aerobic controls, however, offer the more interesting results, and it is on these that the remainder of this brief report will focus.

When the aerobic controls accompanying anaerobic cultures were incubated in outgrowth medium at pH 7.35 or 7.45, OD increased markedly, especially at the higher temperatures (Fig. 2). The minimum temperature at which the increase in OD occurred was lower at pH 7.45 (35°C) than at pH 7.35 (40°C). The increasing temperature requirement with decreasing pH was also evident at pH 7.1 (Fig. 3), where the OD of aerobically incubated suspensions did not increase unless the temperature was 50°C or higher, and at pH 6.75 (Fig. 3), where the minimum temperature for a detectable OD increase was ca. 60°C (no OD increase after 5 h of incubation at pH 6.75, 50°C).

C. perfringens is an anaerobe, and increasing

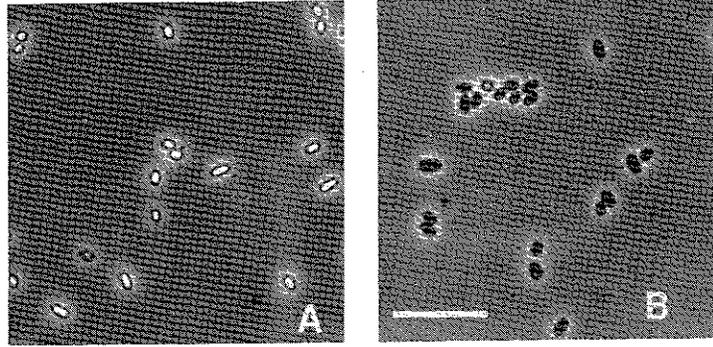


FIG. 1. Phase-contrast photomicrographs of heat-activated (A) and germinated (B) spores of *C. perfringens* NCTC 8238. Spores, activated at 65°C for 15 min, were germinated for 1 h at 35°C in 10 mM KH₂-K₂HPO₄, pH 6.5. Preparations of germinated spores, similar to those in (B), were used as inoculum in subsequent experiments. Marker represents 10 μm.

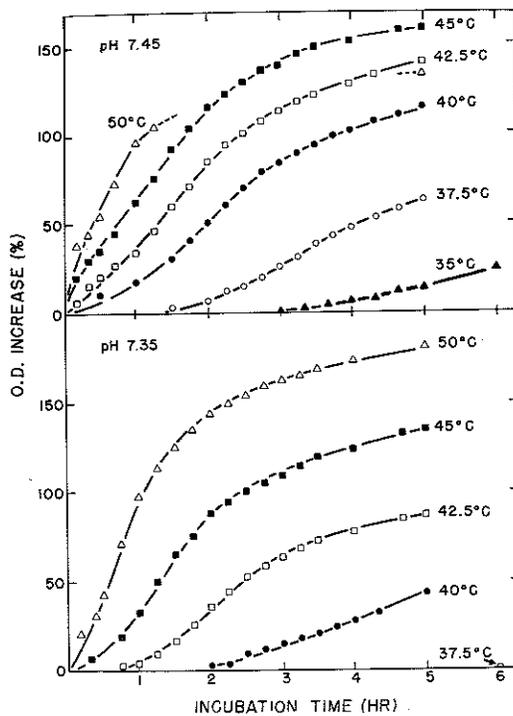


FIG. 2. Increase in OD of suspensions of germinated spores of *C. perfringens* NCTC 8238 incubated aerobically at various temperatures in outgrowth medium at pH 7.45 and pH 7.35. Percentage increase in OD was calculated as $(OD_t - OD_i)/OD_i \times 100$, where OD_i and OD_t were the initial OD and OD after t min, respectively.

OD under aerobic conditions was unexpected. Microscopic observations under phase-contrast optics convinced us that the increase in OD of aerobically incubated suspensions was not attributable to growth, but rather to transformation of some of the phase-dark germinated spores (similar to Fig. 1B), constituting the in-

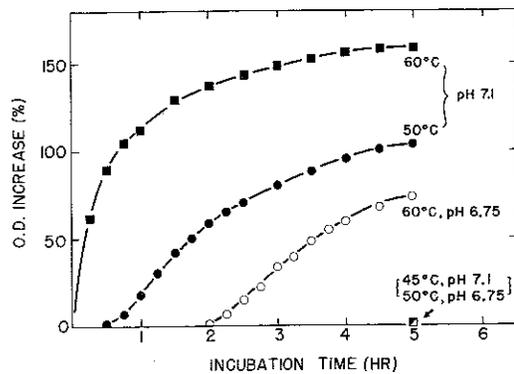


FIG. 3. Increase in OD of suspensions of germinated spores of *C. perfringens* NCTC 8238, incubated aerobically at 50 and 60°C in outgrowth medium at pH 6.75 and 7.1. Percentage increase in OD was calculated as in Fig. 2.

oculum, into phase-bright refractile bodies (Fig. 4), at least superficially resembling resting spores (similar to those in Fig. 1A). As the pH of aerobic incubation increased, the temperature requirement for the transformation from phase-dark to phase-bright (sporelike) bodies decreased. At pH 5.7 or lower, the transformation to phase-bright bodies did not occur even at 60°C; at pH 7.45, refractile bodies were seen after aerobic incubation at temperatures ranging upward from 37.5°C; the minimal temperatures for refractile body formation appeared to be ca. 40, 50, and 60°C at pH 7.35, 7.1, and 6.75, respectively. This appearance of refractile bodies was reflected as an increasing OD (Fig. 2 and 3) during aerobic incubation in outgrowth medium. Refractile bodies were also formed under anaerobic conditions at suitable pH and temperature levels, but their presence was often obscured by the proliferation of vegetative cells. Under anaerobic conditions, growth, with production of

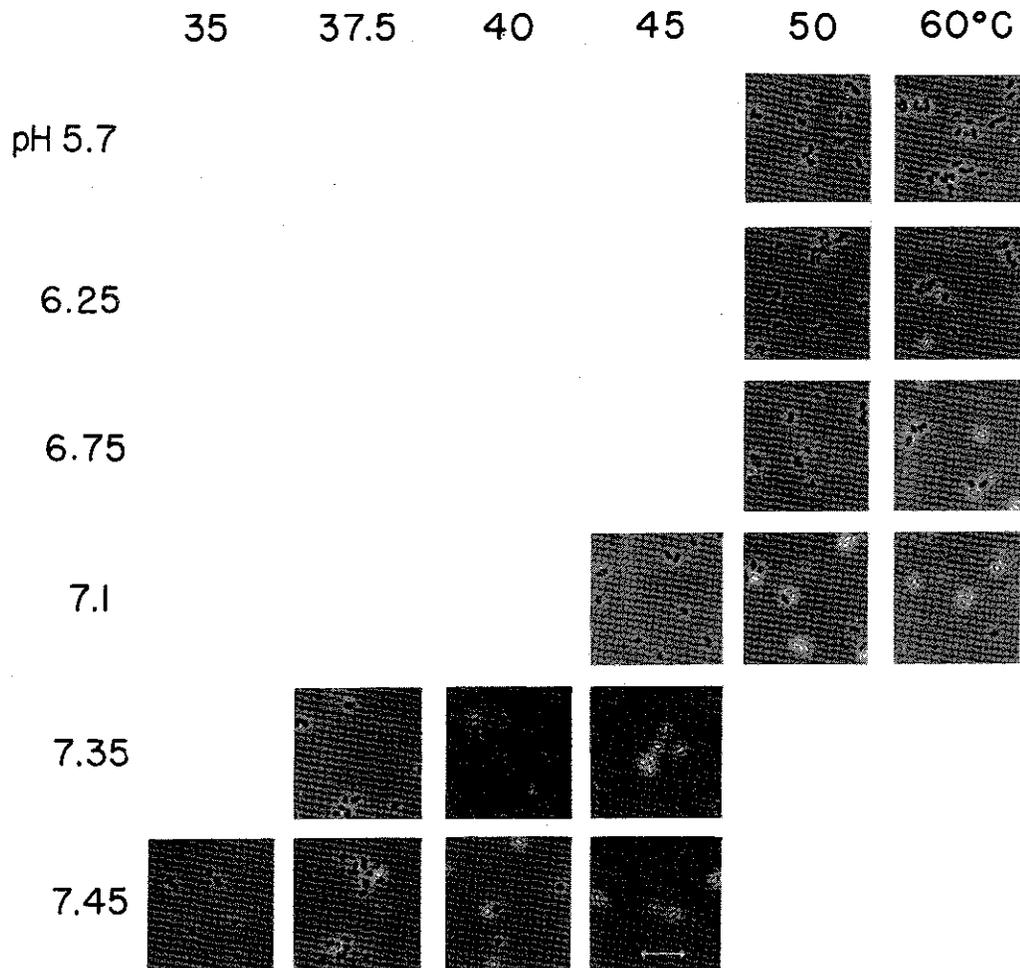


FIG. 4. Formation of refractile bodies from phase-dark germinated spores of *C. perfringens* NCTC 8238, incubated aerobically in outgrowth medium at various temperatures and pH levels. Incubation was for 5 h, except for the 50°C preparations at pH 5.7, 6.25, and 6.75, which were incubated for 7.5, 8.0, and 7.75 h, respectively. Marker represents 10 μ m.

numerous vegetative cells, was evident at temperatures up to 45°C throughout the experimental pH range. Indeed, Smith and Holdeman (4) indicated that *C. perfringens* grows readily at temperatures from 20 to 50°C and at pH levels from 5.5 to 8.0. We have seen some growth at 50°C at pH 6.25 and a lesser amount at pH 6.75, but not at lower or higher pH levels; no growth was observed, at any pH, when the incubation temperature was 60°C or higher.

The OD increase and acquisition of refractility under conditions precluding growth, i.e., aerobic conditions, may have a nutritional basis, since they do not occur in phosphate buffer in the absence of the other (including organic) constituents of the outgrowth medium. The relationship of this phenomenon to other types of cyto-

differentiation (1, 3, 5-7) is, at present, obscure. In the system we have described, the conditions permitting germinated spores of the anaerobe *C. perfringens* to regain refractility without intervening growth and multiplication are notably simple, requiring only manipulation of pH and temperature together with a medium suitable for outgrowth. This system, unlike microcycle sporogenesis, does not appear to be a variant of normal endospore formation. For production of refractile bodies, free from vegetative cells, prevention of outgrowth was desirable and was achieved by maintenance of aerobic conditions.

We make no pretense that this constitutes a completed study. We plan a detailed investigation of the refractile bodies, of the conditions necessary for their formation, of their properties

of dormancy and resistance, and of their capability to germinate and grow. If these refractile, sporelike bodies are indeed hypometabolic, resistant forms with a normal complement of dipicolinic acid and capable of germination, this may be of the utmost fundamental and practical significance.

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