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Sporulation of *Clostridium cylindrosporum* on a Defined, Low-Manganese Medium

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***Clostridium cylindrosporum* HC-1 grew and sporulated well on a defined medium. This is the first demonstration of sporulation of a purinolytic clostridium on a defined medium; manganese levels were below those considered essential for sporulation of most *Bacillus* species. Sporulation appeared to be initiated before exhaustion of the purine substrate.**

Clostridium cylindrosporum and other purinolytic clostridia were recently shown to grow on a defined medium (17), but spores were rare in liquid cultures even after the addition of decoyinine, a sporulation stimulant for *Bacillus subtilis* (7). This result is surprising, as the original description of these bacteria emphasized their sporulation capacity (3). We report here that *C. cylindrosporum* HC-1, the type strain (1), obtained from H. A. Barker, University of California, Berkeley, can grow and sporulate abundantly on a completely defined, clear medium.

The composition of the medium is shown in Table 1. Uric acid was dissolved by heating (14) in approximately a two-thirds final volume of water containing potassium phosphate and sodium phosphate plus 0.4 mM KOH. The other ingredients (17) were added to the resulting urate solution, and the medium was brought to volume by adding water. Deionized glass-distilled water was used for the trace metal experiments; glassware used for these experiments was cleaned in 6 M HCl and rinsed extensively in deionized glass-distilled water. New glassware was used for Se experiments. Iron was provided as ferric sodium EDTA; this form has been used for sporulating anaerobes (13, 15) and does not precipitate with phosphate, an advantage in turbidimetric measurements and in obtaining highly purified spore preparations (Mn contamination of the Calbiochem-Behring preparation used here was 17.1 ppm [17.1 μg/ml], as determined by atomic absorption spectrometry). Thiamine was added at a high concentration; it is sometimes found to stimulate sporulation (6, 12).

The following procedure was used for inoculating and dispensing the medium. A stock spore suspension (0.2 ml; ca. 1.6×10^7 spores per ml) was diluted 1:50 in water, activated-pasteurized at 70°C for 20 min, and then introduced (0.5 ml) into 13 ml of freshly autoclaved medium in 16-mm tubes. Tubes were promptly transferred to GasPak jars and incubated at 37 or 40°C for 24 to 42 h. Cultures were sometimes grown in serum bottles and treated similarly; after sporulation was complete, these serum bottles were capped with sterile rubber-sleeve-type stoppers, stored at 5°C, and used as stock cultures. Viable counts were performed after 48 h in oval tubes with the medium described above supplemented with 2% (wt/vol) agar; a 1-cm seal of uninoculated agar was included to slow the diffusion of oxygen.

Manganese was not added to the medium and is apparently not needed at a high concentration for sporulation as it is for most *Bacillus* species (4, 22). The addition of Mn at levels ranging from 0.1 to 75 μM did not affect final spore yields (ca. 2×10^7 spores per ml). The frequency of sporulation was always >75%. The Mn level of the whole culture (no Mn added) was found by atomic absorption spectrometry to be 0.239 (standard deviation [SD], 0.048) μM. In *Bacillus* species (4, 22), the requirement during sporulation for Mn at greater levels than those needed for vegetative growth is not well understood, and several notable exceptions have been reported (2, 9, 20). A recent proposal (20) postulating an essential role for Mn as a cofactor for phosphoglyceric acid phosphomutase (in the absence of which 3-phosphoglyceric acid accumulates, inhibiting sporulation) may not be valid for organisms not dependent on sugar metabolism (such as *B. fastidiosus* [2]) or for organisms in which Mn is not an obligatory cofactor for phosphoglyceromutase (possibly *C. perfringens* [9]). Exceptional species may also have efficient systems for concentrating Mn (18). Spores formed on the defined medium had only modest heat resistance (D_{85}, approximately 6 min), a value similar to that of spores formed in the presence of Mn levels ranging up to 625 μM (data not shown).

Selenium is required by purinolytic clostridia (17, 21). Required Se concentrations previously reported for these organisms (100 nM) were determined on the basis of favor-

TABLE 1. Defined medium for sporulation of *C. cylindrosporum*

Component	Concn
Uric acid	10 mM
K_2HPO_4	4 mM
Na_2HPO_4	4 mM
KOH	400 μM
$MgSO_4$	140 μM
$CaCl_2$	29 μM
Ferric sodium EDTA	21 μM
Na_2SeO_3	150 nM[a]
Na_2MoO_4	100 nM[a]
Biotin	20 nM
Thiamine hydrochloride	44 μM
$KHCO_3$	20 mM
Resazurin	0.001%
Sodium thioglycolate	2.63 mM[b]

[a] These levels of selenite and molybdate were used to prepare the original stock spore suspensions and for the Mn experiments. Levels in other experiments are stated in the text. The Se content of the uninoculated medium before the addition of selenite was found to be <8.6 nM (C. Hawkes, Fed. Proc. 45:592, 1986).

[b] Added just before autoclaving; the pH was adjusted (if necessary) to 7.1 to 7.4 before thioglycolate was added.

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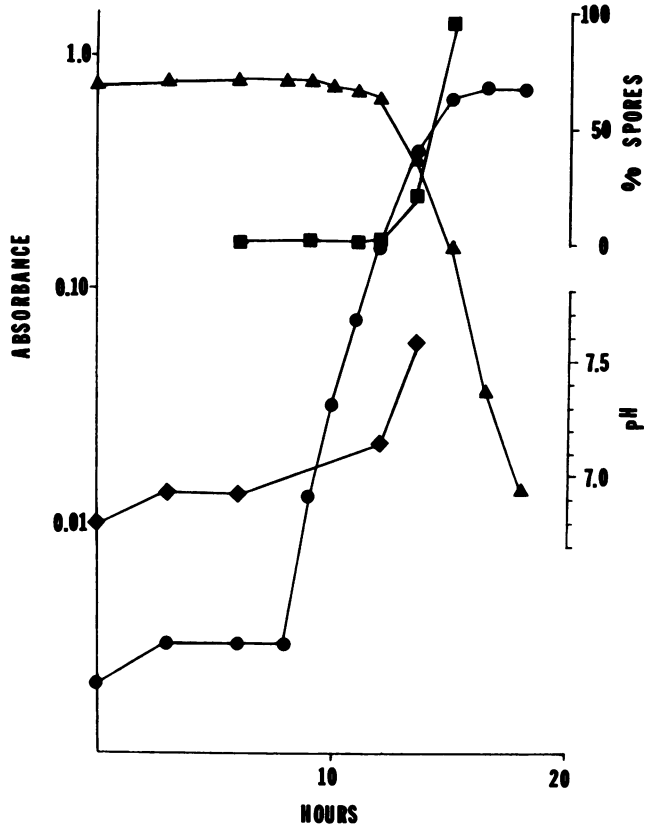

FIG. 1. Time course for growth (A_{540}), uric acid disappearance (A_{290}), pH, and spore formation following inoculation of the defined medium with *C. cylindrosporum* HC-1 spores. Symbols: ●, A_{540}; ▲, A_{290}; ◆, pH; ■, percentage of spores. A_{290} values were obtained after dilution (1:200) in water. Cultures (50 ml; pH 6.8) containing defined medium were inoculated with 2×10^7 spores of heat-shocked strain HC-1 and incubated for 19 h at 37°C. At intervals, 5-ml samples were removed and assayed for growth (A_{540}), uric acid consumption (A_{290} with a 1:200 dilution), pH, and percentage of spores (by phase-contrast microscopy). The defined medium (without added Se) was prepared under a stream of 80% N_2–20% CO_2 and dispensed anaerobically into 160-ml serum bottles (19). $NaHCO_3$ (12 mM) and Na_2S (0.4 mM) were added to the sterile medium by injection as sterile stock solutions just prior to inoculation. Selenium in the defined medium was not measured, but traces of Se may have been introduced through the use of old glassware, house-distilled water, or sparging needles.

able yields of enzyme formation (21), not sporulation. We found that Se levels of 10 nM sometimes resulted in higher spore yields than did Se levels of 100 nM. Of the spores formed in 10 nM Se, about 31% were not fully refractile, as compared with about 18% of those formed in 100 nM Se. At lower Se levels, even greater fractions of the spores were nonrefractile. These Se effects were not demonstrable unless new glassware was used. Molybdate concentrations (1 to 100 nM) did not appear to affect spore formation significantly. Spore formation at 40°C was generally reduced as compared with that at 37°C.

Figure 1 shows growth, sporulation, uric acid utilization, and pH changes after inoculation of the defined medium with spores of *C. cylindrosporum*. An apparent prolonged lag was followed by exponential growth (doubling time, 48 to 51 min), during which uric acid concentrations declined. Final spore counts were 5×10^7 spores per ml (spore frequency, ca. 95%). The lag period was not affected by the addition of yeast extract (0.01 to 0.1%) but decreased to 1.5 to 2 h if a comparable inoculum consisting of vegetative cells was used, suggesting that the medium was not conducive to spore germination. *C. acetobutylicum* (11) and *C. thermosaccharolyticum* (8) have also been reported to sporulate before exhaustion of the primary nutrients, in contrast to the usual type of sporulation in *Bacillus* species (7).

Although the present results indicate that high Se concentrations and high temperatures can lead to reduced spore levels, we are uncertain as to the causes of the extremely poor sporulation reported by Schiefer-Ullrich et al. (17). Sporulation is more sensitive to pH extremes than is vegetative growth (5, 10), and the medium reported here, with increased phosphate, decreased urate, and CO_2 present in the headspace, results in terminal pH values of 8.3 to 8.5 or 7.5 to 7.8 in the method described in the legend to Fig. 1, as compared with pH 9.0 reported by Schiefer-Ullrich et al. (17). The development of asporogenic strains, preventable by routine pasteurization, may also be responsible for very poor sporulation.

The defined medium and conditions described here make possible biochemical studies of the sporulation of *C. cylindrosporum*, a matter of some interest, considering the evidence implicating purine deprivation in the initiation of sporulation (7). Moreover, germination studies of these spores may provide valuable parallels to the interesting results now emerging from studies of the germination of *B. fastidiosus* (16) on uric acid.

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