APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1983, p. 1389–1393
0099-2240/83/041389-05$02.00/0

Vol. 45, No. 4

NOTES

Sporulation of *Clostridium acetobutylicum* P262 in a Defined Medium

SUSAN LONG, DAVID T. JONES, AND DAVID R. WOODS*

Department of Microbiology, University of Cape Town, Rondebosch, 7700, South Africa

Received 19 October 1982/Accepted 17 January 1983

A defined minimal sporulation medium for *Clostridium acetobutylicum* P262, which produces high levels of solvents, is described. The overall sporulation sequence was similar to that of other endospore-forming bacteria. However, we observed a presporulation stage, during which swollen phase-bright cells which contained large amounts of granulose formed. During sporulation, the initiation of spore coat formation occurred before the onset of cortex formation. Other *Clostridium* strains tested showed marked variations in ability to grow and sporulate in various minimal media.

The study of clostridium sporulation has lagged behind the study of aerobic sporeformer sporulation. Two important reasons for this are the technical difficulties of working with strict anaerobes and the complex nutritional requirements of most medically important clostridia. Although some saccharolytic clostridia grow in chemically defined minimal media, only one strain of *Clostridium pasteurianum* has been shown to sporulate well in a simple defined medium (12).

Clostridium acetobutylicum P262 appears to be the only strain used recently for the large-scale industrial production of acetone and butanol in the western world (17). Studies by Jones et al. (9) on the morphological changes which occur in *C. acetobutylicum* P262 during production of acetone, butanol, and ethanol in an industrial fermentation medium indicate that the swollen, phase-bright, presporulation-stage cells (clostridial forms) are involved in the production of acetone and butanol. This has been supported by the isolation of sporulation mutants which either fail to form clostridial stages and produce solvents or form reduced numbers of clostridial stages and produce intermediate levels of solvents (9). A relationship between sporulation and solvent production has also been suggested by Gottschal and Morris (5), who have shown that the loss of spore forming capability during continuous cultivation is associated with a loss of the ability to make solvents; however, solvent-producing cultures exhibit only a low frequency of sporulation. A previous study has reported that good solvent yields are associated with the ability to sporulate well (15). Although sporulation seems to be associated with solvent production by *C. acetobutylicum*, no studies on sporulation in a defined medium have been reported. We developed a chemically defined minimal sporulation medium for *C. acetobutylicum* P262 which will facilitate future studies on the regulation of sporulation and solvent production.

C. acetobutylicum P262 has been described previously (1, 2, 9, 18). ATCC 824 and 10132 and NRRL 527, 592, and 593 were used for comparative studies. All strains were maintained in the form of spore suspensions in sterile distilled water stored at 4°C. Spore suspensions were prepared from cultures grown anaerobically at 34°C on the buffered *Clostridium* basal medium (CBM) described by O'Brien and Morris (14) solidified with 1.5% agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.). After 3 days of incubation, the spores were scraped into distilled water, incubated in 1 mg of lysozyme per ml at 37°C for 30 min, and then washed three times in sterile distilled water. We studied sporulation in buffered CBM, reinforced *Clostridium* medium (RCM; Biolab Chemicals, Pretoria, South Africa), a glucose-mineral salts-biotin minimal medium (GSMM) (8), and the *C. pasteurianum* minimal medium (CPMM) of Mackey and Morris (12). The minimal media were used both with and without the following (per 100 ml of medium): 20 mg of $MgSO_4 \cdot 7H_2O$, 1 mg of $MnSO_4 \cdot 4H_2O$, 1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of *p*-aminobenzoic acid, 0.1 mg of thiamine hydrochloride, and 5 μg of biotin. Sucrose CBM contained 4% sucrose as the carbon source. The *C. acetobutylicum* P262 sporulation minimal me-

dium (CAMM) contained the following (per 100 ml of medium): 6 g of glucose, 0.6 g of $(NH_4)_2HPO_4$, 2 g of $CaCO_3$, 20 mg of $MgSO_4 \cdot 7H_2O$, 1 mg of $MnSO_4 \cdot 4H_2O$, 1 mg of $FeSO_4 \cdot 7H_2O$, 1 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.1 mg of *p*-aminobenzoic acid, 0.1 mg of thiamine hydrochloride, 5 μg of biotin, 0.05 g of cysteine hydrochloride, and 4 ml of the salt solution described by Holdeman et al. (8). $(NH_4)_2HPO_4$ was autoclaved separately and added to the sterile medium. Sterile 1 M potassium phosphate buffer was added to a final concentration of 0.05 M at pH 7.0. Volumes (50-ml) of warm, freshly prepared medium were allowed to reduce overnight in an anaerobic glove box (Forma-Scientific Inc., Marietta, Ohio) containing an atmosphere of oxygen-free N_2, CO_2, and H_2 (70:20:10 [vol/vol]). Solvent production was determined in a molasses fermentation medium (CFM) (9). Spores were activated by heat shock at 75°C for 2 min and then cooled on ice before inoculation of 5 μl of spore suspension into 10 ml of CBM. Sporulation media were inoculated with exponential-phase cells (optical density at 600 nm, 0.45) harvested from CBM and washed once with sterile distilled water. Cultures were incubated at 34°C and agitated every 6 to 8 h by gentle mixing. All manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box. Total bacterial counts, clostridial stage counts, and spore counts were determined with a Thoma counting chamber (Weber Scientific International, Lancing, England) and Zeiss photomicroscope fitted with phase- and interference-contrast optics.

The presence of capsules was determined by negative staining with India ink, the presence of granulose was determined by staining with iodine, and the presence of forespores was determined by the methods of Smith and Ellner (16) and Hoeniger and Headley (7).

The method of Kellenberger et al. (11) was used to fix samples of harvested cells. The fixed cells were dehydrated by passage through a series of acetone solutions of increasing concentration. The organisms were embedded in NC1010 Spurr low-viscosity embedding material (Polaron Equipment Ltd., Hertfordshire, England). Sections were cut with a glass knife on an ultramicrotome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined under a Zeiss 109 electron microscope at 80 kV.

Strain P262 grew well in all complex media tested, but only 20 to 30% sporulation was obtained (Table 1). These low levels of asynchronous sporulation, coupled with the complex nature of the media, severely limited the usefulness of these media for sporulation studies. Of the minimal media, only GSMM and CPMM, which contained the additional salts and growth factors, supported growth of strain P262; however, little or no sporulation occurred. In attempts to obtain a defined minimal medium which would support sporulation, both nutritional and buffering components of the media were varied. These studies resulted in the development of CAMM (see above), which was used in subsequent studies on sporulation. This medium routinely gave good (70 to 80%) levels of sporulation. The most important factor involved in increasing the level of sporulation appeared to be the improvement in the buffering components of CAMM.

In sporulation studies involving another species of butyric acid bacteria, Bowen and Smith (3) found that *C. pasteurianum* sporulation does not take place in a complex medium if the pH of the medium is allowed to fall below 5. In a study on sporulation of the same species in a minimal medium, Mackey and Morris (12) also found that the final pH of the medium has to be controlled to obtain sporulation. During growth of *C. acetobutylicum* P262, the pH of CAMM did not normally fall below 5.2. However, if the pH was allowed to fall to below 5.0, no sporulation occurred. Potassium phosphate and calcium carbonate were found to be more successful buffering agents than sodium bicarbonate, sodium phosphate, calcium lactate, or potassium lactate (unpublished data).

Sporulation of *C. acetobutylicum* and the sequence of morphological changes that lead to the production of clostridial forms and the eventual release of spores were investigated in CAMM (see Fig. 1 through 7). Optical and electron microscopic studies revealed a clearly defined series of stages associated with septum formation, engulfment of the forespore, and maturation and release of the mature spore (Fig. 1 through 7). These stages corresponded closely with the sporulation stages reported for other aerobic and anaerobic species of endospore-forming bacteria (4, 10, 12, 13, 19). In general, the ultrastructural changes that occurred during *C. acetobutylicum* P262 sporulation also resem-

TABLE 1. Sporulation of *C. acetobutylicum* strains in various media

Medium	% of phase-bright spores					
	P262	ATCC 824	ATCC 10132	NRRL 527	NRRL 592	NRRL 593
CBM	20	0	20	15	15	15
Sucrose CBM	30	0	25	30	30	25
RCM	20	0	20	5	5	5
GSMM	<1	<1	<1	0	<1	0
CPMM	0	—[a]	—	—	0	—
CAMM	70–80	10	—	—	20–30	—

[a] —, No growth.

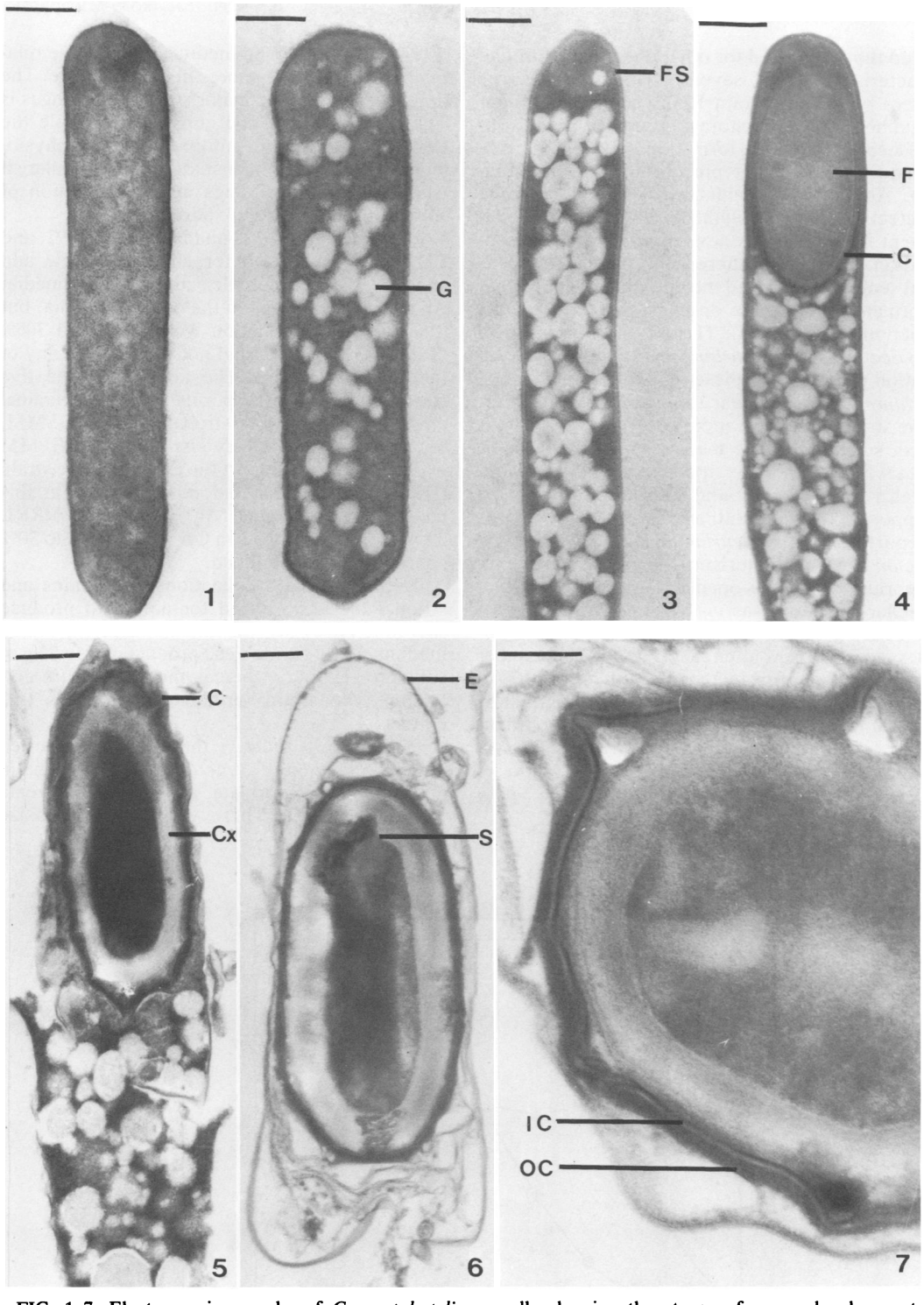

FIG. 1–7. Electron micrographs of *C. acetobutylicum* cells showing the stages of spore development. Abbreviations: N, nuclear material; G, granulose; FS, forespore septum; F, forespore; C, spore coat; IC, inner coat; OC, outer coat; Cx, cortex; S, spore; E, exosporium. (1) Vegetative rods; (2) granulose accumulation; (3) formation of spore septum; (4) completion of engulfment and spore coat deposition; (5) cortex formation and spore maturation; (6) mature spore within its triangular exosporium; (7) detail of spore structure showing inner and outer spore coats. Bars: Fig. 1 through 6, 0.5 μm; Fig. 7, 0.1 μm.

bled those reported for other endospore-forming bacteria (12), but several distinctive features were observed. Strain P262 accumulated large numbers of starch storage granules (granulose) associated with the formation of swollen cigar-shaped phase-bright presporulation cells (Fig. 2). Although accumulation of granulose made detection of axial filaments difficult, axial filament formation was never detected before septation. The major difference in the developmental sequence involved the initiation of spore coat formation before the onset of spore cortex formation (Fig. 4 and 5). This has also been shown to occur in *C. pasteurianum* (12), and the sporulation processes of these two butyric acid *Clostridium* species appear to be similar. However, the structures of the spore coat and the exosporium surrounding the mature spore differ. In *C. acetobutylicum*, the spore coat consisted of clearly defined inner and outer coats (Fig. 7), as opposed to the multilamellar coat structures reported for *C. pasteurianum* (12). *C. acetobutylicum* had a characteristic triangular-shaped exosporium which was open at one end. No multilamellar structure was visible (Fig. 6).

Phase-grey clostridial stages were first observed in CAMM after 25 h (Fig. 8). The number of clostridial forms increased until, by 44 h, 70 to 80% of the cells were swollen phase-bright clostridial forms with dark tips (forespores). Granulose accumulation occurred between 25 and 44 h. The dark forespore converted to a phase-bright forespore between 44 and 52 h. Spore release occurred after 52 h.

CAMM is a defined medium which gave relatively high yields of spores in batch culture. The time sequence of the major structural changes is sufficiently distinct and constant to enable the system to be used for future correlative physiological and biochemical studies. In particular, it will be useful for studies on the regulation of solvent production and sporulation.

ATCC 824 and 10132 and NRRL 527, 592, and 593 were also tested for ability to grow and sporulate on the complex and minimal media. All strains grew well on the complex media, but the levels of sporulation were low (0 to 30%) (Table 1). ATCC 824 did not sporulate in any of the complex media. The abilities of the five strains to grow and sporulate on the minimal media varied. All five strains grew on GSMM, but sporulation was very low or absent. CPMM was only able to support the growth of one strain (NRRL 592), which did not sporulate in this medium. Two strains (ATCC 824 and NRRL 592) were able to grow in CAMM, and 10 to 30% sporulation was obtained.

Both the culture collection type strains and strain P262 were tested for ability to produce solvents in CFM, the molasses fermentation medium (2, 9). Strain P262 produced 15 to 22 g of solvents per liter, whereas the five culture collection type strains produced low levels (<2 g/liter).

These results indicate that there are marked differences among *C. acetobutylicum* strains in ability to grow, sporulate, and produce solvents in various media. These variations emphasize

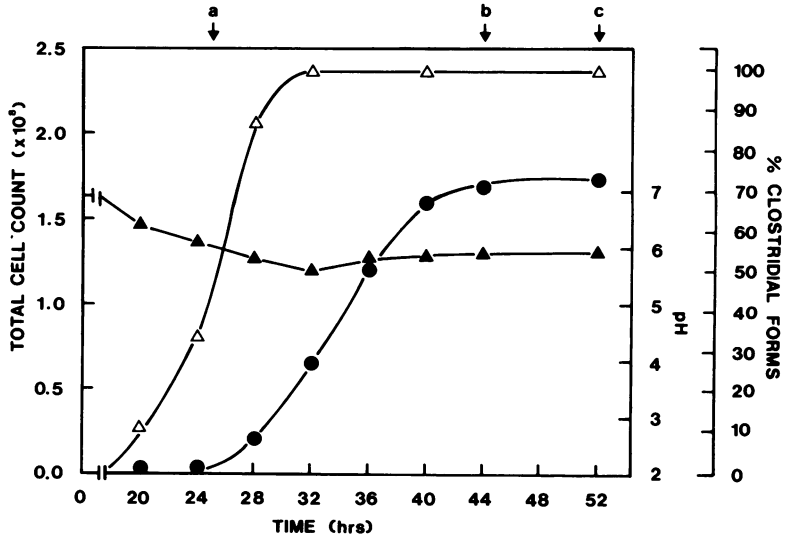

FIG. 8. Growth and sporulation of *C. acetobutylicum* P262 in CAMM. Symbols: △, total cell counts; ▲, pH; ●, percentage of clostridial forms. Times are shown for initiation of formation of clostridial forms (a), formation of phase-bright forespores (b), and spore release (c).

the importance of strain differences and support the recent statement by Gottschalk et al. (6) that, in general, the taxonomy of the genus *Clostridium* is still in an unsatisfactory state. Our results suggest that it will probably be necessary to determine the optimum conditions for each strain in order to obtain high levels of sporulation and solvent production.

LITERATURE CITED

1. **Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods.** 1981. Autolytic activity and an autolysis-deficient mutant of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **42**:929–935.
2. **Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods.** 1979. Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. Appl. Environ. Microbiol. **37**:433–437.
3. **Bowen, J. F., and E. S. Smith.** 1955. Sporulation in *Clostridium pasteurianum*. Food Res. **20**:655–658.
4. **Fitz-James, P., and E. Young.** 1969. Morphology of sporulation, p. 39–72. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press, Inc., London.
5. **Gottschal, J. C., and J. G. Morris.** 1981. Non-production of acetone and butanol by *Clostridium acetobutylicum* during glucose- and ammonia-limitation in continuous culture. Biotechnol. Lett. **3**:525–530.
6. **Gottschalk, G., J. R. Andersen, and H. Hippe.** 1981. The genus *Clostridium* (non-medical aspects), p. 1767–1803. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes, vol. 2. Springer-Verlag, Berlin.
7. **Hoeniger, J. F. M., and C. L. Headley.** 1968. Cytology of spore germination in *Clostridium pectinovorum*. J. Bacteriol. **96**:1835–1847.
8. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed., p. 145. Virginia Polytechnic Institute and State University, Blacksburg, Va.
9. **Jones, D. T., A. van der Westhuizen, S. Long, E. R. Allcock, S. J. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43**:1434–1439.
10. **Kay, D., and S. C. Warren.** 1968. Sporulation in *Bacillus subtilis*: morphological changes. Biochem. J. **109**:819–824.
11. **Kellenberger, E., A. Ryter, and J. Séchaud.** 1958. Electron microscopic study of DNA-containing plasms. Vegetative and mature phage DNA as compared with normal nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
12. **Mackey, B. M., and J. G. Morris.** 1971. Ultrastructural changes during sporulation of *Clostridium pasteurianum*. J. Gen. Microbiol. **66**:1–13.
13. **Murrell, W. G.** 1967. The biochemistry of the bacterial endospore. Adv. Microb. Physiol. **1**:133–251.
14. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
15. **Prescott, S. C., and C. G. Dunn.** 1959. Industrial Microbiology, 3rd ed., p. 250–284. McGraw-Hill Book Co., New York.
16. **Smith, A. G., and P. D. Ellner.** 1957. Cytological observations on the sporulation process of *Clostridium perfringens*. J. Bacteriol. **73**:1–7.
17. **Spivey, M. J.** 1978. The acetone/butanol/ethanol fermentation. Proc. Biochem. **13**:2–5.
18. **Webster, J. R., S. J. Reid, D. T. Jones, and D. R. Woods.** 1981. Purification and characterization of an autolysin from *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **41**:371–374.
19. **Young, M., and J. Mandelstam.** 1979. Early events during bacterial endospore formation. Adv. Microb. Physiol. **20**:102–162.