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Solvent Production and Morphological Changes in *Clostridium acetobutylicum*

D. T. JONES, A. VAN DER WESTHUIZEN, S. LONG, E. R. ALLCOCK, S. J. REID, AND D. R. WOODS*
Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 21 September 1981/Accepted 30 December 1981

The morphological and cytological changes which occurred in *Clostridium acetobutylicum* P262 during the production of acetone, butanol, and ethanol in an industrial fermentation medium were identified and correlated with the growth and physiological changes. The swollen, cigar-shaped clostridial forms were involved in the conversion of acids to neutral solvents, and there was a correlation between the number of clostridial forms and the production of solvents. Sporulation mutants which were unable to form clostridial stages (*cls* mutants) did not produce solvents. Oligosporogenous mutants which showed reduced clostridial stage formation produced intermediate levels of solvents. Sporulation mutants blocked after the clostridial stage, which were unable to form mature spores (*spo* mutants), produced normal levels of solvents.

Although fermentation technology is at present used mainly for the production of complex high-cost organic chemicals, the increasing cost of crude oil has resulted in renewed interest in the production of simple organic chemicals by fermentation processes (18, 22). Earlier in this century, acetone, butanol, and ethanol (ABE) fermentation involving *Clostridium acetobutylicum* was second in importance only to ethanol fermentation, and the potential of ABE fermentation is being reassessed (10, 17). At present, National Chemical Products Ltd., Germiston, South Africa, is one of the few companies still producing acetone and butanol by the traditional ABE fermentation process (17).

Physiological changes associated with solvent production in the ABE process have been well documented (14, 20). However, little has been reported about the relationship between morphological changes and solvent production in the ABE process. We describe the morphological changes which occur during ABE fermentation and the isolation of *C. acetobutylicum* sporulation mutants which were used to elucidate the relationships among morphology, growth, and solvent production.

MATERIALS AND METHODS

Bacterial strain and media. The *C. acetobutylicum* P262 strain which has been utilized and described previously (1, 2, 21) was used. The bacterium was grown at 34°C in the molasses fermentation medium (MFM) of Barber et al. (2), the buffered *Clostridium* basal medium (CBM) of O'Brien and Morris (13), a glucose-mineral salts-biotin minimal medium (7), and beef liver medium. All manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific Inc., Marietta, Ohio). The P262 strain was maintained at 4°C as a clean-spore preparation in distilled water. Sporulation mutants were freeze-dried or kept in beef liver medium. Spores were activated by heat shock at 75°C for 2 min and then cooled on ice before inoculation into CBM. Exponential-phase CBM cultures were then used to inoculate MFM.

Growth and morphology. Total bacterial counts, clostridial stage counts, and spore counts were carried out with a Thoma counting chamber (Weber Scientific International, Lancing, England) and a Zeiss photomicroscope fitted with phase-contrast and interference-contrast optics. Cells were investigated for the presence of capsules by negative staining with India ink, for granulose by staining with iodine, and for forespores by the methods of Smith and Ellner (16) and Hoeniger and Headley (6).

Physiological determinations. The production of ABE was measured by gas chromatography as described previously (2). Titratable acids were determined by titration with 0.02 N NaOH.

Isolation of sporulation mutants. Exponential-phase CBM cultures (5×10^7 cells per ml) of *C. acetobutylicum* P262 were treated with ethyl methane sulfonate (2.5%, vol/vol) for 20 min at 37°C to obtain a 10% survival rate. The washed cells were suspended in CBM, incubated for 18 h, diluted, and plated onto CBM agar containing rifampin (100 μg/ml). Spontaneous rifampin-resistant mutants were selected on gradient plates containing rifampin, as rifampin resistance and altered spore morphology have been shown to be linked in *Bacillus subtilis* (4, 15). Rifampin-resistant colonies were examined to determine whether they were also defective in sporulation. Granulose mutants were isolated by the method of Mackey and Morris (11).

RESULTS

Growth and physiological changes during ABE fermentation. Growth and physiological changes

were monitored in a typical ABE fermentation in MFM (Fig. 1a and b). The volumes used for the laboratory fermentation were scaled down from the industrial-level operation at National Chemical Products, which uses 12 90,000-liter fermentors. Observations were made on laboratory-scale fermentations, but these correlated well with the observations made on samples obtained from the factory. Exponential-phase cells in CBM were diluted 10^4-fold to give an initial cell concentration of 10^5 to 10^6 cells per ml in the MFM. Cultures inoculated in this way were characterized by a very short lag period or no lag period. During exponential growth (3 to 6 h), the doubling time of the culture was about 90 min. From 7 to 8 h the doubling time began to increase, and at 16 to 18 h no further increase in cell number occurred. The period between 7 and 18 h was associated with the production of titratable acids and a decrease in the pH from 6.8 to 5.1. Solvents were only detected after 18 h, when the culture was in the stationary growth phase. Solvent production correlated with a change in cellular morphology and the appear-

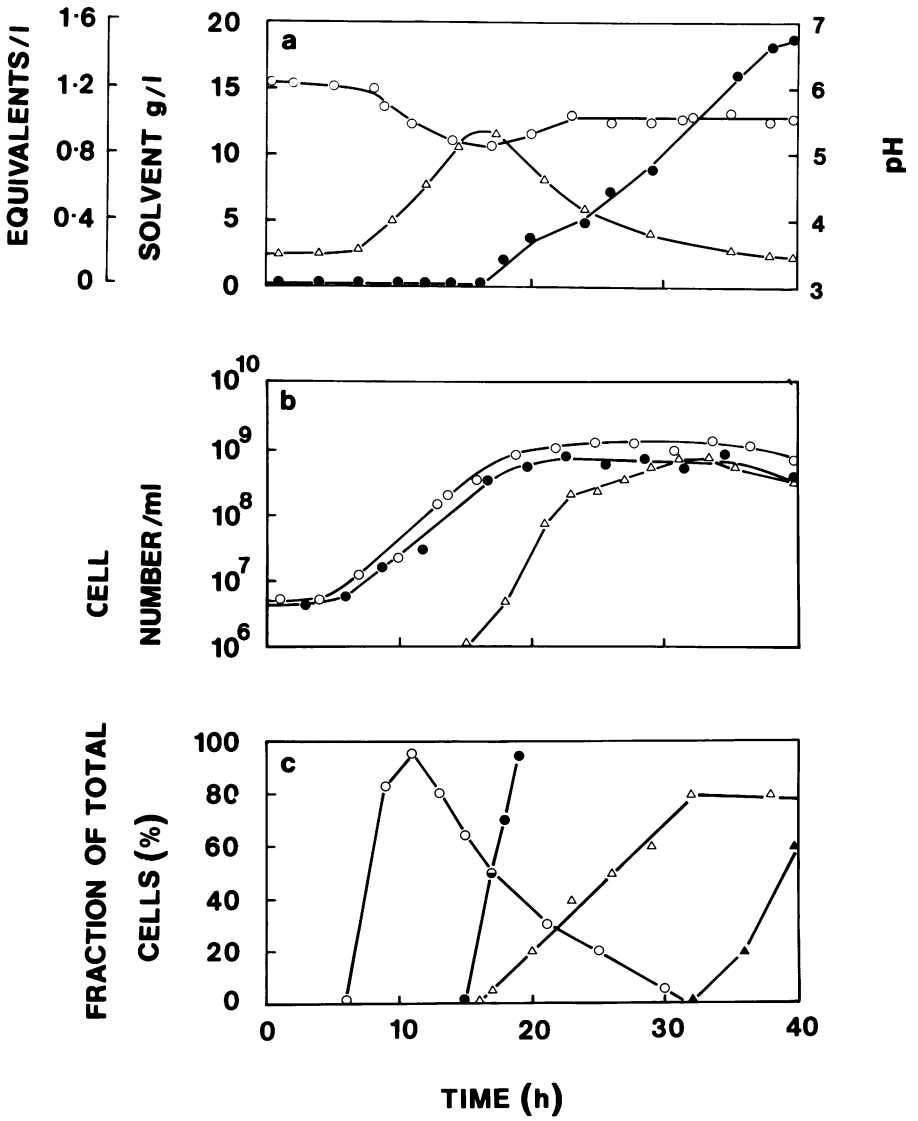

FIG. 1. Physiological (a), growth (b), and morphological (c) changes in *C. acetobutylicum* during ABE fermentation. (a) ○, pH; △, titratable acids; and ●, total solvents. (b) ○, Total cell count; ●, colony-forming units; and △, total clostridial form count. (c) ○, Motility; ●, onset of granulose accumulation; △, appearance of phase-bright clostridial forms; and ▲, appearance of forespores.

ance of clostridial forms (Fig. 1b). During the production of the solvents, the titratable acids decreased and the pH increased to about 5.6. The break in the pH was used to mark the changeover point between the early (active-growth) and late (zero-growth) stages in the fermentation process. Solvent levels increased between 18 and 36 h, before reaching a plateau. During the 18-h period, most of the vegetative rods were converted to clostridial forms.

Morphological and cytological changes during ABE fermentation. After inoculation into MFM, the cells appeared as elongated rods which developed septa and resulted in the formation of long chains of phase-dark, strongly gram-positive cells with sporadic and sluggish motility. About 6 h after inoculation, the chains began to break up and release individual rods which were highly motile (Fig. 2a). At this stage the growth rate was at its maximum and the rods were dividing. Cessation of active growth between 14 and 18 h correlated with a decrease in motility, and at 18 h most of the cells were nonmotile and the rest were sluggishly motile.

Granulose accumulation within the cells was first detected 1 to 2 h before the pH breakpoint, and within 2 h granulose accumulation could be detected in over 90% of the cells (Fig. 1c and 2b). By 30 h the majority of cells (>90%) had formed typical swollen, phase-bright, gram-positive clostridial forms (Fig. 2c and d). The minority of cells (<10%) which did not form a clostridial stage became gram negative and underwent degradative changes. Another cytological change which was associated with the conversion of vegetative rods into clostridial forms was the production of a clearly defined extracellular capsule (Fig. 2e).

In factory fermentations, which were usually stopped after 36 h, sporulation was not normally observed. In fermentations which were allowed to continue, sporulation occurred in a small proportion of the cells. The developmental stages were similar to those reported for other bacilli and clostridial strains (5, 9, 11, 12, 19). In fermentations which produced high levels of solvents, the clostridial forms did not develop further but degenerated before sporulation occurred.

Sporulation mutants. Two groups of mutants were obtained after treatment of *C. acetobutylicum* P262 with ethyl methane sulfonate (Table 1). In the first group the mutants were blocked before stage II and were unable to produce phase-bright, swollen clostridial forms. These were designated *cls* mutants and remained vegetative rods which were unable to synthesize granulose and did not produce capsules or forespore septa. The *cls* mutants which were obtained from rifampin plates (*cls*-1 and *cls*-2) were resistant to rifampin and presumably had mutations in the gene(s) coding for RNA polymerase. The *cls* mutants which were isolated as granulose-negative mutants (*cls*-3 and *cls*-4) were rifampin sensitive and presumably had mutations in the gene(s) coding for the granulose biosynthetic pathway.

The second group of mutants which were blocked after stage II were able to accumulate granulose and produced typical clostridial forms surrounded by a capsule, but were totally or partially blocked in the production of mature spores (Table 1). These were designated *spo* mutants and were either asporogenous (*spo*-1 and *spo*-2) or oligosporogenous (*spo*-3 and *spo*-4), depending on whether their ability to produce mature spores was completely or partially blocked.

In all of the *cls* and *spo* mutants except *cls*-2 the growth rate, vegetative cell development, and final cell concentrations were similar to those of the P262 strain. The *cls*-2 mutant grew more slowly and produced a lower final cell concentration.

Solvent production by sporulation mutants. Solvent production by the eight sporulation mutants and the P262 strain was compared in MFM. In all of the experiments the MFM was inoculated with exponential-phase cells growing in CBM. The P262 strain produced the same levels of solvents in MFM whether inoculated with CBM cultures propagated from spore suspensions or with beef liver medium cultures (Table 1). The four *cls* mutants which were unable to form clostridial stages did not produce solvents. The two asporogenous *spo* mutants, which produced clostridial forms at the same frequency as the P262 strain but were unable to sporulate, produced solvents in amounts similar to those produced by the P262 strain. The two oligosporogenous *spo* mutants, which formed reduced numbers of clostridial stages and spores, produced intermediate levels of solvents.

Solvents were not produced when *C. acetobutylicum* P262 was grown in glucose minimal medium. We showed previously that clostridial forms are not produced in minimal medium (S. Long, D. T. Jones, and D. R. Woods, submitted for publication).

DISCUSSION

Studies on the morphological changes in the ABE fermentation system suggested that the clostridial forms of *C. acetobutylicum* were involved in the conversion of acetate and butyrate to acetone and butanol. This idea was supported by the isolation of sporulation mutants which either failed to form clostridial stages and pro-

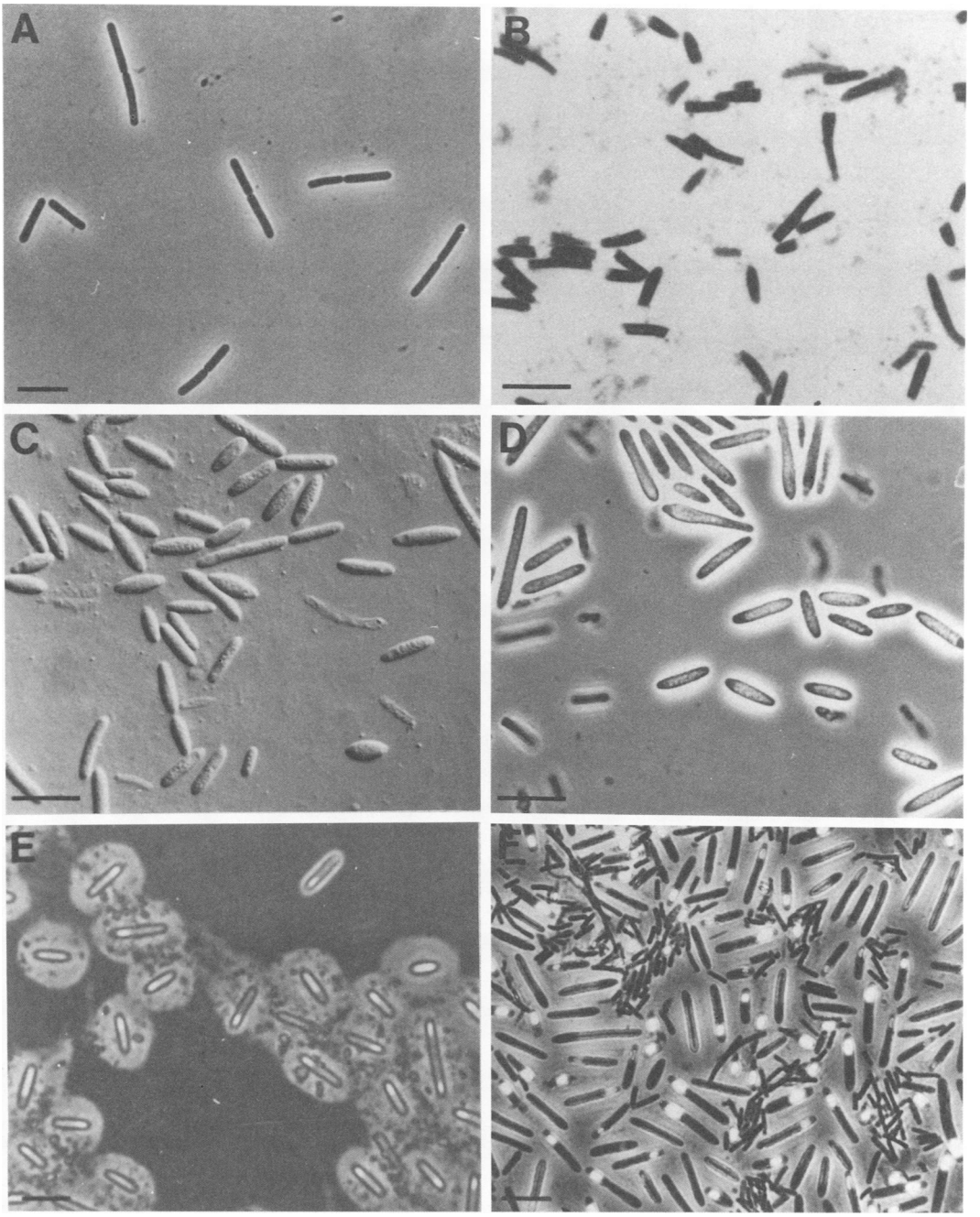

FIG. 2. Photomicrographs of cytological changes in *C. acetobutylicum* during ABE fermentation. (A) Actively growing phase-dark vegetative rods. (B) Iodine-stained clostridial forms showing granulose accumulation and unstained forespores. (C) Swollen, cigar-shaped clostridial forms viewed with interference-contrast optics. (D) Phase-bright clostridial forms with phase-dark forespores. (E) Negatively stained clostridial forms and sporulating rods. (F) Sporulating rods with phase-bright spores. Bar, 10 μm.

TABLE 1. Characteristics of the *C. acetobutylicum* sporulation mutants

Strain	Characteristic[a]							Solvent production[c] (g/liter)
	Clostridial forms	Granulose	Capsule	Septum	Forespore	Mature spore	Rifampin[b]	
P262	++	++	++	++	++	++	s	16.864
cls-1	−	−	−	−	−	−	r	0.168
cls-2	−	−	−	−	−	−	r	0.489
cls-3	−	−	−	−	−	−	s	0.357
cls-4	−	−	−	−	−	−	s	0.196
spo-1	++	++	++	+	−	−	r	16.125
spo-2	++	++	++	+	−	−	r	15.411
spo-3	+	+	+	+	+	+	r	1.077
spo-4	+	+	+	+	+	+	r	2.501

[a] ++, Normal wild-type levels of sporulation features; +, reduced levels of sporulation features; −, no sporulation features.
[b] s, Sensitive to rifampin (20 ng/ml); r, resistant to rifampin (100 μg/ml).
[c] Determined after 48 h.

duced no solvents or formed reduced numbers of clostridial stages and produced intermediate levels of solvents. The identification of a distinct morphological stage associated with solvent production and the isolation of *spo* mutants which can be maintained as clostridial forms are being investigated further in a continuous culture system.

A limiting factor in ABE fermentation is butanol toxicity. One way to overcome this problem is to search for butanol-tolerant strains or mutants. Our results indicate that enrichment or mutation-screening programs must involve the clostridial stage and not the vegetative cell.

Our results confirm those of Davies and Stephenson (3) and O'Brien and Morris (13), who reported that acetate and butyrate, but not butanol and acetone, are produced by *C. acetobutylicum* during exponential growth. *Clostridium thermosaccharolyticum* is similar in that butyric acid is produced as a major fermentation product during exponential growth (8). Induction of sporulation in *C. thermosaccharolyticum* was associated with the formation of glucose 6-phosphate dehydrogenase and ethanol dehydrogenase and the production of ethanol instead of butanol as the major fermentation product.

The identification of the morphological and cytological stages of *C. acetobutylicum* can be used as a simple, practical means of monitoring the progress of ABE fermentation and as an aid in identifying the cause of failed fermentations (17).

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