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Isolation and Characterization of Butanol-Resistant Mutants of *Clostridium acetobutylicum*

MONIQUE HERMANN,[1] FRANÇOISE FAYOLLE,[1] RÉMY MARCHAL,[1] LAURENCE PODVIN,[1] MADELEINE SEBALD,[2] AND JEAN-PAUL VANDECASTEELE[1]*

Direction Biotechnologie et Environnement, Institut Français du Pétrole, 92506 Rueil-Malmaison,[1] and Unité des Anaérobies, Institut Pasteur, 75015 Paris,[2] France

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In a wild-type strain of *Clostridium acetobutylicum* isolated from soil, solvent production appeared limited by butanol toxicity. Butanol-resistant mutants have been obtained which produced significantly higher solvent concentrations (about 30%) than the wild-type strain. Some other physiological differences were observed between a selected resistant mutant and the wild-type strain at the level of solvent resistance and sporulation.

The possibilities offered by the acetone-butanol fermentation for the valorization of biomass have led to a renewed interest in this fermentation. These possibilities have justified a research and development program which provided the framework for the present study.

As is well known, an important limitation of this fermentation is the total solvent concentration obtained, which frequently approaches 20 g liter^{-1}, and it is usually accepted that this limitation results from butanol toxicity (16, 18). In the present work, mutants resistant to butanol were isolated, and some of their physiological properties were described. In particular, their capacity for solvent production was examined. A preliminary report of this work has been presented elsewhere (8).

MATERIALS AND METHODS

General microbiological conditions. All microbiological work was performed under conditions of sterility and strict anaerobiosis. Anaerobic handling and incubation of the cultures were performed in an anaerobic glove box (Forma Scientific) with a controlled atmosphere (10% H_2, 10% CO_2, 80% N_2) or in sealed serum vials or pressure tubes by the anaerobic technique of Hungate (10) modified by Balch and Wolfe (2).

Strain origin and maintenance. *Clostridium acetobutylicum* 903 was isolated from soil by enrichment culture on Jerusalem artichoke medium by P. Kayser (Institut National Agronomique, Paris, France). For maintenance, cultures of this strain and of the mutants derived from it were propagated in anaerobic test tubes on prereduced potato or Jerusalem artichoke medium containing (per liter) 250 g of cooked potatoes (or Jerusalem artichoke tubers), 10 g of glucose, 2 g of ammonium sulfate, and 2 g of calcium carbonate.

Isolation of butanol-resistant mutants. PY medium was used and prepared as described by Holdeman et al. (9) or by Smibert and Krieg (20), except that vitamin K_1 and hemin were omitted. The carbon source was 10 g of glucose liter^{-1}. Exponential-phase cultures of PY medium of *C. acetobutylicum* 903 were plated on PY agar plates (0.1 ml plate^{-1}) containing various amounts (5, 7, 10, and 12 mg ml^{-1}) of *n*-butanol. A crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) was added at the center of each plate. The plates were then incubated at 34°C for 48 h. Colonies growing at butanol concentrations which did not allow growth of the wild-type strain were selected and purified before testing their solvent production.

Growth inhibition studies. The medium used contained (per liter of distilled water): CH_3COOH, 2.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4$, 10 mg; $MnSO_4$, 10 mg; yeast extract, 0.5 g; glucose, 30 g. The pH was adjusted to 6.7 by addition of ammonia. The cultures were carried out in sealed anaerobic tubes (18 by 150 mm) containing 5 ml of medium. Addition of oxygen-free solvents was performed in the anaerobic chamber by injection with a syringe through the rubber stoppers of the sterile tubes. These tubes were inoculated at a 5% level with 5% 16-h-old culture on the same medium and incubated at 35°C under static conditions. Growth was estimated by measurement of A_{600} with a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) spectrophotometer.

Solvent production experiments. Preliminary tests were conducted at 34°C in anaerobic tubes under static conditions, and the medium used (medium M) contained (per liter of distilled water): $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4$, 10 mg; $MnSO_4$, 10 mg; yeast extract, 4 g. The carbon source was glucose or hydrolyzed Jerusalem artichoke juice prepared as described below (total sugar concentration, 60 g liter^{-1}). It was supplemented with 3 g of $CaCO_3$ liter^{-1} to limit the pH decrease during growth.

Fermentation experiments were carried out in 6-liter laboratory fermenters (Biolafitte, St. Germain en Laye, France) containing 4 liters (final volume) of acid-hydrolyzed Jerusalem artichoke juice supplemented with 3 g of $(NH_4)_2SO_4$ liter^{-1} and 3 g of $CaCO_3$ liter^{-1}. Jerusalem artichoke juice was obtained by pressing fresh, sliced Jerusalem artichoke tubers. The juice was hydrolyzed by H_2SO_4 (45 min at 90°C at pH 2) and then neutralized to pH 6.8 by $Ca(OH)_2$. After sterilization (40 min at 120°C) of the fermenter containing the medium, anaerobic conditions were maintained by a continuous flow of sterile nitrogen gas in the fermenter headspace until inoculation, which was performed with 200 ml of an anaerobic 20-h-old flask culture of the same medium. The fermentation was carried out at 35°C with slow stirring. The initial pH was set at 6.8 and was not regulated throughout the fermentation, which was usually terminated within 36 to 40 h.

Fermentation progress was monitored by measurement of

* Corresponding author.

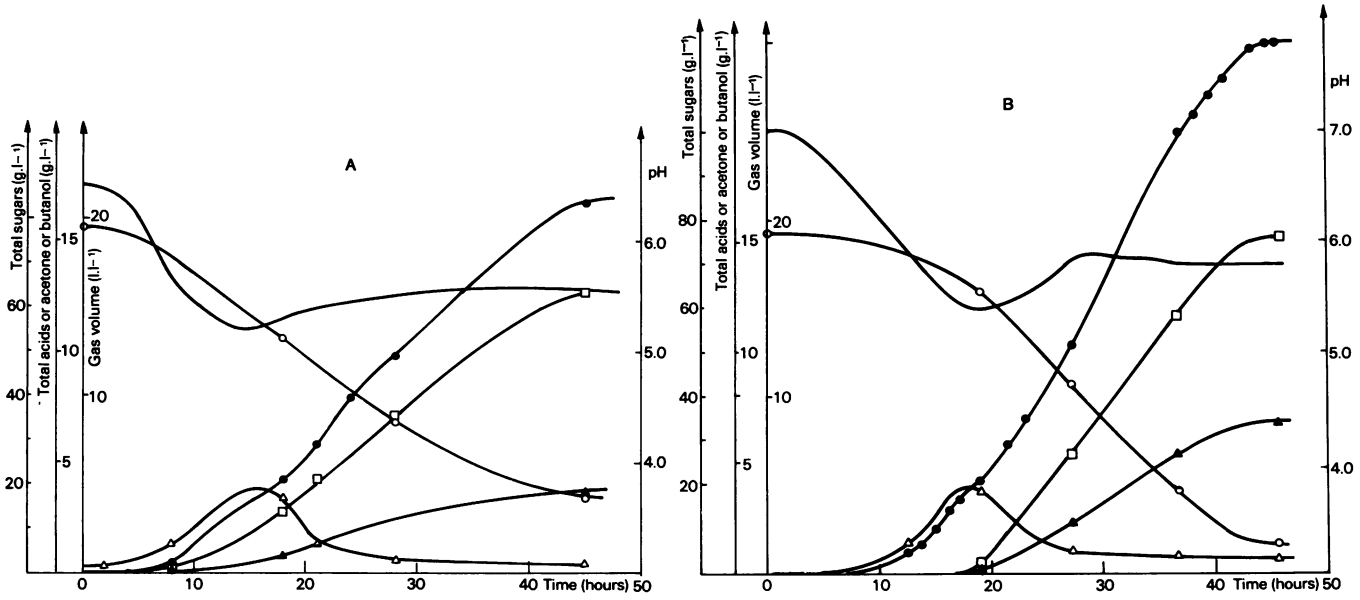

FIG. 1. Production of solvents by *C. acetobutylicum* in a laboratory fermenter. (A) Strain 903; (B) strain 904. The carbon source was hydrolyzed Jerusalem artichoke juice. Experimental conditions were as described in Materials and Methods. Symbols: ——, pH; ○——○, total sugars (fructose plus glucose); △——△, total acids (acetic plus butyric); □——□, butanol; ▲——▲, acetone; ●——●, total gases produced (H_2 plus CO_2).

the volume of gases produced (gas volumetric counter of 1 dm^3; Flonic, Colombes, France), and samples were taken for determination of sugars, acids, and solvents.

Sporulation. The sporulation medium was medium D of Sacks and Thompson (19). Precultures of the bacteria were performed on the medium of Davies and Stephenson (6). While in exponential growth, these were used to inoculate at a 10% level flasks containing 125 ml of medium D. These static cultures were incubated at 34°C in an anaerobic chamber. Samples were taken at different times and used for the determination of the total viable cells and the heat-resistant spores. Viable cell counts were done by plating after dilution portions of the samples on the medium of Davies and Stephenson (6) supplemented with agar. Determination of the heat-resistant spores was carried out by plating on the same medium portions which had been heated for 10 min at 80°C.

Sporulation rates are expressed as the percentage of heat-resistant bacteria at the time of measurement with respect to the total viable population at the time of maximum growth. Similar determinations were performed on cultures grown on media described below (Jerusalem artichoke, potato, and medium M-glucose).

Analytical methods. The presence of sugars, acids, and solvents was determined on centrifuged culture samples.

Assay of sugars (fructose and glucose) was performed by the enzymatic-spectrophotometric method involving hexokinase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase (4), which allows the individual determinations of both sugars (kit from Boehringer GmbH, Mannheim, Federal Republic of Germany).

The concentrations of solvents and acids were measured by flame ionization gas chromatography. The presence of solvents (butanol, acetone, and ethanol) was determined by the method of Barber et al. (3). The presence of acetic and butyric acids was determined on a column (8 mm by 2 m) packed with 25% neopentyl glycoladipate on 110/120 mesh Chromosorb W acid washed at a temperature of 150°C.

RESULTS

Isolation of butanol-resistant mutants. Butanol-resistant mutants of strain 903 were obtained as described in Materials and Methods in an effort to select better solvent-producing strains. After 48 h of incubation, no growth inhibition was observed on plates containing 5 and 7 mg of butanol ml^{-1}. A clear background with no growth was observed on plates with 12 mg of butanol ml^{-1}. Butanol-resistant colonies were obtained around the zone of inhibition by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on plates containing 10 mg of butanol ml^{-1}. Several of these mutants were picked and purified on PY plates. Their resistance to butanol was tested again before measuring their solvent production. No spontaneous mutant was obtained on control plates without *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine after 48 h of incubation. Another mutagen, ethyl methane sulfonate, was tested and yielded no butanol-resistant mutant.

Solvent production by butanol-resistant mutants. Screening for better solvent producers among butanol-resistant mutants was first carried out in test tubes with two carbon sources, glucose or hydrolyzed Jerusalem artichoke juice. With either carbon source, many mutants were found which produced solvent concentrations significantly higher than those produced by the wild-type strain (data not shown).

More detailed comparative experiments were performed in laboratory fermenters with wild-type strain 903 and a selected butanol-resistant mutant (strain 904). Similar fermentation patterns were obtained in these conditions and are illustrated in Fig. 1 with a medium containing, as carbohydrate source, Jerusalem artichoke, a potentially interesting crop for fermentation. A classical fermentation pattern is apparent, with a first stage of growth and acid production, followed by a second stage of solvent production with reutilization of part of the acids produced. Determination of gas evolution usually correlates well with solvent production and is convenient for estimation of the course of the fermentation. The total fermentation time was generally below 48 h

TABLE 1. Solvent production in laboratory fermenters by the wild-type strain 903 and the butanol-resistant strain 904 of *C. acetobutylicum*[a]

Strain and expt no.	Production (g liter^{-1}) of:			Butanol/acetone ratio	Production of acetic and butyric acids (g liter^{-1})	Total gas produced (liter liter^{-1})	Gas balance[c]	Sugars (g liter^{-1})		Solvent yield[d]
	Butanol	Acetone	Total solvents[b]					Utilized	Residual	
903										
1	12.8	3.8	16.6	3.36	0.3	21.25	0.93	55	12	0.30
2	12.0	3.2	15.2	3.75	1.5	20.5	0.92	47	24	0.32
3	10.4	7.1	17.5	1.42	1.5	22.5	1.29	56	0.5	0.31
4	11.1	4.4	15.5	2.53	1.2	23	0.93	50	30	0.31
5	11.3	2.9	14.2	3.89	2.0	17.1	1.05	40	13	0.35
904										
6	15.2	6.9	22.1	2.2	0.7	30	1.02	70.5	6.5	0.31
7	13.0	6.1	19.1	2.13	1.5	28	0.99	60.6	5.7	0.31
8	13.1	7.2	20.3	1.82	3.5	36.75	0.91	59.6	12.0	0.34
9	13.5	6.0	19.5	2.25	0.6	27.5	0.98	51.6	11.1	0.38
10	13.0	6.1	19.1	2.13	ND[e]	26	1.01	56.2	12.3	0.34

[a] Fermentation was conducted on hydrolyzed Jerusalem artichoke juice as described in Materials and Methods.
[b] Ethanol production (≤0.3 g liter^{-1}) is not included.
[c] Ratio of the theoretical gas production (calculated from the measured concentrations of solvents and acids with the stoichiometric equations of product formation from sugars) to the measured gas production.
[d] Mass ratio of the total solvents produced to the sugars consumed.
[e] ND, Not determined.

for both strains. As ethanol production usually was below 0.3 g liter^{-1}, only the curves for butanol and acetone are presented.

Representative results obtained in these fermentation conditions for five different runs with strain 903 and butanol-resistant mutant 904 are given in Table 1. Gas balances and yields which allow evaluation of the internal coherence of the data are presented. Clearly, better solvent productions were obtained with strain 904 (19 to 22 g liter^{-1}) than with strain 903 (14 to 17.5 g liter^{-1}). This point is also illustrated in Table 2, which presents the average solvent productions calculated for both strains on a large series of fermentations. The data also show that the average butanol/acetone ratios, despite some dispersion in the case of strain 903, were significantly higher for strain 903 than for strain 904.

Growth inhibition by solvents. The influence of various concentrations of butanol and other solvents on the growth curves of strains 903 and 904 was compared. The growth curves obtained in the presence of butanol are shown in Fig. 2. Two distinct inhibitory effects were observed. First, the maximal growth rate was reduced by high solvent concentrations. With butanol, severe inhibition was already observed at concentrations below 10 g liter^{-1}, whereas with the three other solvents tested, concentrations of 40 g liter^{-1} or higher were necessary to result in strong growth inhibition (Fig. 3). Solvents were also found to affect the final growth yield of the culture. At solvent concentrations which resulted in strong reduction of the growth rate, the final growth yield also was strongly decreased (Table 3). Butanol-resistant mutant 904 was more resistant than strain 903 to the four solvents tested, butanol, acetone, ethanol, and methanol. Depending on the solvent tested, the differences between strains were more apparent on one or the other type of inhibitory effect considered. In the case of acetone for example, no significant difference on the inhibition of growth yield was observed (Table 3).

TABLE 2. Comparison of solvent production performances of *C. acetobutylicum* 903 and 904[a]

Strain[b]	Avg production (SD) (g liter^{-1}) of:			Avg butanol/acetone ratio (SD)
	Total solvents	Butanol	Acetone	
903	14.7 (1.9)	11.0 (1.1)	3.7 (1.2)	3.22 (0.76)
904	20.7 (1.0)	14.1 (0.8)	6.6 (0.6)	2.16 (0.24)

[a] The fermentation conditions were as described in Table 1.
[b] For 903, the average of 16 fermentations was taken; for 904, the average of 14 fermentations was taken.

Sporulation studies. Sporulation of the mutant strain 904 was compared with that of strain 903 on a sporulation medium (medium D) previously described for *Clostridium perfringens* (19). Determinations of viable cells and heat-resistant spores were carried out on the cultures of the two strains, and the results are shown in Fig. 4. In both cases, the number of heat-resistant spores, which was nil at the beginning of the culture, increased rapidly at the end of the growth phase, after which the viability of vegetative cells decreased considerably. However, the percentages of sporulated cells after 120 h of incubation were 2.8% for strain 903 and 0.08% for mutant strain 904.

The differences in sporulation rates between the two strains were even more apparent in similar experiments conducted on culture media routinely used in this laboratory for acetone-butanol fermentation. The sporulation rates obtained with these media were higher for strain 903 than with medium D but remained very low for strain 904 (Table 4). The loss of viability observed after the end of the growth phase, as expressed in the viability rates presented in Table 4, was also greater with strain 904 than with strain 903. In addition, in all media tested, strain 904 presented a higher growth than strain 903, as shown by the maximal cell counts presented in Fig. 4 and Table 4.

DISCUSSION

Butanol-resistant mutants of *C. acetobutylicum* have been recently obtained by methods not involving mutagenesis either by a serial enrichment procedure (13) or by simple spreading on agar slope plates with a butanol concentration gradient (M. Zygmunt, Ph.D. thesis, University of Lille, Lille, France, 1983). With our method, the mutant yield in the absence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was quite poor.

Preliminary results showed that a significant proportion of butanol-resistant mutants grown in tubes produced more

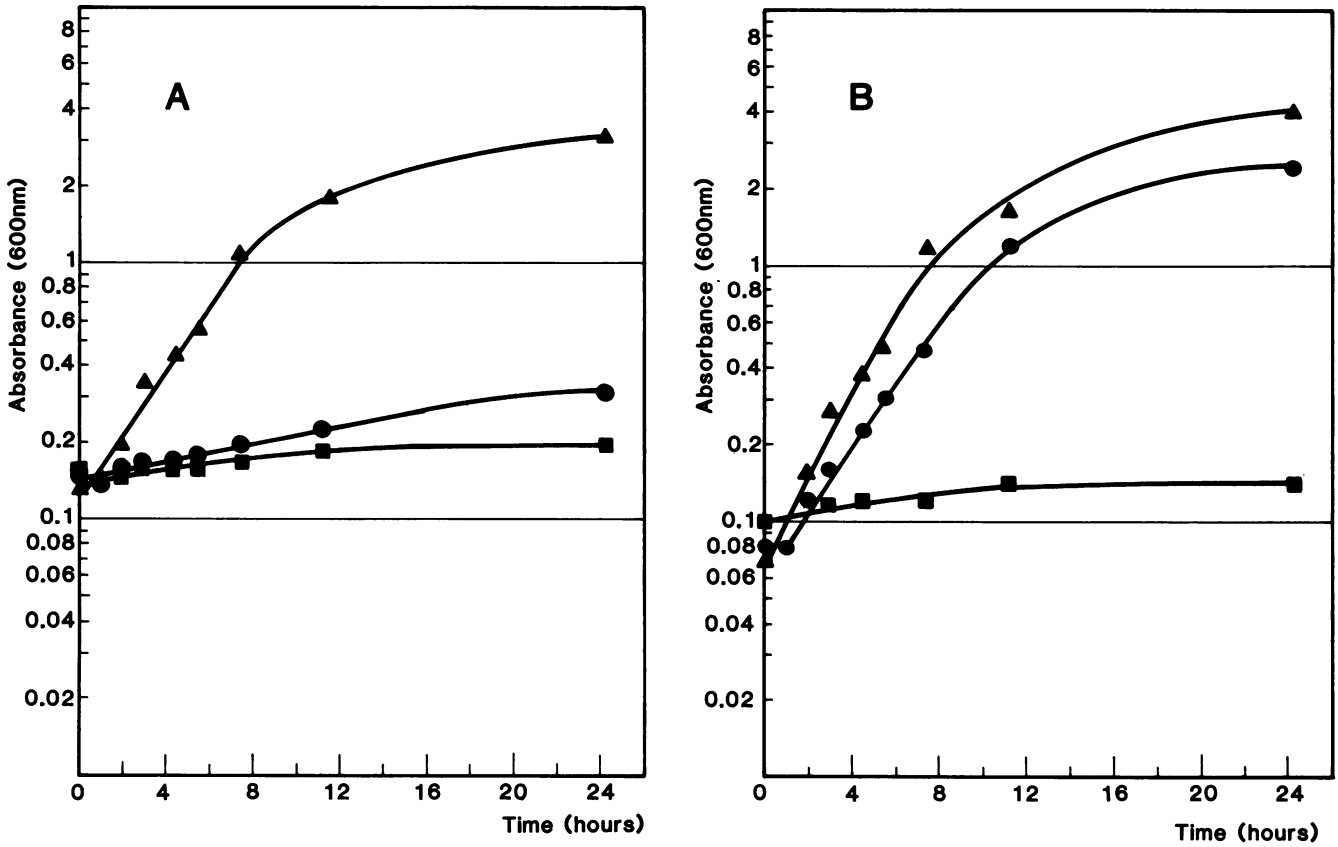

FIG. 2. Effect of added butanol on growth of *C. acetobutylicum* wild-type strain 903 and butanol-resistant mutant 904. (A) Strain 903; (B) strain 904. Culture conditions were as described in Materials and Methods. Symbols: ▲, no butanol added; ●, 8 g of butanol liter^{-1}; ■, 10 g of butanol liter^{-1}.

solvents than the wild-type strain (8). However, more complete assessment of the respective capacities of wild-type and butanol-resistant strains for solvent production required experiments under a variety of conditions since performance is strongly dependent on culture conditions. Emphasis has been placed here on cultures in fermenters in which conditions (pH profile and sugar utilization, for example) can be well determined and which also yield higher and more reproducible results. The results presented above show that, under the conditions used, strain 904 produced 30 to 40% more solvents than strain 903. Although still-higher performances were obtained with strain 904 immediately after isolation, the subsequent results obtained for solvent production and other physiological characteristics reported here were found stable over a period of several years in the absence of selective pressure. The reproducibility of the fermentation performance in particular was found satisfactory in tests, including a series of pilot runs in a 3,000-liter fermenter. The productivities repeatedly obtained at this level (20 to 22 g of solvents liter^{-1} in 36 h) are among the highest reported for butanol-acetone fermentation.

As shown by the differences in butanol/acetone ratios, acetone accounted for a relatively larger part of the increase in solvent production by strain 904, but butanol production also was clearly increased. These results are different from those of Lin and Blaschek (13), who obtained with butanol-resistant mutant SA-1 more butanol but except a very high substrate concentrations somewhat less total solvents than with the wild-type strain ATCC 824. Culture conditions may explain at least partially these different results. In our case, higher sugar concentrations did not increase the solvent yield but merely resulted (Table 1) in a partial utilization of the carbon source. In the culture of strain ATCC 824 on starch, however, amylase activity (and thus carbon source availability) appears, as pointed out by the authors, a limiting factor of solvent production, thus interfering with solvent yield and possibly with solvent distribution.

With both strains 903 and 904, inhibitory effects of added butanol became important only at concentrations approaching that of complete inhibition. In the case of other solvents, inhibitory effects are more gradual. In all cases, not only is the maximal growth rate lowered as already reported (13, 16), but the final growth yield is also clearly decreased at high solvent concentrations. Although not readily interpreted, this effect also has been recently reported in the case of ethanol inhibition in *Clostridium thermohydrosulfuricum* (15). This effect on growth yield makes final growth measurements at 24 h a reliable way to determine the inhibitory capacity of the added solvent, which can then be expressed by the minimal inhibitory concentration above which no growth is observed.

The results indicate that strain 903 presents a resistance to butanol a little lower than that of strain ATCC 824, which is strongly inhibited by 10 to 11 g of butanol liter^{-1} (13, 16, 18). The higher resistance to ethanol of strain 903 is also in agreement with the results of Moreira et al. (16). The increase in resistance to butanol is lower than that reported by Lin and Blaschek (13), but the relationship between

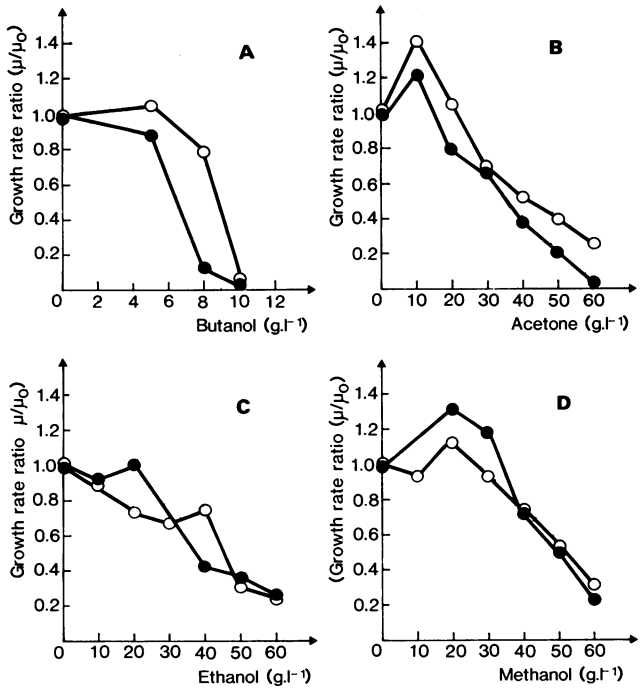

FIG. 3. Inhibition of the growth rate of *C. acetobutylicum* wild-type strain 903 and butanol-resistant mutant 904 by solvents. Culture conditions were as described in Materials and Methods. The solvents tested were butanol (A), acetone (B), ethanol (C), and methanol (D). Their effects are expressed as the ratio of the maximal growth rates obtained from the growth curves, μ/μ_0, where μ is with solvent and μ_0 is without solvent. Average μ_0 values in these conditions were 0.0055 and 0.006 min^{-1} for strains 903 and 904, respectively. Symbols: ●, strain 903; ○, strain 904.

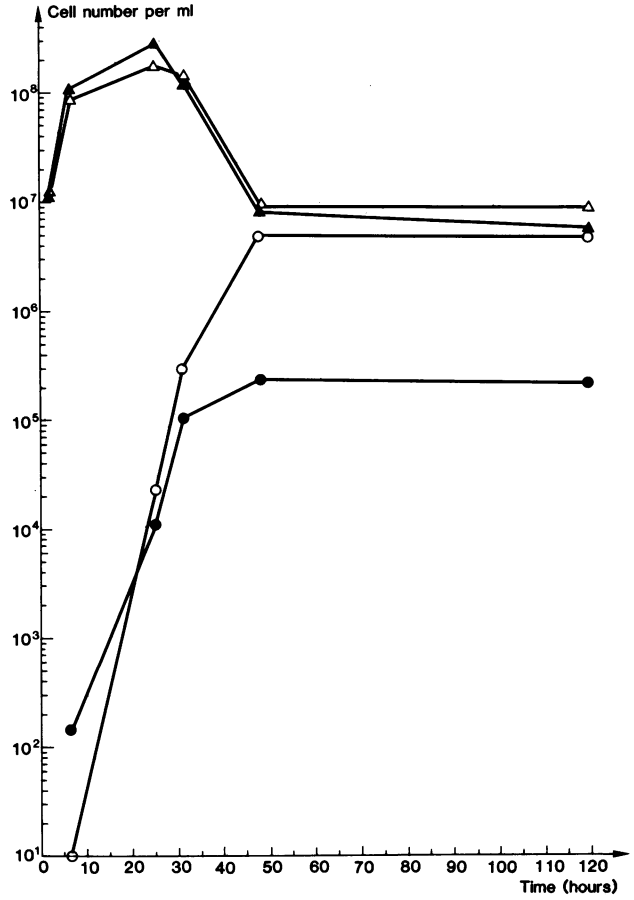

FIG. 4. Time course of cell viability and sporulation in cultures of *C. acetobutylicum* wild-type strain 903 and butanol-resistant mutant 904. The culture was carried out on D medium as described in Materials and Methods. Total viable cell count for strain 903 (△) and strain 904 (▲) is shown. Heat-resistant spore count for strain 903 (○) and strain 904 (●) is shown.

solvent production capability and resistance to butanol is probably not simple. In the case of ethanol production, for example, in which mutants presenting a resistance to ethanol increased by factor of severalfold have recently been reported (1), there was no direct correlation between these two aspects, probably because of the complexity of the mechanism of resistance involved. The important point shown here is that an increase in resistance to butanol as found in strain 904 however limited may bring about a clearly significant increase in solvent production. Our results also indicate that the increased resistance of strain 904 is not specific to butanol but also extends to other alcohols, e.g., ethanol and methanol. This suggests that the mutation involved is of a general nature, possibly a change in the lipid composition of the membrane, which is well known to be influenced by solvents as shown in studies of the effect of ethanol on various microorganisms (5, 7, 11, 17). For example, a modification of the membrane structure which would make it more resistant to the fluidizing effect of solvents would be in line with the recently reported fluidizing effect of butanol on the lipid regions of *C. acetobutylicum* (21).

The fact that strain 904 is an oligosporogenous mutant raises the question of the relationship between solvent production and sporulation. Solvent production starts at about the pH breakpoint and takes place during a period in which growth as judged by viable cell count slows down and stops (Fig. 1). We observed (data not shown) that this period of solvent production coincides with the onset of sporulation as characterized by morphological changes and resistance to chloroform of the bacteria. Heat resistance, characteristic of mature spores, appears later when solvent production has

TABLE 3. Inhibition of growth yield by solvents in *C. acetobutylicum*

Solvent concn (g $liter^{-1}$)	Strain	% Inhibition of growth yield[a] by added solvent			
		Butanol	Ethanol	Methanol	Acetone
0	903	0	0	0	0
	904	0	0	0	0
8	903	94	ND	ND	ND
	904	42	ND	ND	ND
10	903	99	ND	ND	ND
	904	99	ND	ND	ND
30	903	ND	ND	ND	80
	904	ND	ND	ND	86
40	903	ND	95	61	97
	904	ND	66	52	96
50	903	ND	96	95	99
	904	ND	97	78	97
60	903	ND	97	97	ND
	904	ND	97	96	ND

[a] Growth yield is expressed as A_{600} at 24 h − A_{600} at 0 h.
[b] ND, Not determined.

TABLE 4. Sporulation and viability rates of *C. acetobutylicum* 903 and 904 upon growth on various media

Culture media	Strain					
	903			904		
	Maximum viable counts (cells ml^{-1})	Sporulation rate[a] (%)	Viability rate[b] (%)	Maximum viable counts (cells ml^{-1})	Sporulation rate[a] (%)	Viability rate[b] (%)
Jerusalem artichoke pulp	9.5×10^8	16.8	21	2×10^9	0.0018	0.085
Potato	2.3×10^8	47.8	87	8×10^8	0.031	3.75
Glucose-M	2.5×10^8	30	36	9.5×10^8	0.15	1.05

[a] Measured at 74 h and expressed as defined in Materials and Methods.
[b] Measured at 74 h and expressed as the percentage of viable cells present at that time with respect to the number present at maximum growth.

ceased. During the latter period, the viable cell count also decreases.

This decrease is much more pronounced with strain 904, and probably reflects the lower sporulation rate of this strain, as bacteria which do not complete sporulation undergo lysis. These observations clearly show that complete sporulation is not necessary for solvent production but also indicate that the bacterial population which produces solvents undergoes the physiological changes of the initial stages of sporulation. These results are consistent with those of Jones et al. (12) and support the same general conclusions. The fact that a mutant selected for its resistance to butanol is also a sporulation mutant is interesting but does not necessarily imply a direct relationship between solvent production and sporulation, as it may be a result of the pleiotropic nature of a membrane mutation. It can be noted here that no obvious relationship between sporulation and solvent production was observed among sporulation mutants of *C. acetobutylicum* P262 (12, 14).

An interesting observation, however, was that strain 904 grew somewhat more rapidly than strain 903 and reached higher cell densities on all media tested. The possible relationship of this property with sporulation rates and solvent production capability is presently unclear. It should be noted that butanol-resistant mutant *C. acetobutylicum* SA-1 (13) also presented better growth kinetics than the wild-type strain from which it was derived.

Another point of interest, which is presently under investigation, is the characterization of the membrane lipid composition of butanol-resistant mutants.

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