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Direct Selection of *Clostridium acetobutylicum* Fermentation Mutants by a Proton Suicide Method

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Clostridium acetobutylicum **ATCC 10132 mutants altered in acetic acid synthesis or in the shift to solventogenesis were directly selected by a proton suicide method after mutagenic treatment, by using bromide and bromate as selective agents. The mutants were characterized according to their solvent and acid production. On the selection plates they differed in colony phenotype from the parent strain.**

Clostridium acetobutylicum metabolizes glucose to acetone, butanol, and ethanol (9). This microorganism produces acetic and butyric acids and ATP during its exponential growth phase and then switches to solvent production. The switch to the solventogenic pathway involves acid reutilization for the synthesis of alcohols and acetone (4).

The molecular mechanism involved in this transition and its regulation is not well understood. Mutants suitable for the study of this phenomenon have been isolated. Dürre et al. (2) and Rogers and Palosaari (8) directly selected mutants altered in alcohol dehydrogenases. Mutants altered in the acid recycling to solventogenesis were isolated by screening sporulation-defective and low-acid-producing mutants (8).

Winkelman and Clark (10) developed a proton suicide method for the selection of fermentation mutants of *Escherichia coli* based on lethal effects derived from acid synthesis. In this procedure, bromate and bromide sodium salts that react to release toxic bromine in an acid environment are added to the growth medium. Thus, acid production becomes lethal to the cells, and the survivors are expected to have mutations affecting acid synthesis; in the case of *C. acetobutylicum*, mutations could also affect acid reutilization.

The aim of this work was to directly select mutants affected in acid synthesis or reutilization by means of this method.

(Some of the results described in this paper were presented in abstract form [P. H. Cueto and B. S. Méndez, Abstr. V Meet. PAABS Southern Conf. 1988, abstr. no. 85, p. 43].)

All mutant strains were derived from *C. acetobutylicum* ATCC 10132. The bacteria were grown at 37°C either in clostridial basal medium (7) or in synthetic medium (MM) (6) supplemented with $CaCl_2 \cdot 2H_2O$ (10 mg liter^{-1}); thiamine (2 mg liter^{-1}; cysteine (1.1 g liter^{-1}); and resazurin (5 mg liter^{-1}). The method of Hungate (3) was used for liquid cultures. Cultures were plated in an anaerobic hood in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Spores were kept in potato broth (1) at 4°C.

To evaluate the lethal concentrations of bromate and bromide salts, the salt solutions were autoclaved separately and added to solid MM to give the desired concentrations. Cultures were prepared as follows. A stock culture (0.5 ml) was 1/10 diluted in MM, and the diluted culture was heated for 5 min at 80°C and incubated to an optical density at 600 nm of 0.5; two successive transfers were done in MM with a 10% inoculum before 0.1 ml of the culture was plated on plates containing different amounts of sodium bromide and sodium bromate. The lowest combined salt concentration that inhibited growth was considered the lethal concentration.

To formulate the medium suitable for the selection of mutants altered in the shift to solventogenesis, the glucose concentration in solid MM was 48 g liter^{-1}, to ensure high enough acetate and butyrate concentrations to reach the solventogenic phase (5, 6).

The mutagenesis was performed on 12.5 mM NaBr–2.4 mM $NaBrO_3$ and 15.2 mM NaBr–3.0 mM $NaBrO_3$. The pH of the medium was adjusted to 7. Samples (0.1 ml) of cultures prepared as indicated above were plated on these plates, and the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine crystal was added. After incubation, the opaque colonies on the selection medium were purified twice. Sixty colonies were picked and kept as stock cultures.

The cultures for fermentation product determination were prepared as follows. Stock cultures (0.5 ml) of wild-type and mutant strains were diluted 1/10 in clostridial basal medium, heat-shocked, and incubated for 5 days. A Gow Mac Series 550 gas chromatograph equipped with a flame ionization detector was used. Acetone, ethanol, and butanol were

TABLE 1. Lethal NaBr and $NaBrO_3$ concentrations at different initial pHs

Salt concn (mM)		Growth at pH[a]		
NaBr	$NaBrO_3$	6.0	6.5	7.0
0	0	+++	+++	+++
3.1	0.6	+++	+++	+++
6.1	1.2	+++	+++	+++
9.2	1.8	+	++	++
12.5	2.4	−	+	++
15.2	3.0		−	+
18.2	3.6			−

[a] +++, Normal lawn; ++, translucent lawn; +, translucent isolated colonies; −, no growth.

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TABLE 2. Solvent and acid production in Br^--BrO_3^--resistant mutants

Group	Strain	Solvent concn (mmol liter^{-1})[a]			Acid concn (mmol liter^{-1})[a]		Max OD_{600}[b]
		Ethanol	Acetone	Butanol	Acetic	Butyric	
I	ATCC 10132	2.0	16.5	40.5	5.5	7.0	1.2
	UBA 2	3.2	27.3	47.1	2.2	4.2	1.5
	UBA 48	4.3	25.5	52.3	3.9	3.9	1.5
	UBA 131	2.7	23.2	46.5	2.5	2.6	2.0
	UBA 140	2.9	26.7	47.9	4.0	2.7	1.5
II	UBA 49	0.8	0.2	1.9	1.7	7.6	0.4
	UBA 93	1.1	0.02	0.5	9.5	25.5	1.2
	UBA 106	0.5	0.6	1.6	1.8	9.2	0.6
	UBA 121	1.5	0.7	1.1	1.7	9.1	0.5
	UBA 146	1.2	0.5	0.9	2.7	6.0	0.4
III	UBA 30	2.0	8.4	21.1	10.8	10.2	1.1
	UBA 104	1.9	11.7	26.7	9.5	15.1	1.3
	UBA 130	0.8	3.0	16.8	10.7	13.9	2.0

[a] Values represent the average of two determinations for the same culture.
[b] Max OD_{600}, Maximum optical density at 600 nm.

separated in a 1.80-m glass column packed with 1% Sp-1000 on Carbopack (80/100 mesh). For the determination of acids, the culture supernatants were acidified with formic acid (37 g liter^{-1}, final concentration). Acetate and butyrate were separated in a 1.80-m glass column packed with 10% Sp-1000–1% H_3PO_4 on Chromosorb WAW (100/120 mesh). The standard error of the measurements was 9.1%.

The combined effect of bromide plus bromate on *C. acetobutylicum* growth was measured at different pHs. The results are shown in Table 1. A parallel control performed with NaCl instead of NaBr allowed us to discard the possibility of an osmotic inhibition. These data confirmed the toxic effect of protons. White opaque colonies surrounded by a translucent lawn appeared close to the inhibition zone on the mutagenesis plates. We did not observe white colonies on the plates without the mutagen. The total number of opaque colonies was 1,574 on plates inoculated with 6.5×10^8 cells.

Fifty-seven resistant strains were screened for solvent production, as alterations in any gene involved in acid synthesis or their reutilization would give solvent production different from that of the wild-type strain. Those that showed a solvent production phenotype different from that of the wild type were grouped in three types: 7 high-, 10 low-, and 29 intermediate-producing mutants (types I, II, and III, respectively). We chose 12 strains at random from these different phenotypes and partially characterized them by their solvent and acid production and the maximal optical density reached by the cultures at 10 g of glucose liter^{-1}, which is enough to allow the shift to solventogenesis (Table 2).

Type I mutants presented a high butanol production correlated with a low acid concentration. These phenotypes could indicate mutations which lead to a more active acid recycling or which affect its regulation in a way in which solventogenesis could be induced by a lower inducer concentration.

Type II mutants, except for UBA 93, were low acetic acid producers and could not achieve full growth. This fact is coincident with the lack of ATP formation in the pathway leading to the formation of acetate (4). Normal butyric acid levels were reached in these four mutants, so we could rule out the possibility of mutations in the glycolytic pathway to acetyl coenzyme A.

UBA 93 and the type III mutants showed higher acid and lower butanol production than the wild type and could be defective in acid recycling. A possible explanation for the selection of these four mutants by the proton suicide method is that *C. acetobutylicum* reutilizes acids. So we cannot exclude with our present data that these mutants could produce less total acids than the wild-type strain. Kinetic experiments are under way in our laboratory to elucidate this.

Finally, we observed that these mutants exhibited an opaque colony phenotype different from that of the translucent colonies of the wild-type strain in the presence of the selective agent. This fact would allow identification of the wild-type genes involved in acid synthesis or in the shift to solventogenesis, provided that they could eventually be transferred to these mutants, by screening for the different colony phenotypes of recombinants on plates containing bromide and bromate sodium salts.

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LITERATURE CITED

1. **Calam, C. T.** 1980. Isolation of *Clostridium acetobutylicum* strains producing butanol and acetone. Biotechnol. Lett. **2:** 111–116.
2. **Dürre, P., A. Kuhn, and G. Gottschalk.** 1986. Treatment with allyl alcohol selects specifically for mutants of *Clostridium acetobutylicum* defective in butanol synthesis. FEMS Microbiol. Lett. **36:**77–81.
3. **Hungate, R. E.** 1950. The anerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14:**1–49.
4. **Jones, D. T., and D. R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. **50:**484–524.
5. **Long, S., D. T. Jones, and D. R. Woods.** 1984. Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P 262. Appl. Microbiol. Biotechnol. **20:**256–261.
6. **Monot, F., J. R. Martin, H. Petitdemange, and R. Gay.** 1982. Acetone and butanol production by *Clostridium acetobutylicum*

in a synthetic medium. Appl. Environ. Microbiol. **44**:1318–1324.
7. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
8. **Rogers, P., and N. Palosaari.** 1987. *Clostridium acetobutylicum* mutants that produce butyraldehyde and altered quantities of solvents. Appl. Environ. Microbiol. **53**:2761–2766.
9. **Smith, L. D. S., and G. Hobbs.** 1974. *Clostridium*, p. 560. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.
10. **Winkelman, J. W., and D. P. Clark.** 1984. Proton suicide: general method for direct selection of sugar transport and fermentation-defective mutants. J. Bacteriol. **160**:687–690.