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Treatment with allyl alcohol selects specifically for mutants of *Clostridium acetobutylicum* defective in butanol synthesis

(Acetone-butanol fermentation; allyl alcohol; butanol dehydrogenase; butyraldehyde dehydrogenase; hydrogenase; *Clostridium acetobutylicum*)

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1. SUMMARY

Treatment of *Clostridium acetobutylicum* with allyl alcohol allowed the selection of mutants which were unable to produce *n*-butanol, whereas the synthesis of acetone and ethanol was unaffected. Enzymatic investigations revealed that all mutant strains were devoid of butyraldehyde dehydrogenase or showed a very low activity of this enzyme as compared to the wild type.

2. INTRODUCTION

Despite the renewed interest in *C. acetobutylicum*, relatively little is known about the regulation of solvent production in this organism [1]. In batch culture the fermentation can be divided into 2 distinct physiological phases. In the first, acidogenic stage, exponential growth is observed and carbohydrates are degraded to acetate and butyrate, thus decreasing the pH of the culture to below 5.0. During the second, solventogenic phase, acetone and *n*-butanol are formed and the pH value raises again [2]. The onset of solvent forma-

tion was studied in detail in continuous culture experiments; it required a low pH, certain threshold concentrations of butyrate and/or acetate, and a suitable growth-limiting factor such as phosphate or sulfate [3,4], whereas ammonium and magnesium limitation proved to be less effective [4–7].

However, nothing is known about the translation of these environmental factors into signals acting at the molecular level. Such studies would be greatly facilitated by mutants in the acidogenic and/or solventogenic pathways. Unfortunately, genetic studies with *C. acetobutylicum* are scarce. Spontaneous mutations resulted in butanol-tolerant [8] or asporogenous [9] strains. Artificial mutagenesis using ethyl methanesulfonate or nitrosoguanidine yielded auxotrophic [10,11], antibiotic-resistant [12,13], butanol-tolerant [13,14], autolysis-deficient [15], and asporogenous [16,17] mutants, respectively. Several strains blocked in a certain sporulation stage proved also to be deficient in total solvent production [12,16,17]. In this paper we report the specific selection of spontaneous mutants that are defective in *n*-butanol formation, but can still produce acetone and ethanol.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth media

C. acetobutylicum DSM1732 was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G., and used throughout this study.

The organism was grown in the clostridial basal medium (CBM) described by [18] modified by omission of MnSO_4 . Solvent production in batch cultures was ensured by the addition of clarified corn steep liquor as described [13,19,20].

3.2. Enzyme assays

For preparation of cell extracts, 500 ml cultures were grown until solvent synthesis started as revealed by gas chromatography. The cells were harvested under strictly anaerobic conditions (tightly sealing centrifuge bottles, all manual operations in an anaerobic chamber (Mecaplex, Switzerland)). The pellet was resuspended in 3 ml Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (0.1 M, pH: 7.5) containing 1 mM dithioerythritol (DTE). Cells were broken by one passage through a French pressure cell (77 MPa). This suspension was centrifuged at $95\,000 \times g$ for 60 min. The supernatant was used for all enzymatic investigations.

The assay mixture for measuring butanol dehydrogenase activity contained in a final volume of 1.03 ml: 77 mM Tris-HCl buffer (pH 7.8), 0.23 mM NADPH, cell extract (0.5–2 mg protein), and 10 μl butyraldehyde solution (pure butyraldehyde was diluted in ethanol (1:5) and an equal volume of H_2O was added). The reaction mixture for the determination of butyraldehyde dehydrogenase contained in a final volume of 1.04 ml: 67 mM Tris-maleate buffer (pH: 6.0), 72 mM semicarbazide hydrochloride, 1 mM DTE, 0.27 mM NADH, cell extract (0.5–2 mg protein), and 0.1 mM butyryl-CoA. Butyraldehyde dehydrogenase proved to be very oxygen-labile, whereas butanol dehydrogenase was only partially inactivated under aerobic conditions. The absorption change at 365 nm was followed in both cases. Hydrogenase activity was determined in a mixture containing in a final volume of 0.9 ml: 89 mM Tris-HCl buffer (pH: 8.5), 0.002% methylene blue, and cell extract (20 μg protein). The cuvettes were flushed with

hydrogen for 15 min, before the reaction was started. The reduction of methylene blue at 578 nm was followed [21]. For calculation of enzyme activity a molar extinction coefficient of $\epsilon_{570} = 13.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was used. Protein concentrations in cell extracts were measured according to [22].

3.3. Analysis of fermentation products

Solvents and acids were determined gas chromatographically as described [3].

4. RESULTS

4.1. Selection of non-butanol-producing mutants (bol^-)

Liquid cultures of *C. acetobutylicum* were supplemented with varying amounts of allyl alcohol (0.02–0.5 M) and grown to maximal absorbance. No growth inhibition was observed with concentrations up to 0.38 M. Using 0.5 M allyl alcohol, only about 20% of the absorbance of control cultures without supplement was measured. Gas chromatographic analyses of the fermentation products showed that merely trace amounts or no *n*-butanol at all were formed, whereas acetate, butyrate, acetone, and ethanol were found in about the same quantities as with the wild type. Butyraldehyde was never detected. After a second transfer in CBM + 0.35 M allyl alcohol, *n*-butanol production ceased completely. However, when aliquots of these liquid cultures were used to inoculate media without allyl alcohol, production of this solvent was re-established.

In order to isolate stable mutants, strain DSM1732 was grown in liquid CBM + 0.35 M allyl alcohol and transferred 4 times into the same medium. Aliquots were then plated onto plain CBM plates and grown at 37°C for 2 days. 22 single colonies were randomly picked and checked for their fermentation products (Table 1). All strains were unable to produce *n*-butanol in contrast to the wild type. Depending on the strain an impaired or even improved synthesis of the other compounds could be observed. The phenotypical marker of butanol-non-production seemed to be very stable, since up to now no revertants have

Table 1

Fermentation analyses of allyl alcohol-resistant strains

Complete fermentation balances were not given because of the rather complex composition of the medium. The abbreviation CAG stands for *C. acetobutylicum* Göttingen. The other 12 mutants strains (CAG11–CAG22) proved to have a very similar fermentation pattern and data are therefore omitted.

Strain	Fermentation products (mM)				
	Acetate	Butyrate	Acetone	Butanol	Ethanol
DSM1732 (wild type)	10.9	17.4	1.5	5.7	4.7
CAG1	9.3	20.5	1.1	0	4.6
CAG2	14.7	19.2	3.3	0	6.2
CAG3	9.8	25.4	0.6	0	3.7
CAG4	9.4	23.3	0.6	0	3.3
CAG5	17.8	30.2	1.0	0	3.7
CAG6	19.7	26.4	1.7	0	3.0
CAG7	11.8	24.3	1.3	0	4.0
CAG8	13.8	20.9	1.4	0	3.9
CAG9	9.2	22.8	0.2	0	3.6
CAG10	9.9	21.6	0.8	0	3.2

been found following repeated growth in the absence of allyl alcohol.

4.2. Enzymatic characterization of *bol*⁻-mutants

10 mutants and the wild type were tested for the presence of the two enzymes responsible for *n*-butanol formation, i.e., butanol dehydrogenase and butyraldehyde dehydrogenase (Table 2). The specific activity of hydrogenase was measured as an internal control of strict anaerobiosis, since this

enzyme is oxygen-labile. It is obvious that all mutants were devoid of butyraldehyde dehydrogenase or exhibited only very low specific activities (10–17% as compared to the wild type). Butanol dehydrogenase on the other hand was present in all strains, with CAG6 and CAG8 showing a somewhat lower specific activity (47 and 60%, respectively) than the wild type. However, hydrogenase activity was higher in the mutants compared to strain DSM1732, the only

Table 2

Enzymatic characterization of *bol*⁻-mutants

Strain	Relevant phenotype ^a	Specific activity (U/mg protein)		
		Butanol dehydrogenase	Butyraldehyde dehydrogenase	Hydrogenase
DSM1732 (wild type)	<i>bol</i> ⁺ , AAS	0.015	0.029	5.7
CAG1	<i>bol</i> ⁻ , AAR	0.033	0.005	13.2
CAG2	<i>bol</i> ⁻ , AAR	0.024	0 ^b	7.6
CAG3	<i>bol</i> ⁻ , AAR	0.019	0.004	1.0
CAG4	<i>bol</i> ⁻ , AAR	0.039	0	13.8
CAG5	<i>bol</i> ⁻ , AAR	0.020	0	13.3
CAG6	<i>bol</i> ⁻ , AAR	0.007	0.003	8.9
CAG7	<i>bol</i> ⁻ , AAR	0.020	0.003	22.8
CAG8	<i>bol</i> ⁻ , AAR	0.009	0	4.8
CAG9	<i>bol</i> ⁻ , AAR	0.011	0.005	8.2
CAG10	<i>bol</i> ⁻ , AAR	0.019	0.002	8.9

^a *bol* stands for butanol, AAS means allyl alcohol-sensitive, AAR stands for allyl alcohol-resistant.

^b 0 means below detection limit (0.0004 U/mg protein)

exceptions being CAG3 and CAG8. Cell extracts of mutants did not react with allyl alcohol, using NAD^+ or NADP^+ as coenzymes, respectively. The wild type, however, oxidized allyl alcohol with a specific activity of 0.002 U/mg protein in a test system containing Tris-HCl buffer (pH 7.8) and semicarbazide hydrochloride. Only NAD^+ could be used as electron acceptor, NADP^+ being completely inactive.

4.3. Investigations of the sporulation ability of *bol*⁻-mutants

Mutants grown on solid media were checked microscopically for the presence of spores. Additionally, the iodine staining procedure of [9] was used for visualization of spore formation. It was found that the *bol*⁻-mutation did not affect the ability of the cells to sporulate.

5. DISCUSSION

Allyl alcohol has been used effectively to isolate mutants of *Escherichia coli* [23] and yeast [24–26] defective in alcohol dehydrogenase. The compound acts as a suicide substrate being oxidized to the toxic aldehyde acrolein by action of the dehydrogenase [27]. Surprisingly, spontaneous mutations leading to allyl alcohol resistance of *C. acetobutylicum* affected neither ethanol nor butanol dehydrogenase. However, all mutants had lost butyraldehyde dehydrogenase activity. This might point to a higher degree of unspecificity of this enzyme compared to the former two. Such an explanation is supported by the fact that cell extracts of the wild type were able to oxidize allyl alcohol using NAD^+ as coenzyme, whereas mutants lacking the NAD^+ -dependent butyraldehyde dehydrogenase could not perform such a reaction.

The isolation of allyl alcohol-resistant mutants that produced only low amounts of butanol has been mentioned earlier [1]. However, no data are given and it seems that these strains differ from those described here, since they were still able to synthesize all solvents.

In recent years data have accumulated suggesting that initiation of sporulation and of solvent

production share common regulatory features [1,9,12,16,17,28]. The results reported here do not disprove this assumption. All mutants characterized showed a defined genetic lesion concerning butyraldehyde dehydrogenase. An effect on sporulation could have been only expected if the mutation occurred in a regulatory sequence common to all solvent producing enzymes and not in a structural gene. The precise location of the mutation(s) will be subject of further investigations.

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