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^{31}P Nuclear Magnetic Resonance Studies of Acetic Acid Inhibition of Ethanol Production by Strains of *Zymomonas mobilis*

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Abstract *In vivo* ^{31}P Nuclear Magnetic Resonance (^{31}P NMR) and metabolic studies were carried out on an acetic acid tolerant mutant, *Zymomonas mobilis* ZM4/Ac^R, and compared to those of the parent strain, *Z. mobilis* ZM4, to evaluate possible mechanisms of acetic acid resistance. This investigation was initiated to determine whether or not the mutant strain might be used as a suitable recombinant host for ethanol production from lignocellulose hydrolysates containing various inhibitory compounds. ZM4/Ac^R showed multiple resistance to other lignocellulosic toxic compounds such as syringaldehyde, furfural, hydroxymethyl furfural, vanillin, and vanillic acid. The mutant strain was resistant to higher concentrations of ethanol or lower pH in the presence of sodium acetate, compared to ZM4 which showed more additive inhibition. *In vivo* ^{31}P NMR studies revealed that intracellular acidification and de-energization were two mechanisms by which acetic acid exerted its inhibitory effect. For ZM4/Ac^R, the internal pH and the energy status were less affected by sodium acetate compared to the parent strain. This resistance to pH change and de-energization caused by acetic acid is a possible explanation for the development of resistance by this strain.

Key words: Acetic acid inhibition, ^{31}P NMR, ethanol production, *Zymomonas mobilis*

Ethanol is an important industrial chemical, and its interest in fuel ethanol has increased over the past two decades following the oil shock of the late 1970s and the early 1980s. Recently, ethanol has been promoted as a "green fuel" and has received wide-spread support, because of its benefits in reducing vehicle emissions and minimizing greenhouse gas production. For efforts directed towards its development as an alternative transportation fuel to be successful, ethanol must be produced from renewable biomass at economical cost. The rapid and efficient fermentation of the pentose sugars found in lignocellulosic feedstocks is an absolute requirement for the economical conversion of biomass to ethanol [10]. In response to this challenge, recombinant strains of *Zymomonas mobilis* [2, 33], as well as *Escherichia coli* [16, 24], *Klebsiella oxytoca* [23], and *Saccharomyces cerevisiae* [4, 6, 31], have been developed. As well as their ethanol producing abilities, other important issues need to be considered, for these recombinant bacteria and yeasts including strain stability (particularly in long-term continuous culture), resistance to inhibitors in the lignocellulosic hydrolysates as well as potential for contamination control during extended process operation [7].

Z. mobilis has attracted a strong interest for fuel ethanol production, because of its higher specific rates of sugar uptake and ethanol production, higher ethanol tolerance, and higher ethanol conversion efficiencies when compared to yeasts [11, 15, 28, 29]. The bacterium converts glucose to ethanol using the Entner-Doudoroff pathway and is able to grow in media containing up to 40% glucose. However, *Z. mobilis* can only ferment glucose, sucrose, and fructose, and lacks the pentose metabolism pathway necessary to ferment xylose. Recently, the successful cloning of enzymes for xylose uptake and assimilation in *Z. mobilis* has been reported [33]. These genetically engineered strains can convert the pentose sugar, xylose, to ethanol by the combined use of the Entner-Doudoroff and pentose metabolism pathways *via* the cloned enzymes xylose isomerase, xylulokinase, transketolase, and transaldolase. In a further study, the cloning of three additional enzymes for arabinose utilization has been reported [2].

Ethanol fermentation with the recombinant strains using hemicellulose hydrolysates can be substantially inhibited by toxic substances produced during their hydrolysis. Among the identified inhibitory compounds, acetate has been shown to be the most significant at the concentrations produced in the hydrolysates [27]. Furthermore, the ratio

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of acetate to fermentable sugars is likely to be higher when these hydrolysates are derived from hardwoods [12].

In order to develop effective ethanol fermentation processes using lignocellulose hydrolysates, it will be necessary to utilize acetic acid tolerant recombinant strains. For this purpose, an acetic acid tolerant mutant of *Z. mobilis* ZM4 has been isolated as a possible host strain, and its ethanol production kinetics have been characterized [7].

In the present study, ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopic and metabolic evaluations have been carried out on the acetate tolerant mutant, *Z. mobilis* ZM4/Ac^R, and compared to those on *Z. mobilis* ZM4. These studies were designed to elucidate possible mechanisms of acetate resistance and to determine the suitability of the mutant strain as a potential recombinant host for ethanol production from lignocellulose hydrolysates containing inhibitory compounds.

MATERIALS AND METHODS

Organism and Culture Maintenance

Zymomonas mobilis ZM4 (ATCC 31821) and a mutant with decreased acetic acid sensitivity derived from ZM4 (ZM4/Ac^R) were used in this work [7]. For long-term storage, these strains were kept at −70°C in 150 g/l glycerol. For use in experiments, the strains were maintained on agar plates containing 20 g glucose, 10 g yeast extract (Oxoid), and 20 g agar (Oxoid No. 1) per liter at pH 5.4. Colonies were grown on this medium for 3 days at 30°C, and then stored at 4°C for no longer than 2 weeks before use as inocula in liquid media.

Culture Conditions

The first seeded medium contained 25 g glucose, 10 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, and 2 g KH_2PO_4 per liter. The second seed culture medium was identical in composition to the first seed medium except that the glucose concentration was increased to 50 g/l. The main culture medium was identical to the second seed medium except for the yeast extract concentration, 5 g/l. Glucose and phosphate were autoclaved separately from the ingredients and the final pH (approximately 5.6) was not adjusted. A single colony of ZM4 or ZM4/Ac^R was transferred from the stock culture plate to 10 ml of the first seed culture medium in a 15-ml cap tube and incubated statically for 24 h. The culture was transferred to 140 ml of the second seed medium in a 250-ml flask. After 15 h of static incubation, the culture was inoculated into 1,350 ml of the main fermentation medium in a 2-l culture vessel. The main culture was carried out under nonaerated conditions, however mild agitation was provided in order to maintain a homogeneous culture. All the cultures were carried out at controlled 30°C and pH 5.5.

Resting Cell Experiments

Resting cell experiments were used with MES buffer medium to evaluate the inhibitory effect of acetic acid and other toxic compounds on the metabolism of the parent and mutant strains. Cells were grown in batch culture to the late exponential growth phase, as determined by optical density and sugar concentration measurements. The cells were harvested by centrifugation at 3,000 ×g for 15 min at 4°C, then washed in 100 mM MES buffer (pH 4, 4.5, 5, 5.5, or 6) containing 1 g KH_2PO_4 and 1 g $MgCl_2 \cdot 6H_2O$ per liter. The cells were finally concentrated to about 3.4×10^{10} cells/ml in a small volume of MES buffer. The resulting cell suspension was kept on ice until being used for experiments. A 5.1 ml cell suspension was incubated with 1.2 ml inhibitory compounds (as stated in Results), and 2.8 ml 900 mM glucose in a 12-ml test tube at 30°C. During the incubation, each 1-ml experimental suspension was harvested by centrifugation (3 min at 2,000 ×g) at every 10 min and the supernatant was kept at −20°C until determination of glucose and ethanol concentrations.

Analytical Methods

Biomass was determined by centrifuging the cells (10 min at 2,000 ×g) from a known volume of culture sample, washing twice with distilled water, then drying to constant weight at 105°C.

Glucose was determined on a YSI 2300 STAT Plus analyzer (Yellow Springs Instrument Co., U.S.A).

Ethanol was quantified by gas chromatography (Packard, model 427) using a Porapak Q column (4 mm ID×2 m, 100/120 mesh) operated isothermally with N_2 as a carrier gas and a flame ionization detector. Peak areas were determined with an integrator.

In Vivo ^{31}P Nuclear Magnetic Resonance Spectroscopy

The preparation of cells for NMR studies was the same as for the resting cell experiments except for the final density of cell suspension (about 1.7×10^{11} cells/ml), because *in vivo* ^{31}P NMR studies are generally limited by the relative low sensitivity of this technique [20]. The resulting cell suspension was kept on ice until its use for NMR experiments. Samples for in vivo measurements contained (final volume 4.0 ml): 2.92 ml cell suspension, 0.31 ml D_2O, 0.05 ml triethylphosphate (TEP), 0.31 ml sodium acetate solution, and 0.41 ml of 2.7 M glucose. All NMR measurements were performed at 30°C. Spectra were obtained with a Bruker DMX-500 spectrometer, operating in the Fourier transform mode, using a 10-mm broadband multinuclear probe. ^{31}P NMR spectra were recorded at 202.46 MHz with a recycle time of 1.0 s and a flip angle of 60°. NMR spectra were acquired in 5-min blocks of 300 scans using composite pulse ^1H decoupling in a bilevel scheme with 2-W decoupler power during acquisition.

Calculation of Kinetic Parameters

For the resting cell experiments, where growth was very limited, the specific glucose uptake rate ($q_{glucose}$) and specific ethanol production rate ($q_{ethanol}$) were calculated based on the formula:

$$q_{glucose}=(1/x_{av})\,(\Delta s/\Delta t) \text{ and } q_{ethanol}=(1/x_{av})\,(\Delta p/\Delta t)$$

where Δs and Δp are the changes in the glucose and ethanol concentrations, respectively, over the time period Δt, and x_{av} is the average biomass concentration over Δt. The Δt value was usually 1–1.5 h. The ethanol yield, $Y_{p/s}$, was calculated by dividing $q_{ethanol}$ by $q_{glucose}$.

RESULTS

Effect of Lignocellulosic Inhibitory Compounds on Glucose Metabolism

Effects of lignocellulosic inhibitory compounds were compared, at levels envisioned for a pretreated hardwood liquid hydrolysate [21, 27], on the specific rates of glucose utilization and ethanol production of ZM4 and ZM4/Ac^R (Table 1). Acetic acid was shown to be the most inhibitory compound at these concentrations, followed by syringaldehyde and furfural on the fermentation of ZM4. Hydroxymethyl furfural, vanillin, and vanillic acid did not show any inhibitory effects at the relevant concentrations.

The presence of most compounds except furfural resulted in an increase in both specific rates of glucose utilization and ethanol production of ZM4/Ac^R, presumably due to their increasing uncoupling effect on this acetic acid resistant mutant. Interestingly, the addition of 10.9 g/l sodium acetate (8 g/l acetic acid equivalent) caused both rates to increase by more than 60% for this mutant strain. The addition of furfural resulted in an increase in specific glucose utilization rate, but a decrease in specific ethanol production rate, and therefore, a lower ethanol yield.

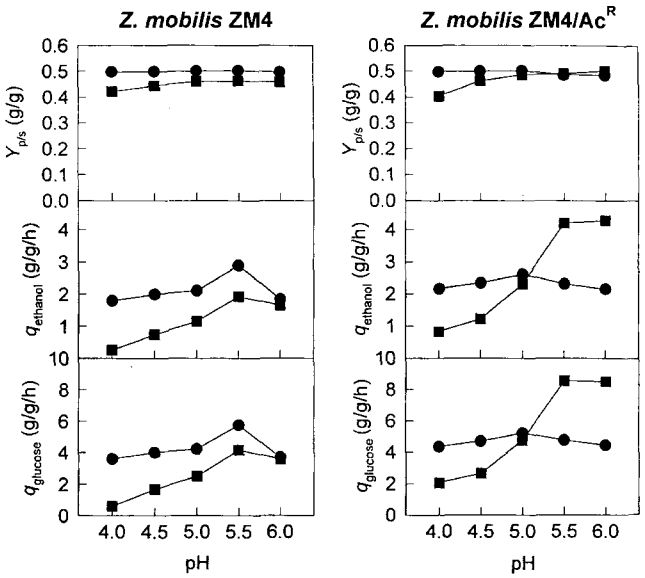

Fig. 1. pH dependence of effect of 10.9 g/l sodium acetate on the glucose fermentation rates of *Z. mobilis* ZM4 and ZM4/Ac^R in MES buffer medium at 30°C.
MES buffer (100 mM) medium contained 50 g glucose, 1 g KH_2PO_4, and 1 g $MgCl_2 \cdot 6H_2O$ per liter. ■ with 10.9 g/l sodium acetate, ● without sodium acetate.

pH Dependence of Effect of Acetic Acid

Effects of acetic acid in the pH range from 4.0 to 6.0 on the specific rates of glucose utilization and ethanol production of ZM4 and ZM4/Ac^R were compared (Fig. 1). The addition of 10.9 g/l sodium acetate caused a decrease in both specific

Table 1. Effect of lignocelluosic toxic compounds on the glucose utilization rate and ethanol production rate of *Z. mobilis* ZM4 and *Z. mobilis* ZM4/Ac^R in MES buffer medium (pH 5.5) at 30°C.

Strain	Sample	Concentration (g/l)	$q_{glucose}$ (g/g/h)	$q_{ethanol}$ (g/g/h)	$Y_{p/s}$ (g/g)
ZM4	Control		5.22	2.60	0.50
	Na-acetate	10.9	3.88	1.79	0.46
	Furfural	0.3	5.07	2.39	0.47
	Hydroxymethyl furfural	0.9	5.22	2.59	0.50
	Vanillin	0.043	5.39	2.67	0.50
	Vanillic acid	0.084	5.22	2.53	0.48
	Syringaldehyde	0.13	4.59	2.28	0.50
ZM4/Ac^R	Control		5.20	2.55	0.49
	Na-acetate	10.9	8.57	4.20	0.49
	Furfural	0.3	5.52	2.54	0.46
	Hydroxymethyl furfural	0.9	5.82	2.88	0.49
	Vanillin	0.043	5.43	2.65	0.49
	Vanillic acid	0.084	5.57	2.73	0.49
	Syringaldehyde	0.13	5.54	2.71	0.49

MES buffer (100 mM) medium contained 50 g glucose, 1 g KH_2PO_4, and 1 g $MgCl_2 \cdot 6H_2O$ per liter.

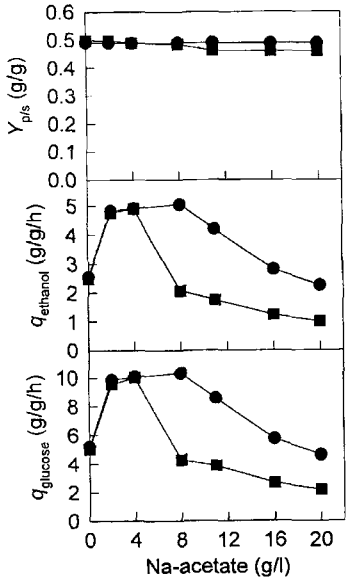

Fig. 2. Effect of sodium acetate concentrations on the glucose fermentation rates of *Z. mobilis* ZM4 and ZM4/Ac^R in MES buffer medium at 30°C.
MES buffer (100 mM, pH 5.5) medium contained 50 g glucose, 1 g KH_2PO_4, and 1 g $MgCl_2 \cdot 6H_2O$ per liter. ■ *Z. mobilis* ZM4, ● *Z. mobilis* ZM4/Ac^R.

rates of glucose utilization and ethanol production of ZM4 at lower pH values. The ethanol yield from glucose was lower also at the lower pH values of 4.0 or 4.5.

However, ZM4/Ac^R showed a dual response. Addition of 10.9 g/l sodium acetate caused an increase in the specific rates at pH values higher than 5.0; however, decreasing the pH below 5.0 resulted in a decrease in both specific rates. The ethanol yield also fell as pH decreased.

Effect of Acetic Acid Concentration

Effects of different acetic acid concentrations on the fermentation rates of ZM4 and ZM4/Ac^R at pH 5.5 were analyzed (Fig. 2). The specific rates of glucose utilization and ethanol production of ZM4 increased about two-fold with the addition of 2 g/l and 4 g/l sodium acetate. However, increasing the concentration of sodium acetate from 8 g/l to 20 g/l caused a significant decrease of both specific rates which was approximately proportional to the increasing of acetate concentrations.

Both specific rates of glucose utilization and ethanol production of ZM4/Ac^R were increased with increasing sodium acetate concentration up to 8 g/l, after which there was a decline. However, at the higher concentrations of sodium acetate, 11, 16, or 20 g/l, the fermentation rates of ZM4/Ac^R were significantly higher than those of ZM4.

Ethanol yields of ZM4/Ac^R from glucose were not changed by the addition of sodium acetate under these experimental conditions, however those of ZM4 were decreased slightly by the increase of acetate levels.

Combined Effects of Sodium Acetate and Ethanol

The effects of 10.9 g/l sodium acetate with or without the addition of ethanol (10–50 g/l) at pH 5.5 were studied for both strains in order to determine the fermentation stability of ZM4/Ac^R at these conditions (Table 2). Increasing concentrations of ethanol up to 50 g/l resulted in a similar effect on the fermentation rates of both strains, ZM4 and ZM4/Ac^R. Both specific rates of glucose utilization and ethanol production were decreased by the increasing concentration of ethanol. The ethanol yields of ZM4 were slightly decreased, however those of ZM4/Ac^R were not affected by the addition of ethanol.

Table 2. Effect of ethanol with or without the addition of acetic acid on the glucose utilization rate and ethanol production rate of *Z. mobilis* ZM4 and *Z. mobilis* ZM4/Ac^R in MES buffer medium (pH 5.5) at 30°C.

Strain	Sample	$q_{glucose}$ (g/g/h)	$q_{ethanol}$ (g/g/h)	$Y_{p/s}$ (g/g)
ZM4	Control	5.22	2.60	0.50
	10.9 g/l Na-acetate	3.88	1.79	0.46
	10 g/l ethanol	5.21	2.58	0.50
	25 g/l ethanol	4.93	2.42	0.49
	50 g/l ethanol	4.44	2.18	0.49
	10.9 g Na-acetate and 10 g ethanol/l	3.73	1.72	0.46
	10.9 g Na-acetate and 25 g ethanol/l	2.92	1.29	0.44
	10.9 g Na-acetate and 50 g ethanol/l	2.46	1.02	0.42
ZM4/Ac^R	Control	5.20	2.55	0.49
	10.9 g/l Na-acetate	8.57	4.20	0.49
	10 g/l ethanol	5.22	2.56	0.49
	25 g/l ethanol	5.10	2.49	0.49
	50 g/l ethanol	4.48	2.19	0.49
	10.9 g Na-acetate and 10 g ethanol/l	8.40	4.08	0.49
	10.9 g Na-acetate and 25 g ethanol/l	7.54	3.57	0.47
	10.9 g Na-acetate and 50 g ethanol/l	5.66	2.38	0.42

MES buffer (100 mM) medium contained 50 g glucose, 1 g KH_2PO_4, and 1 g $MgCl_2 \cdot 6H_2O$ per liter.

The addition of 10.9 g/l sodium acetate with ethanol appeared to cause an additive inhibition of fermentation rates for ZM4. Ethanol yields were decreased also by the increasing concentration of ethanol in the presence of sodium acetate. However, ZM4/Ac^R showed a relatively high resistance to the increasing ethanol concentration in the presence of sodium acetate. The addition of 10.9 g/l sodium acetate resulted in partially uncoupled metabolism with increasing specific fermentation rates due to acetic acid counterbalancing the inhibitory effects of ethanol on ZM4/Ac^R. ZM4/Ac^R showed higher specific glucose uptake rates even in the presence of both 10.9 g/l sodium acetate and 50 g/l ethanol, compared to the control experiments with no addition of inhibitors. However, the ethanol yields were decreased by the increase of ethanol concentration in the presence of sodium acetate.

Phosphorous NMR Study at pH 5.5

Figure 3A shows a time profile of four characteristic ^{31}P NMR spectra from a typical experiment for the metabolism of glucose by ZM4/Ac^R at pH 5.5. Relatively broad intracellular resonances of sugar phosphate, intracellular phosphate, nucleoside diphosphate, NADH, and uridine diphospho-sugars

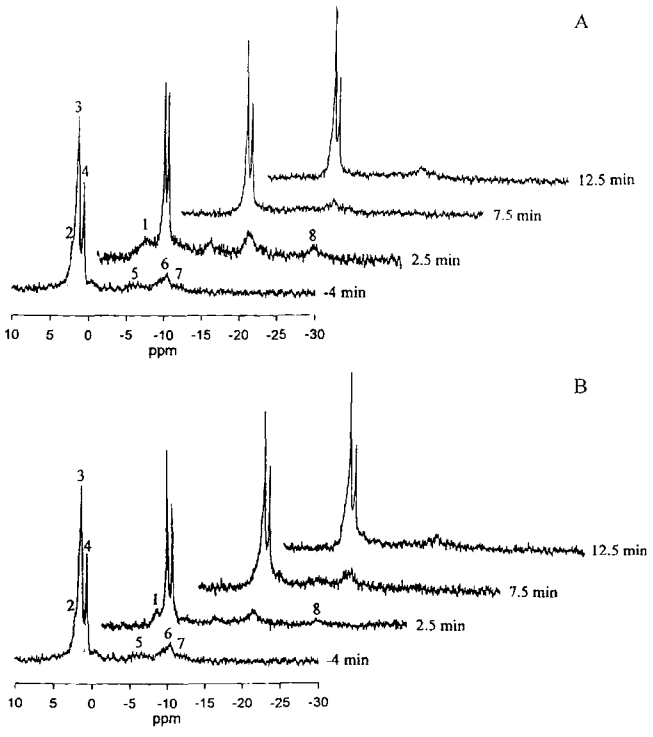

Fig. 3. ^{31}P NMR spectra of *Z. mobilis* ZM4/Ac^R (10^{11} cells/ml suspended in MES buffer, pH 5.5) after addition of 277 mM glucose at 30°C in the absence (A) and presence (B) of 10.9 g/l sodium acetate.

MES buffer (100 mM) contained 1 g KH_2PO_4 and 1 g $MgCl_2 \cdot 6H_2O$ per liter. *Assignment:* 1: sugar phosphate; 2: intracellular phosphate; 3: extracellular phosphate; 4: Triethyl phosphate as the internal standard; 5: NDP; 6: NAD and NADP; 7: UDP-sugar; 8: β-NTP. The resonance of the α- and γ-NTP phosphate groups overlapped with the NDP signals.

were evident before glucose addition. The resonances with the smaller linewidths were from extracellular inorganic phosphate and TEP (the standard; 0.44 ppm). After the cells had begun to metabolize glucose, the intracellular resonance of nucleotide triphosphates (NTP) appeared at −5.0 ppm (NTP-γ), −10.0 ppm (NTP-α), and −18.4 ppm (NTP-β), although no NTP resonances were observed in the resting cells before the addition of glucose. After the addition of glucose, there was a rapid build-up of sugar phosphates, with a concomitant decrease in the internal phosphate resonance. The disappearance of the internal phosphate resonance was probably due to the rapid use of phosphate for sugar phosphates production and to a pH shift to lower pH which caused the internal phosphate resonance to overlap with that of the external phosphate. Between 2.5 min and 7.5 min, sugar phosphate levels decreased markedly, whereas the total inorganic phosphate level increased rapidly. The ^{31}P NMR spectra of ZM4/Ac^R were similar to those of ZM4.

Figure 3B shows a time profile of four characteristic ^{31}P NMR spectra from a typical experiment for the metabolism of glucose by ZM4/Ac^R at pH 5.5 in the presence of 10.9 g/l sodium acetate. After the cells had begun to metabolize glucose, there was a rapid build-up of sugar phosphates, with a concomitant decrease in the internal phosphate resonances. Between 2.5 min and 7.5 min, sugar phosphate levels decreased markedly, whereas the levels of NAD$^+$ plus NADH increased and the total inorganic phosphate level was unchanged. It is interesting to notice that the intracellular resonance of NTP increased only slightly during glucose metabolism, in spite of the fact that glucose was rapidly metabolized to ethanol and that ATP would have been produced during glucose metabolism (1 mol of ATP theoretically being produced per 1 mol of glucose). This means that ATP was rapidly utilized by this strain in the presence of acetate.

Phosphorous NMR Study at pH 4.0

In vivo ^{31}P NMR spectra of ZM4 actively metabolizing glucose at pH 4.0 showed slightly reduced levels of intracellular sugar phosphate and NTP (Fig. 4A) compared to those of ZM4 at pH 5.5 (data not shown, but similar to ZM4/Ac^R). This result suggests that ZM4 was less energized and with a lower rate of glucose metabolism at pH 4 compared to pH 5.5. After the addition of 10.9 g/l sodium acetate at pH 4.0, levels of sugar phosphate and NTP were markedly decreased (Fig. 4B). The resonances of sugar phosphates shifted upfield, and overlapped with the phosphate resonance. This shift of resonance was probably due to the decrease of internal pH [pH(int)] caused by the presence of acetic acid. Total inorganic phosphate peaks of the cell suspension with sodium acetate were higher than those of control experiment without addition of sodium acetate, indicating that less phosphate was utilized for NTP production and sugar phosphate formation under these conditions.

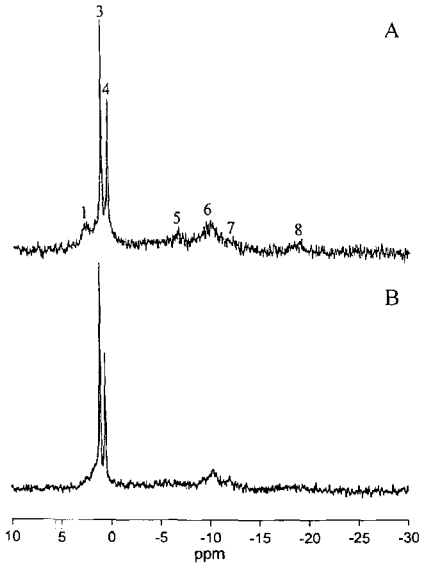

Fig. 4. Comparison of ^{31}P NMR spectra of *Z. mobilis* ZM4 (10^{11} cells/ml suspended in MES buffer, pH 4.0) actively metabolizing glucose after addition of 277 mM glucose at 30°C in the absence (A) and presence (B) of 10.9 g/l sodium acetate. Spectra were obtained at 2.5 min, when the cells actively metabolized glucose. MES buffer (100 mM) contained 1 g KH_2PO_4 and 1 g $MgCl_2 \cdot 6H_2O$ per liter. For assignment see Fig. 3.

In vivo ^{31}P NMR spectra of ZM4/Ac^R actively metabolizing glucose at pH 4.0 also showed slightly reduced levels of intracellular sugar phosphates and NTP (Fig. 5A) compared to those of ZM4/Ac^R at pH 5.5 (see Fig. 3A). These results suggest that ZM4/Ac^R was less energized and the rate of glucose metabolism was lower at pH 4 compared to pH 5.5. As a result of the addition of 10.9 g/l sodium acetate at pH 4.0, levels of sugar phosphates were slightly reduced, although those of NTPs were markedly decreased (Fig. 5B). The slight decrease in the levels of sugar phosphates of ZM4/Ac^R compared to ZM4, following the addition of sodium acetate at pH 4.0, indicated that only ZM4/Ac^R could continue to actively metabolize glucose in the presence of 10.9 g sodium acetate/l at lower pH. Resonances of sugar phosphates were shifted upfield for both strains, however the shift of sugar phosphate resonance of ZM4/Ac^R was less than that of ZM4, indicating that internal pH of ZM4/Ac^R was less affected by the addition of sodium acetate.

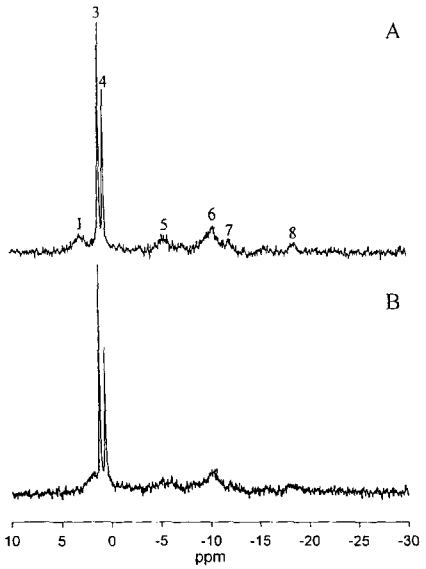

Fig. 5. Comparison of ^{31}P NMR spectra of *Z. mobilis* ZM4/Ac^R (10^{11} cells/ml suspended in MES buffer, pH 4.0) actively metabolizing glucose after addition of 277 mM glucose at 30°C in the absence (A) and presence (B) of 10.9 g/l sodium acetate. Spectra were obtained at 2.5 min, when the cells actively metabolized glucose. MES buffer (100 mM) contained 1 g KH_2PO_4 and 1 g $MgCl_2 \cdot 6H_2O$ per liter. For assignment see Fig. 3.

DISCUSSION

Acetic acid (HAc), the most inhibitory compound produced during the hydrolysis of hemicellulose [27], is a weak acid with a pKa of 4.75 which dissociates in a pH-dependant fashion, into anionic 'acetate' (Ac^-) and a proton (H^+). The uncharged form of acetic acid is soluble in the lipids of the cell membrane [22]. By virtue of its ability to freely traverse the cell membrane, the protonated form of acetic acid acts as an electroneutral permeant species. It has been suggested that the protonated acetic acid causes its inhibitory effect by interference with the homeostatic mechanisms related to the maintenance of a constant intracellular pH, resulting in decrease in growth and metabolism, as seen also with other weak acids [5, 25]. There have been other reports also about the pH-dependant inhibitory effect of acetic acid on *Zymomonas* cells. As pH was decreased, the inhibitory effect of acetic acid on strains ZM4 and ZM6 increased, resulting in reduced growth rate and cell yield [7, 14]. Therefore, it was relevant to isolate an acetic acid tolerant mutant for efficient production of ethanol from lignocellulosic hydrolysates containing high concentrations of acetic acid. As reported recently by our group, an acetic acid tolerant mutant, *Z. mobilis* ZM4/Ac^R, isolated in an acetate-enriched continuous culture following NTG mutagenesis of ZM4, could grow in the presence of 20 g/l sodium acetate, although the growth of *Z. mobilis* ZM4 was inhibited above 12 g/l sodium acetate at pH 5.0 [7]. Although some fermentation kinetic work has been done using ZM4/Ac^R, the biochemical basis for enhanced acetate tolerance in ZM4/Ac^R remains unknown but is of considerable interest. In this study, possible mechanisms of acetate inhibition and enhanced acetate tolerance have been elucidated using *in vivo* ^{31}P NMR.

Although acetate has proved to be the most significant inhibitory compound in hemicellulose hydrolysates, other compounds such as furfural, hydroxymethyl furfural, vanillin,

and vanillic acid have been shown to be toxic at the relevant concentrations [3, 27, 32]. Therefore, the effects of lignocellulosic toxic compounds on fermentation parameters of ZM4/Ac^R were compared to those of ZM4. The effect of inhibitors was tested at concentrations found when hardwoods were hydrolyzed with dilute sulfuric acid under high pressure [21, 27]. Our results with resting cell experiments showed that ZM4/Ac^R has multiple resistance to lignocellulosic toxic compounds. Although the precise mode of action and resistance mechanism of ZM4/Ac^R remain only partly understood, the present study shows that the enhanced multiple resistance of the mutant is significant in view of the high level of compounds found in many lignocellulosic hydrolysates.

ZM4/Ac^R showed dual responses, *viz* either stimulation or inhibition in the presence of acetic acid according to pH, however ZM4 showed a stronger pH-dependant inhibition. The specific fermentation rates of ZM4/Ac^R were less affected at the lower pHs in the presence of sodium acetate, compared to the parent strain ZM4. The stimulation effect of acetic acid at pHs above 5.0 may be due to an increased level of maintenance metabolism induced by acetic acid. There have been other reports about the uncoupling effect of acetic acid at low concentrations, which is a consequence of increased maintenance energy requirements [13, 14, 19]. To prevent acidification of the cytoplasm, the cell must divert more energy to maintenance and less to growth. In this way, the undissociated forms of acetic acid may act to uncouple anabolism (growth) from catabolism (generation of ATP from glucose metabolism).

For efficient production of ethanol from concentrated lignocellulosic hydrolysates, the producing strain should be tolerant to both inhibitory compounds and ethanol. From this point of view, ZM4/Ac^R may be a very relevant strain, because it showed a relatively high resistance to ethanol in the presence of acetic acid, compared to ZM4 which showed a more additive inhibition.

The mechanism of acetic acid inhibition of ethanol fermentation was studied by ^{31}P NMR. ^{31}P NMR can provide noninvasive information pertaining to cellular metabolism [8, 20], because the technique provides information on the energy status of the cells, by virtue of its ability to identify the various nucleotide phosphates and other energy-rich compounds therein, as well as to characterize the intracellular pH from the chemical shifts of internal phosphate and other phosphorylated metabolites [1, 9, 18, 30]. Our *in vivo* ^{31}P NMR studies support the hypothesis that acetic acid causes a significant inhibitory effect by acidification of the cytoplasm. Intracellular acidification could explain the slower rates of glucose metabolism in the presence of acetic acid. Cytoplasmic enzymes might also be adversely affected by a decrease in the internal pH caused by the acidification. Furthermore, we have shown in both strains of *Z. mobilis* that intracellular levels of sugar phosphates and NTP were markedly decreased by the addition of sodium acetate. This decrease of energy compounds by the addition of sodium acetate suggests that intracellular de-energization may also be one of the principal mechanisms by which acetic acid exerts its toxic effect. Such inhibitory mechanisms of sodium acetate found in *Zymomonas mobilis* are similar to those reported for the yeasts, *Pachysolen tannophilus* and *Pichia stipitis*. It has been suggested that intracellular de-energization and acidification may be mechanisms by which the main degradation products (furfural, hydroxymethylfurfural, acetic acid) derived from acid hydrolysis of hemicellulosic materials exert their toxic effect on the yeast cells, although whether this de-energization is a direct effect of these compounds on cellular membranes or a more indirect effect on cellular metabolism remains to be investigated [17].

It is interesting to note that levels of intracellular sugar phosphates were only slightly reduced (about 10%) for ZM4/Ac^R, although those of NTP were significantly decreased (about 50%) by the addition of 10.9 g/l sodium acetate at pH 4.0 during glucose metabolism. This compared to ZM4, for which both levels were significantly reduced (about 40% and 75%, respectively). Also, the pH(int) of ZM4/Ac^R was less affected than that of ZM4 in the presence of acetic acid. These results are not surprising in light of the fact that the specific growth rates, specific glucose uptake rates, and specific ethanol production rates of ZM4/Ac^R were much higher than those of ZM4 in the presence of sodium acetate. These results indicate that glucose was more actively metabolized in ZM4/Ac^R compared to ZM4, and ATP produced through the Entner-Doudoroff pathway was used more rapidly in the presence of acetic acid. This rapid utilization of ATP in the presence of acetic acid resulted, most likely, from an increase in maintenance energy requirements. Maintenance of pH(int) can be energetically expensive, resulting in the membrane H^+-ATPase consuming a high percentage of the total cellular ATP [26]. It is possible then that the maintenance of pH(int) in the presence of acetic acid causes significant depletion of the cellular ATP levels. Such a depletion of ATP may restrict growth of *Z. mobilis* and also cause a decrease in the biomass yield. It has been reported that addition of 12 g/l sodium acetate to ZM4 culture caused 40% and 65% decrease of specific growth rate and biomass yield from glucose, respectively [7].

Through this study using ^{31}P NMR, we have shown that intracellular acidification and de-energization are the major mechanisms by which acetic acid exerts its toxic effects. Furthermore, the enhanced resistance of ZM4/Ac^R against acetic acid is likely to result from the increased resistance to cytoplasmic acidification and de-energization induced by acetic acid in the mutant strain. ZM4/Ac^R appears to be an attractive host to develop new recombinant strains for ethanol production from lignocellulosic hydrolysates containing toxic compounds, because this mutant shows multiple resistance

to a combination of ethanol and toxic compounds as well as enhanced acetic acid resistance.

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