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Nuclear Magnetic Resonance Studies of Acetic Acid Inhibition of Rec *Zymomonas mobilis* ZM4(pZB5)

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Abstract

The fermentation characteristics and effects of lignocellulosic toxic compounds on recombinant *Zymomonas mobilis* ZM4(pZB5), which is capable of converting both glucose and xylose to ethanol, and its parental strain, ZM4, were characterized using ^{13}C and ^{31}P nuclear magnetic resonance (NMR) in vivo. From the ^{31}P NMR data, the levels of nucleoside triphosphates (NTP) of ZM(pZB5) using xylose were lower than those of glucose. This can be related to the intrinsically slower assimilation and/or metabolism of xylose compared to glucose and is evidence of a less energized state of ZM4(pZB5) cells during xylose fermentation. Acetic acid was shown to be strongly inhibitory to ZM4(pZB5) on xylose medium, with xylose utilization being completely inhibited at pH 5.0 or lower in the presence of 10.9 g/L of sodium acetate. From the ^{31}P NMR results, the addition of sodium acetate caused decreased NTP and sugar phosphates, together with acidification of the cytoplasm. Intracellular deenergization and acidification appear to be the major mechanisms by which acetic acid exerts its toxic effects on this recombinant strain.

Index Entries: Recombinant *Zymomonas*; xylose fermentation; ^{13}C nuclear magnetic resonance; ^{31}P nuclear magnetic resonance; acetic acid inhibition.

Introduction

Zymomonas mobilis has attracted wide 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 *(1–4)*. This Gram-negative bacterium converts glucose to ethanol using the Entner-Doudoroff pathway and is able to grow

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in media containing up to 40% glucose. However, *Z. mobilis* can only ferment glucose, sucrose, and fructose and lacks the pentose phosphate pathway necessary to ferment xylose. Recently, metabolic engineering of *Z. mobilis* has been reported *(5)*, with the genetically engineered strains able to now convert xylose to ethanol by the combined use of the Entner-Doudoroff pathway and the cloned enzymes xylose isomerase, xylulokinase, transketolase, and transaldolase for xylose assimilation and metabolism.

The ethanol fermentation efficiency of the recombinant strains can be substantially hindered by toxic substances produced during the hydrolysis of the various lignocellulosic materials *(6)*. Among the compounds identified, acetic acid has been shown to cause significant inhibition at the concentrations produced in the hydrolysates. The ratio of acetate to fermentable sugar is particularly high when these are derived from hardwoods *(7)*. Although there have been several reports about acetate inhibition on ethanol fermentation, little has been learned about the mechanism of this inhibition.

In recent years, noninvasive nuclear magnetic resonance (NMR) methods have been developed that allow us not only to identify the presence of particular metabolites within a biological sample but also to monitor reaction rates, enzyme activities, and membrane transport in vivo *(8–11)*. Despite the low levels of the carbon-13 isotope (1.1%), natural-abundance ^{13}C NMR studies have proven useful for monitoring the progress of fermentation processes *(8–11)*. ^{31}P NMR can also provide noninvasive information pertaining to cellular metabolism *(12)*. This technique provides information on the energy status of the cells, by virtue of its ability to quantify levels of nucleoside triphosphates and other energy-rich compounds, as well as to identify changes in the intracellular pH from the chemical shifts of internal phosphate and other phosphorylated metabolites *(13–15)*.

In the present study, the effect of acetate inhibition on recombinant *Z. mobilis* ZM4(pZB5), which is capable of converting both glucose and xylose to ethanol, has been characterized using ^{13}C and ^{31}P NMR in vivo, and a mechanism has been proposed to explain its inhibitory action.

Materials and Methods

Organism and Culture Maintenance

Z. mobilis ZM4 (ATCC 31821) and a recombinant *Z. mobilis* ZM4(pZB5) strain that contains *Escherichia coli* genes for xylose assimilation (xylose isomerase and xylulokinase) and pentose metabolism (transketolase and transaldolase) on the plasmid pZB5, together with the gene for tetracycline resistance, were used in this study *(5)*. The recombinant strain was kindly provided by Dr. Min Zhang under a Material Transfer Agreement with the National Renewable Energy Laboratory (NREL), Golden, CO. For long-term storage, these strains were kept at –70°C in 200 g/L of glycerol. For use in experiments, the strains were maintained on agar plates containing 20 g/L

of glucose for ZM4 (or 20 g/L of xylose for ZM4[pZB5]), 10 g/L of yeast extract (Oxoid), and 20 g/L of agar (Oxoid No. 1) at pH 5.4. Tetracycline was added aseptically to the sterile media at a concentration of 10 mg/L as a selection pressure for plasmid (pZB5) maintenance. Colonies were grown on this medium at 30°C and then stored at 4°C for no longer than 2 wk before use as inocula in liquid media.

Culture Conditions

First-seed medium contained 25 g/L of glucose (or 25 g/L of xylose), 10 g/L of yeast extract, 1 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of $(NH_4)_2SO_4$, and 2 g/L of KH_2PO_4. Second-seed culture medium was identical in composition to the first-seed medium. Main culture medium was identical to the second-seed medium except for glucose or xylose concentrations (50 g/L each) and yeast extract concentration (5 g/L). Glucose (or xylose) or phosphate were autoclaved separately from the ingredients and the final pH (approx 5.6) was not adjusted. Tetracycline was added aseptically to the sterile media at a concentration of 10 mg/L as a selection pressure for plasmid (pZB5) maintenance. A single colony of ZM4 or ZM4(pZB5) was transferred from the stock culture plate to 10 mL of first-seed culture medium in a 15-mL cap tube and incubated statically for 24 h for ZM4 or 72 h for ZM4(pZB5). The culture was transferred to 140 mL of second-seed medium in a 250-mL flask. After 15 h for ZM4 or 48 h for ZM4(pZB5) of static incubation, the culture was inoculated into 1350 mL of main fermentation medium in a 2-L controlled culture vessel (LH Engineering, Maidenhead, Berks, UK). Cultivation was carried out under nonaerated conditions; however, mild agitation (150 rpm) was provided in order to maintain a homogeneous culture. All the cultures were carried out at controlled conditions of 30°C and pH 6.0, unless otherwise stated.

Preparation of Cells for NMR Spectroscopy

Cells were grown in batch culture to late exponential growth phase, as determined by measurements of optical density and sugar concentration. They were harvested by centrifugation at 3000*g* for 15 min at 4°C, and then washed in 100 m*M* MES buffer (pH 4.0, 4.5, 5.0, 5.5, or 6.0) containing 0.1% KH_2PO_4 and 0.1% $MgCl_2 \cdot 6H_2O$. The cells for ^{13}C NMR were concentrated to about 30 g/L of dry cell weight in a small volume of MES buffer. The resulting cell suspension was kept on ice until use. Samples for in vivo ^{13}C NMR measurements contained (final volume 4.33 mL) 2 mL of cell suspension, 0.33 mL of D_2O, 0.05 mL of 50 m*M* dimethyl sulfoxide, 0.61 mL of inhibitory compounds (as stated in Results), and 1.34 mL of 900 m*M* xylose or glucose.

In vivo ^{31}P NMR studies are generally limited by the relatively low sensitivity of this technique *(16)*. To overcome this limitation, the cells were more highly concentrated (1.7×10^{11} cells/mL). Samples for in vivo ^{31}P NMR measurements contained (final volume 4.0 mL) 2.92 mL of cell suspension,

0.31 mL of D_2O, 0.05 mL of triethylphosphate (TEP), 0.31 mL of sodium acetate solution, and 0.41 mL of 2.7 *M* xylose or glucose.

In Vivo NMR Spectroscopy

All NMR measurements were performed at 30°C. Spectra were obtained with a Bruker (Bruker Analytic Gmbh, Karlsruhe, Germany) 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°. ^{31}P NMR spectra were acquired in 5-min blocks of 300 scans using composite pulse ^{1}H decoupling in a bilevel scheme with 2-W decoupler power during acquisition. Natural-abundance proton-decoupled ^{13}C NMR spectra were recorded as just described except that the frequency was 125.75 MHz and the recycle time was 2 s. Spectra were acquired in blocks of 300 scans (10 min).

Results

^{13}C NMR Studies of Sugar Uptake and Ethanol Production Kinetics

Carbon-13 natural-abundance NMR studies of xylose fermentation by *Z. mobilis* ZM4(pZB5) were performed in order to analyze quantitatively xylose uptake and ethanol production rates in vivo and *in situ*. Figure 1A shows a kinetic spectrum of xylose utilization obtained with a cell suspension of recombinant *Z. mobilis* ZM4(pZB5) as a function of incubation time. Figure 1B shows data for ethanol production. By monitoring the decrease of the C-1 resonances for the α- and β-anomers of xylose and the concomitant increase in ethanol peaks with time, it was possible to monitor the course of xylose fermentation in real time in the NMR tube. The same approach was used to monitor glucose fermentation (data not shown). Peak areas were determined by integration using the Bruker NMR software, multiefp, and integration, and the concentrations of sugars (xylose or glucose) and ethanol were calculated from the integration results.

Effect of Lignocellulosic Inhibitory Compounds on Xylose Metabolism of Recombinant ZM4(pZB5)

The effects of lignocellulosic inhibitory compounds, at levels reported previously for a pretreated-hardwood liquid hydrolysate *(6,17)*, on specific rates of xylose utilization and ethanol production of ZM4(pZB5) were analyzed using ^{13}C NMR (Table 1). Sodium acetate (protonated form as acetic acid) was shown to be the most inhibitory compound at the concentration tested (10.9 g/L of sodium acetate; 8.0 g/L of acetic acid equivalent), followed by vanillin, syringaldehyde, hydroxymethylfurfural (HMF), and furfural. Vanillic acid did not show any inhibitory effects for these experimental conditions. It was found that these inhibitory compounds did not affect ethanol yields, although they reduced both rates of xylose utilization and ethanol production.

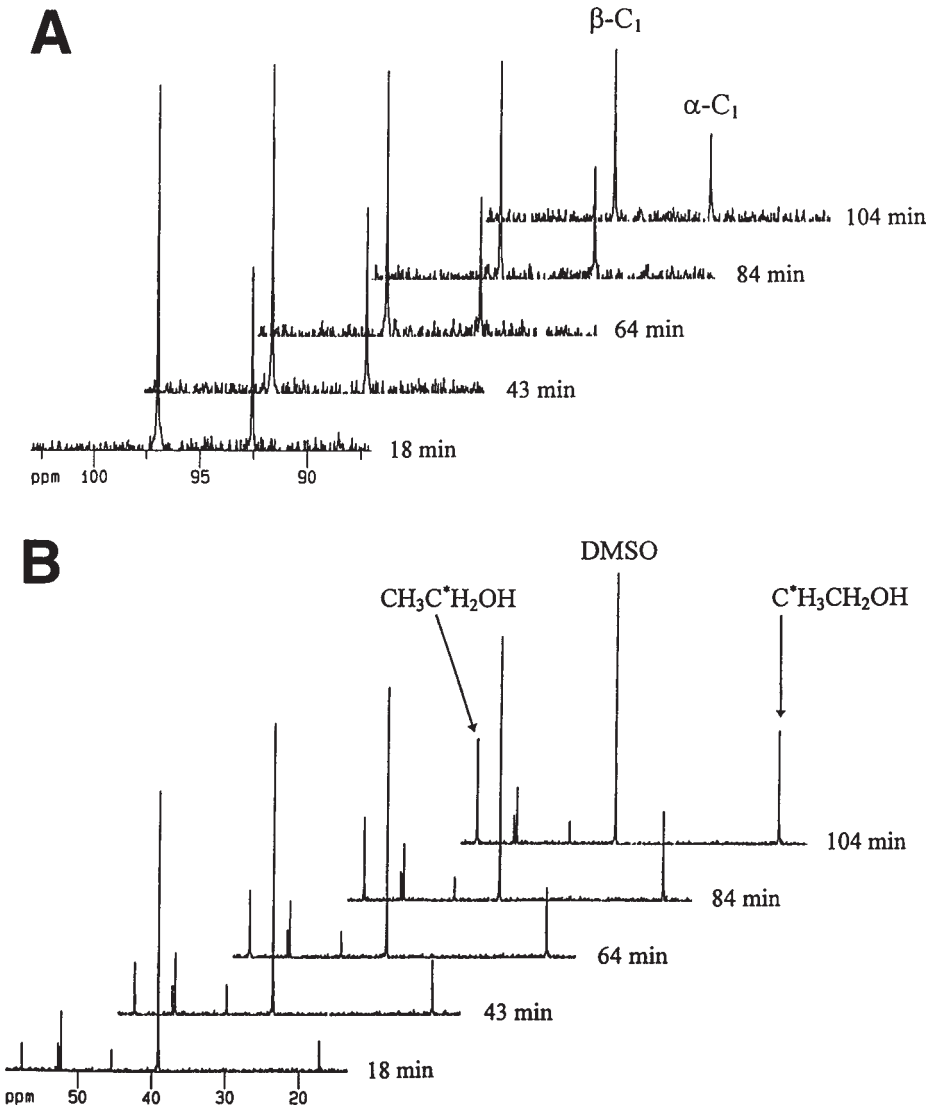

Fig. 1. Uptake of xylose **(A)** and production of ethanol **(B)** by *Z. mobilis* ZM4(pZB5) monitored with ^{13}C NMR. Xylose (277 m*M*) was added to a cell suspension and NMR measurements were immediately started. The signals of the β-C_1 of xylose appeared at 96.99 ppm and that of the α-C_1 of xylose at 92.59 ppm. The signals of the C_1 of ethanol appeared at 57.8 ppm and that of the C_2 of ethanol at 17.18 ppm.

pH Dependence of Inhibitory Effect of Acetate Addition

The effect of sodium acetate addition (10.9 g/L of sodium acetate) in the pH range from 4.0 to 6.0 on the specific rates of xylose utilization and ethanol production of ZM4(pZB5) was analyzed using ^{13}C NMR (Fig. 2A). The optimum pH for xylose utilization and ethanol production of ZM4(pZB5) for the control experiment (without the addition of sodium acetate) was 5.5.

Table 1
Effects of Lignocellulosic Inhibitory Compounds on Specific Rates of Xylose Uptake and Ethanol Production and Ethanol Yield of *Z. mobilis* ZM4(pZB5) at 30°C and Initial pH 6.0 Determined by ^{13}C NMR

Compound	Compound concentration (g/L)	Xylose utilization rate (g xylose/[g cell·h])	Ethanol production rate (g ethanol/[g cell·h])	Ethanol yield (g ethanol/g xylose)
Control		1.58	0.63	0.40
Sodium acetate	10.9	1.15	0.46	0.40
Furfural	0.3	1.40	0.53	0.38
HMF	0.9	1.36	0.54	0.40
Vanillin	0.043	1.24	0.49	0.40
Vanillic acid	0.084	1.57	0.63	0.40
Syringaldehyde	0.13	1.24	0.50	0.40

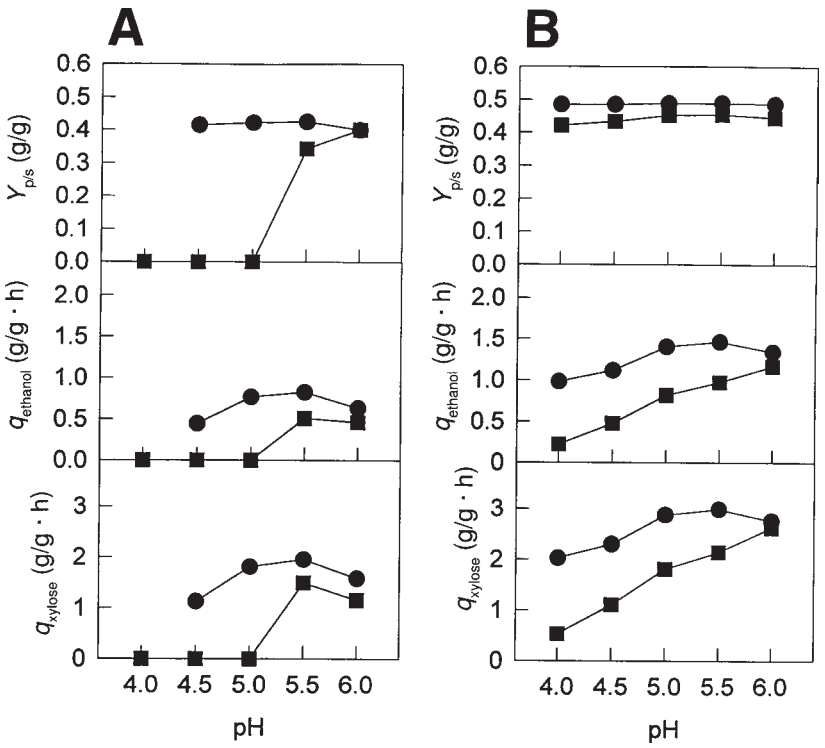

Fig. 2. pH dependence of effect of 10.9 g/L of sodium acetate on the xylose fermentation **(A)** and glucose fermentation **(B)** rates of *Z. mobilis* ZM4(pZB5). ■, with 10.9 g/L of sodium acetate; ●, without sodium acetate.

The addition of 10.9 g/L of sodium acetate caused a decrease in both specific rates of xylose utilization and ethanol production at pH 5.5 and 6.0. However, ethanol yield was not affected at pH 6.0, although it was decreased at pH 5.5. The addition of 10.9 g/L of sodium acetate caused complete inhibition of xylose utilization and ethanol production at a pH of 5.0 and lower.

Interestingly, the xylose metabolism of recombinant ZM4(pZB5) was highly sensitive to acetate at a pH <5.0. The acetate sensitivity of glucose metabolism of ZM4(pZB5) for the same experimental conditions was determined also for comparison (Fig. 2B). In this latter case, the optimum pH for specific glucose utilization and ethanol production rates of ZM4(pZB5) was 5.5, the same value as for xylose metabolism. Decreasing the pH caused a decrease in both specific rates. However, ethanol yields from glucose consumed were largely unaffected. The effect of 10.9 g/L of sodium acetate addition on glucose metabolism was also found to be pH dependent. Although decreasing the pH caused a decrease in both specific rates of glucose utilization and ethanol production, ZM4(pZB5) utilized glucose and produced ethanol at a pH <5.0 in the presence of 10.9 g/L of sodium acetate. Ethanol yields were also decreased by lowering the pH.

The effect of acetate addition on ZM4 in the pH range from 4.0 to 6.0 on the specific rates of glucose utilization and ethanol production showed results similar to those for glucose metabolism by ZM4(pZB5) measured in the NMR sample tube (data not shown). The addition of 10.9 g/L of sodium acetate caused a decrease in both specific rates of glucose utilization and ethanol production of ZM4 at lower pH values. The ethanol yields from glucose were decreased also at the lower pH values.

Note, however, that although specific rates and yields achieved on glucose and xylose media in an NMR sample tube provide valuable comparative data, they cannot be compared with the maximal rates and yields achieved in controlled fermentations. In the former case, a cell suspension (approx 30 g/L) in a small sample tube (4.33 mL) was used, with the cell in MES buffer (plus phosphate) and no other added nutrients except for the carbon source.

^{31}P NMR Studies of Sugar Metabolism

Figure 3A shows four characteristic in vivo ^{31}P NMR spectra from a typical experiment of xylose fermentation by ZM4(pZB5). Relatively broad intracellular resonances of sugar phosphates, inorganic phosphate, nucleoside diphosphates, NADH, and uridine diphospho-sugars were detected before xylose 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 xylose, the intracellular resonances of nucleoside triphosphates (NTP) appeared at –18.4 ppm (NTP-β), -10.0 ppm (NTP-α), and -5.0 ppm (NTP-γ), although no NTP resonances were observed before the addition of xylose. However, NTP signals were small and were not detectable after 17.5 min. Because in vivo resonances of NTP are broad, it is generally difficult to differentiate between adenosine triphosphate (ATP) and the other NTPs in the spectra. In most cells, more than 90% of NTP consists of ATP *(18)*. Within 2.5 min after xylose addition, the intensity of sugar phosphate increased about fourfold, whereas the internal inorganic phosphate signal decreased strongly. Sugar phosphates returned to their initial level after 15 min.

Interestingly, NTP signals during xylose fermentation were very low, suggesting the less-energized status of recombinant ZM4(pZB5) using xylose as a sole carbon source. Therefore, it was necessary to evaluate ^{31}P NMR spectra of ZM4(pZB5) using xylose with those of ZM4(pZB5) using glucose in order to compare their energy status. Figure 3B shows four characteristic in vivo ^{31}P NMR spectra from a typical experiment of glucose fermentation of ZM4(pZB5). After the cells had begun to metabolize glucose, the intracellular resonances of NTP appeared at -18.4 ppm (NTP-β), -10.0 ppm (NTP-α), and -5.0 ppm (NTP-γ), although no NTP resonances were observed before the addition of glucose. After the addition of glucose, there was a rapid buildup of intracellular sugar phosphate, with a concomitant decrease in the intracellular inorganic phosphate level. The disappear-

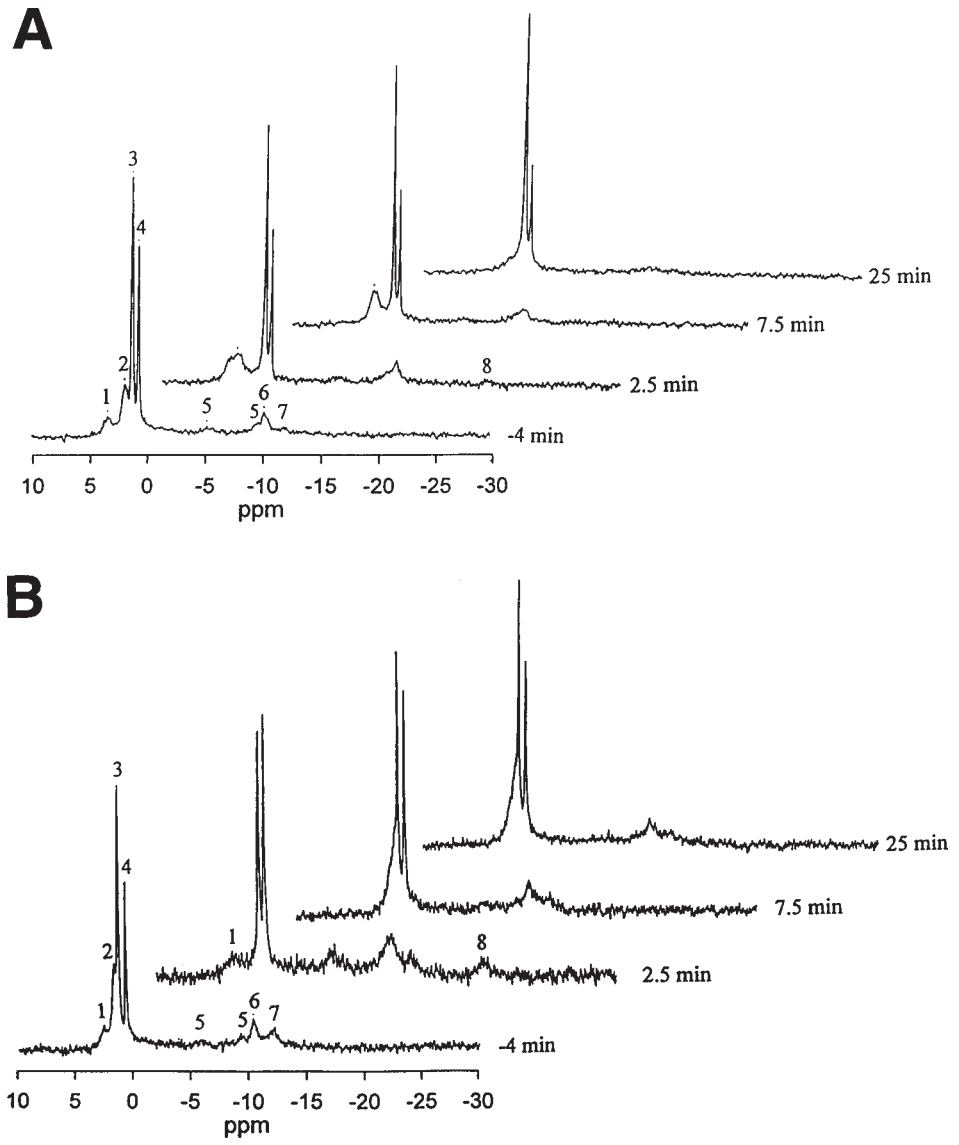

Fig. 3. ^{31}P NMR spectra of *Z. mobilis* ZM4(pZB5) after addition of 277 m*M* xylose **(A)** and after addition of 277 m*M* glucose **(B)** at 30°C and pH 5.5. 1, Sugar phosphates; 2, intracellular phosphate; 3, extracellular phosphate; 4, triethyl phosphate as the internal standard; 5, NDP; 6, NAD and NADP; 7, UDP sugars; 8, β-NTP. The resonance of the α- and γ-NTP phosphate groups overlapped with the NDP signals.

ance of internal phosphate resonance was probably owing to rapid use of phosphate to make sugar phosphates and to a pH shift to lower pH that resulted in the internal phosphate resonance overlapping with the external phosphate resonance. Between 2.5 and 7.5 min, the level of sugar phosphates decreased, whereas the level of total inorganic phosphate (intracellular and extracellular) increased.

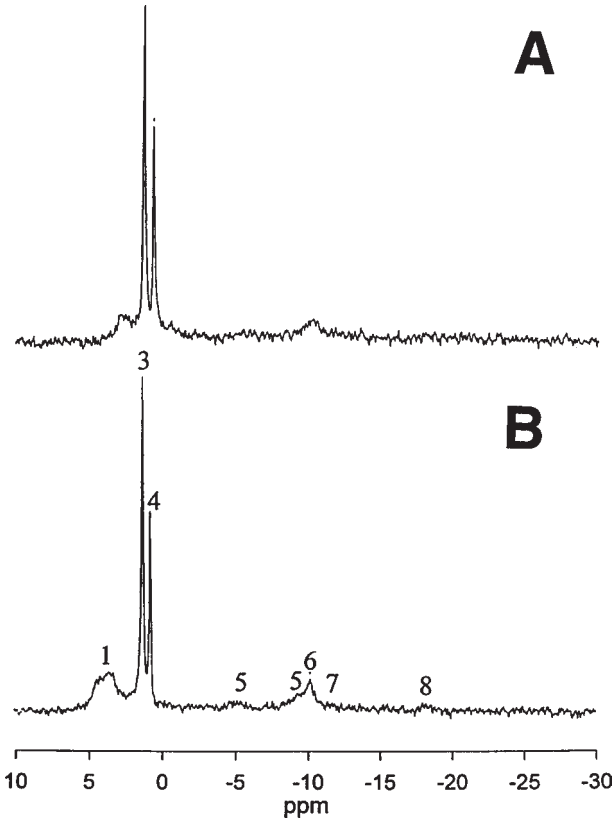

Fig. 4. Comparison of ^{31}P NMR spectra of *Z. mobilis* ZM4(pZB5) at 2.5 min after addition of 277 m*M* xylose at 30°C and pH 5.5 in the presence **(A)** and absence **(B)** of 10.9 g/L of sodium acetate. For assignment of numbers *see* Fig. 3.

^{31}P NMR spectra of glucose fermentation of parental strain ZM4 were similar to those of ZM4(pZB5) using glucose (data not shown). When comparing the spectra of recombinant ZM4(pZB5) using xylose with those of ZM4(pZB5) or ZM4(pZB5) using glucose, it was clear that xylose-fermenting ZM4(pZB5) cells were less energized than glucose-fermenting ZM4(pZB5) or ZM4 cells.

^{31}P NMR Studies of Acetate Inhibition

To elucidate the mechanism of acetate inhibition on ethanol fermentation by *Zymomonas* strains, ^{31}P NMR spectroscopic analyses were conducted. In vivo ^{31}P NMR spectra of ZM4(pZB5) metabolizing xylose in the presence of 10.9 g/L of sodium acetate at pH 5.5 showed different levels of sugar phosphates and NTP formation as compared to control with no acetate addition (Fig. 4). Figure 4A shows representative data obtained from a cell suspension incubated with sodium acetate. The initial formation of sugar phosphates was reduced to 30% as compared to the control, and less NTP was formed (Fig. 4B). These results clearly

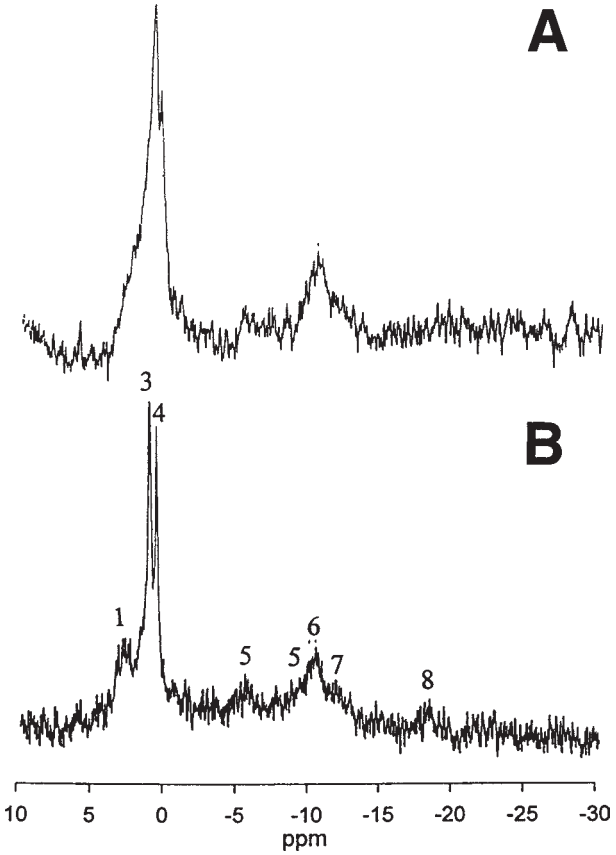

Fig. 5. Comparison of ^{31}P NMR spectra of *Z. mobilis* ZM4 (pZB5) at 2.5 min after addition of 277 m*M* glucose at 30°C and pH 5.5 in the presence **(A)** and absence **(B)** of 10.9 g/L of sodium acetate. For assignment of numbers *see* Fig. 3.

showed that acetate caused a significant decrease in formation of NTP and sugar phosphates.

In vivo ^{31}P NMR spectra of ZM4(pZB5) metabolizing glucose in the presence of 10.9 g/L of sodium acetate at pH 5.5 were compared to control spectra without sodium acetate (Fig. 5). Figure 5A shows representative data obtained from a cell suspension of ZM4(pZB5) incubated with sodium acetate. The resonances of sugar phosphates (Fig. 5A) were somewhat less than those of the control experiment (Fig. 5B) and shifted upfield, therefore overlapping with the inorganic phosphate resonance. This shift of resonance was probably owing to the decrease in internal pH caused by the presence of acetic acid.

In vivo ^{31}P NMR spectra of ZM4(pZB5) metabolizing xylose in the presence of 10.9 g/L of sodium acetate at pH 4.5 showed only small increases in levels of sugar phosphates and NTP. These ^{31}P NMR results are consistent with the kinetic results of xylose utilization and ethanol production, which showed significant inhibition for these conditions.

Discussion

Sugar metabolism of ZM4(pZB5) has been studied by ^{13}C NMR, which can monitor the production of specific metabolites in vivo *(8–11)*, and ^{31}P NMR, which provides information on the energy status of the cells *(13–16)*. Our in vivo ^{31}P NMR studies have shown that the levels of NTP of ZM4(pZB5) using xylose was less than those of ZM4(pZB5) using glucose (Fig. 3A,B). This is evidence of intrinsically slower xylose uptake and/or metabolism and a less-energized state of ZM4(pZB5) cells during xylose fermentation. It has been reported previously that the specific rates of growth, substrate utilization, and ethanol production, as well as the growth yield of recombinant *Z. mobilis* strains on xylose, are lower than those on glucose *(5,19)*. Also, it has been shown that a typical fermentation with ZM4(pZB5) growing on mixtures of glucose and xylose follows two-phase growth kinetics, with the initial uptake of glucose and xylose being followed by slower growth on xylose after glucose depletion. After glucose depletion, the cell growth rate decreases gradually to zero, with the slower uptake of remaining xylose and ethanol production continuing in apparent uncoupled metabolism *(19)*. From our ^{31}P NMR studies, it would suggest that the reduced growth of ZM4(pZB5) on xylose in the second phase of the growth kinetics may be limited by the availability of intracellular ATP.

It has been reported that ethanol production by bacteria and yeast on lignocellulosic hydrolysates can be substantially hindered by inhibitory substances produced during hydrolysis *(6,20,21)*. Acetate and furfural were reported to be strongly inhibitory to recombinant *Z. mobilis* CP4(pZB5). In addition, syringaldehyde, vanillin, and HMF were shown to have moderately inhibitory effects at the concentrations determined in the hydrolysates *(6)*. The inhibitory effects of these compounds on ZM4(pZB5) have been found to be similar to those reported for CP4(pZB5) (Table 1). Most inhibitory compounds did not affect the ethanol yield of ZM4(pZB5), although they reduced both the rates of xylose utilization and ethanol production. The present results suggest that one of these compounds, acetate, may have a significant inhibitory effect on the cloned enzymes involved in pentose assimilation and metabolism and/or on limiting ATP availability.

For the likely concentrations of the identified inhibitory compounds, acetate has been shown to be most toxic. The strong inhibitory effect of acetate/acetic acid suggests that efforts to minimize toxicity should be directed to removing the acetic acid formed during the hydrolysis of the lignocellulosic substrates or to developing a fermentation strategy to overcome this inhibitory effect. As one approach to minimizing this inhibitory effect, an acetate-tolerant mutant of ZM4 has been developed that is capable of fermentation at pH 5.0 in the presence of 20 g/L of sodium acetate *(22)*.

The inhibitory effect of acetate can be directly related to the pH of the medium. As pH is decreased, the inhibitory effect of acetate (as acetic acid) on strains of *Z. mobilis* has been shown to increase, resulting in reduced growth rate and cell yield *(22–24)*. This effect results from the pH-depen-

dent nature of the dissociation of acetic acid. In the present study, it was shown that the effect of pH on the glucose metabolism of ZM4(pZB5) is similar to that of ZM4 in the presence of sodium acetate. However, xylose metabolism of ZM4(pZB5) was found to be highly sensitive to acetate addition at a pH <5.0.

The mechanism of acetic acid inhibition has been studied by ^{31}P NMR (Figs. 4 and 5). Our in vivo ^{31}P NMR studies have confirmed the suggestion that acetic acid causes its inhibitory effect by acidification of the cytoplasm *(25,26)*. We have shown also that both intracellular levels of sugar phosphates and NTP of ZM4(pZB5) for growth on xylose were markedly decreased by the addition of sodium acetate. This decrease in NTP suggests that intracellular deenergization is also one of the mechanisms by which acetic acid exerts its toxic effect. These inhibitory mechanisms suggested for *Z. mobilis* are similar to those proposed for *Pachysolen tannophilus* and *Pichia stipitis* by Lohmeier-Vogel et al. *(27)*, who suggested that intracellular deenergization and acidification were the major mechanisms by which the main degradation products (furfural, HMF, acetic acid) derived from acid hydrolysis of lignocellulosics exerted their toxic effect on yeast cells. With the recombinant *Zymomonas* strains utilizing xylose, it is possible also that the cloned enzymes are relatively sensitive to these inhibitory components.

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