0273-2289/1559-0291 (Online)/06/134/0015/$30.00

Enhancement of Acid Tolerance in *Zymomonas mobilis* by a Proton-Buffering Peptide

David J. Baumler,[1] Kai F. Hung,[2] Jeffrey L. Bose,[2] Boris M. Vykhodets,[2] Chorng M. Cheng,[2] Kwang-Cheol Jeong,[2] and Charles W. Kaspar*,[1–3]

[1]Cellular and Molecular Biology; [2]Food Microbiology and Toxicology; and [3]Molecular and Environmental Toxicology, University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706-1187, E-mail: cwkaspar@facstaff.wisc.edu

Received November 22, 2005; Revised January 6, 2006; Accepted January 26, 2006

Abstract

A portion of the *cbpA* gene from *Escherichia coli* K-12 encoding a 24 amino acid proton-buffering peptide (Pbp) was cloned via the shuttle vector pJB99 into *E. coli* JM105 and subsequently into *Zymomonas mobilis* CP4. Expression of Pbp was confirmed in both JM105 and CP4 by HPLC. *Z. mobilis* CP4 carrying pJB99-2 (Pbp) exhibited increased acid tolerance ($p < 0.05$) in acidified TSB (HCl [pH 3.0] or acetic acid [pH 3.5]), glycine-HCl buffer (pH 3.0), and sodium acetate-acetic acid buffer (pH 3.5) in comparison to the parent strain (CP4) and CP4 with pJB99 (control plasmid). Although the expression of Pbp influenced survival at a low pH, the minimum growth pH was unaffected. Growth of *Z. mobilis* in the presence of ampicillin also significantly increased acid tolerance by an unknown mechanism. Results from this study demonstrate that the production of a peptide with a high proportion of basic amino acids can contribute to protection from low pH and weak organic acids such as acetic acid.

Index Entries: *Z. mobilis*; acid tolerance; CbpA; ampicillin; pH homeostasis.

Introduction

A great deal of research has been directed toward an economical biomass to ethanol conversion scheme as a renewable energy source

*Author to whom all correspondence and reprint requests should be addressed.

(for recent reviews, *see* Dien et al. *[1]* and Jeffries and Jin *[2]*). There are a number of candidate ethanol-producing organisms that have potential for this industrial process (*Saccharomyces cerevisiae*, *Pichia stipidis*, *Candida shehatae*, *Candida tropicalis*, *Schizosaccharomyces pombe*, *Pachysolen tannophilus*, *Klebsiella oxytoca*, *Erwina chrysanthemi*, *Escherichia coli, Zymomonas mobilis*, *Lactobacillus* species, and *Clostridium* species), but none have all of the desirable traits of the ideal ethanologen *(1,3)*. *Z. mobilis* is the most efficient ethanol producer among the candidates with the production of 1.5 to 1.9 mol ethanol from each mol glucose *(4)*, but it is limited by the relatively narrow spectrum of carbon sources *(5)* that are metabolized (glucose, fructose, and sucrose). *Z. mobilis* has been genetically engineered to expand its substrate utilization by introduction of a plasmid encoding for xylose metabolism (xylose isomerase, xylulokinase, transaldolase, and transketolase) *(6)*; however, the original engineered strain displayed an acid sensitive phenotype (M. Finkelstein, personal communication).

The acid-sensitive phenotype was found to be associated with the *tetA* marker on the engineered plasmid; therefore, the genes for xylose metabolism were inserted into the chromosome of *Z. mobilis* CP4. A similar approach was used to expand the capability of *Z. mobilis* strain AX101 to co-ferment glucose, xylose, and arabinose, because conversion of these three sugars to ethanol is pivotal for complete bioconversion of herbaceous crops *(7)*. Even with expanded capabilities for substrate utilization, a major obstacle in batch fermentations are the cidal effects of acetic acid, commonly present in hydrolysates, and also produced as a minor byproduct of glucose metabolism *(4)*. In addition, growth on xylose increases the sensitivity of engineered *Z. mobilis* strains to acetic acid *(8)*. NMR studies with *Z. mobilis* determined that acetic acid inhibition is a result of deenergization and acidification of the cytoplasm *(9)*. Some increased tolerance of xylose-fermenting *Z. mobilis* strains to acetic acid has been achieved through culturing the strain in continuously higher concentrations of hydrolysate *(10)*, but the co-fermenting AX101 strain remained sensitive to acetic acid, especially in the presence of ethanol *(1)*.

In this study, the acid tolerance of *Z. mobilis* CP4 was studied and increased by cloning a portion of the acid-induced *cbpA* gene from *E. coli (11)* [proton-buffering peptide (Pbp) (33.4% of amino acids capable of occupying one or more protons)] into *Z. mobilis* CP4. The effects of the antibiotic resistance genes *bla* (ampicillin^r) and *tetA* (tetracycline^r) on acid tolerance in *Z. mobilis* are also discussed.

Materials and Methods

Bacterial Strains and Culture Media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth *(12)* with shaking (150 rpm) at 37°C and strains were stored in nutrient broth (Difco Laboratories, Detroit, MI) supplemented with 10% glycerol at –70°C. *Z. mobilis*

Table 1
Plasmids and Bacterial Strains Used in This Study

Strain or plasmid	Relevant characteristics	Source and/or reference
Strains		
E. coli K-12	Non-pathogenic	Lab collection
E. coli JM105	Host for plasmid replication	Lab collection
Z. mobilis CP4	Isolate from fermenting sugarcane in Recife, Brazil	*(6)*
Plasmids		
pBR-322	Ap^r, Tc^r	Lab collection
pKK-223-3	Ap^r	Pharmacia
pZB-186	*tet A*(C), Cm^r	*(6)*
pJB99	Ap^r, Δ*tet A* (C), Δ*cat* (Cm^r), pZB-186	This study
pJB99-2	pJB99 with P_{tac}-*pbp*	This study

strains were grown in RM broth *(6)* without shaking at 30°C and stored in RM broth supplemented with 20% glycerol at –70°C. Growth of cultures was monitored spectrophotometrically at 600 nm using a Bioscreen C Analyzer (Thermolabsystems, Helsinki, Finland). For *E. coli* log-phase cultures, 5 µL of an 18-h culture was used to inoculate 5 mL of LB broth (with ampicillin for all pJB99-containing strains) and incubated with shaking (150 rpm) at 37°C and harvested at $A_{600} = 0.4 \pm 0.1$. For *Z. mobilis*, strains grown in antibiotics had slower growth rates than the parent strain. Therefore, 10 µL of the parent strain (CP4), or 100 µL of CP4 strains either grown in the presence of Amp or containing pZB-186, pJB99, or pJB99-2, from 24-h cultures were used to inoculate 10 mL of RM broth and incubated at 30°C for 14 h ($A_{600} = 0.5 \pm 0.1$). Antibiotics (Sigma Chemical Company, St. Louis, MO) were added to media when appropriate: 20 µg/mL tetracycline, 100 µg/mL ampicillin for *E. coli* and the CP4 parent strain, and 200 µg ampicillin/mL for pJB99-containing CP4 strains. All pH measurements were made using an Accumet model 10 pH meter (Fisher Scientific, Pittsburgh, PA) with the provided Accumet combination pH electrode (gel-filled) and standardized using pH 7.0 and 4.0 buffer solutions (Fisher).

General Genetic Methods

Procedures for the isolation of genomic DNA were carried out as described by Sambrook et al. *(12)*. Plasmid DNA was isolated using the QIAprep Spin Minikit and protocol (Qiagen Inc., Chatsworth, CA). The preparation of competent cells and transformations of *E. coli* were carried out as described previously *(12)* with the minor modification that transformants were recovered on tryptic soy agar (TSA; Difco) containing the appropriate antibiotic for each respective plasmid-containing strain (pZB-186, 20 µg/mL tetracycline; pJB99 or pJB99-2, 100 µg/mL ampicillin).

Z. mobilis competent cells and transformations were carried out following the *E. coli* protocol *(12)*, with the exceptions that RM medium was used and transformants were recovered on RM agar containing the antibiotic for each respective plasmid-containing strain (pZB-186, 20 μg/mL tetracycline; pJB-99 or pJB99-2, 200 μg/mL ampicillin), and the modification of 37°C incubation for 5 min instead of 42°C for 90 s. Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA). Linear fragments and plasmid DNA were compared to a DNA ladder (1 Kb) or Supercoiled ladder (Promega Corp., Madison, WI) and then purified from 1% agarose gels (Gibco BRL, Grand Island, NY) using the Geneclean II kit (Bio101, Inc., Vista, CA). Primary DNA cloning and manipulation were conducted in *E. coli* JM105. All PCR amplifications of DNA were performed using an Amplitron II Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA) with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles. All reagents and Taq DNA polymerase were used as recommended by the manufacturer (Perkin-Elmer Co., Foster City, CA).

Construction of Shuttle Vector pJB99 and pJB99-2

The plasmid pZB-186 (Tc^r, Cm^r) *(6)* was digested with *Cla*I and *Nde*I, and the 2504-bp fragment comprising *tet* A(C) was removed. The β-lactamase gene from pBR-322 with the addition of *Cla*I and *Nde*I sites was amplified by PCR using the primers ampnde-1 (5′ GGACTTCCATATGGTAAA CTTGGTC 3′) and ampcla-2 (5′ CCATCGATGGACAATAACCCTGAT 3′). The resulting fragment was digested with *Cla*I and *Nde*I, and ligated into pZB-186 (Tc^s, Cm^r). The resulting vector pZA (Tc^s, Cm^r, Ap^r) was digested with *Pvu*II to remove the 412-bp fragment containing the promoter region for chloramphenicol resistance, and the vector self ligated to yield pJB99 (Tc^s, Cm^s, Ap^r) (Fig. 1A).

The nucleotide sequence encoding for Pbp (MGVKPTDDLKTIKTA YRRLARKYH) was amplified by PCR from the *cbpA* gene of *E. coli* K-12 using the primers *cbpa-1* (5′ GCCGGAATTCATGGGCGTGAAACC GACGGA 3′) and *cbpa*-2 (5′ AAAACTGCAGTCAATGGTATTTGCGGGC 3′). The amplified 100-bp fragment was digested with *Eco*RI and *Pst*I and ligated into pKK-223-3. pKK-223-3(*pbp*) was then digested with *Ssp*I, and the blunt-end fragment (*pbp* with the P_{tac} promoter) was ligated into a *Pvu*II-digested pJB99 to yield pJB99-2 (5690 bp). The insertion of the P_{tac}-*pbp* fusion into pJB99 was confirmed by agarose gel (1.5%) electrophoresis following transformation into *E. coli* JM105 (Fig. 1B). Additionally, the DNA sequence and insertion of *pbp* in pJB99-2 was confirmed by DNA sequencing using the primer 5′TGTTGACAATTAATCATCGGCTC3′ (University of Wisconsin-Madison Biotechnology Center). After purification from *E. coli* JM105, plasmids were transformed into *Z. mobilis* CP4 as described in general genetic methods. *E. coli* JM105 and *Z. mobilis* CP4 transformants were analyzed by agarose-gel electrophoresis to confirm the presence of the plasmid.

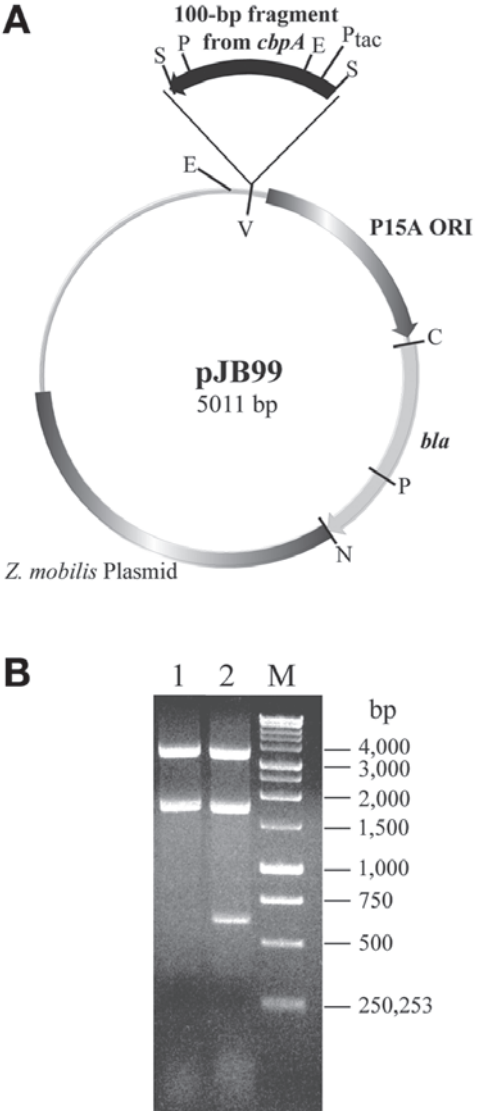

Fig. 1. **(A)** Restriction map of shuttle vector pJB99 displaying the region and orientation for the inserted fragment of Ptac and a portion of *cbp*A, which resulted in pJB99-2. C, *Cla*I; E, *Eco*RI; N, *Nde*I; P, *Pst*I; S, *Ssp*I; V, *Pvu*II. **(B)** Agarose gel electrophoresis of digested plasmids following restriction endonuclease digestion with *Eco*RI and *Pst*I. Lanes 1, pJB99; 2, pJB99-2. Molecular size markers were run in lane M; sizes are shown to the right.

Pbp Expression

Cultures of *E. coli* were grown to log-phase ($A_{600} = 0.4 \pm 0.1$) and IPTG added to a final concentration of 2.5 m*M* 1 h prior to harvest; whereas, *Z. mobilis* cultures were grown to log-phase ($A_{600} = 0.5 \pm 0.1$) without IPTG. Protein was extracted following the manufacturer's instructions using the

B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). *E. coli* protein extracts were passed through a Centricon-30 Microconcentrator (Amicon, Danvers, MA) to harvest proteins smaller than 30,000 molecular weight. *E. coli* and *Z. mobilis* protein extracts (10 µL) were analyzed using a Discovery BIO Wide Pore C18 HPLC column (Supelco, Bellefonte, PA) and separated by high-performance liquid chromatograph (System Gold 125, Beckman Coulter, Fullerton, CA) equipped with a UV detector (200–300 nm, 168 System Gold, Beckman), with the following conditions: solvent A (0.1% trifluoroacetic acid [TFA] [Pierce] [vol/vol] in HPLC-grade H_2O [Sigma]), solvent B (0.08% TFA [vol/vol] in 50% acetonitrile [Pierce] [vol/vol] and 50% H_2O), and a flow rate of 0.2 mL/min. For the initial 35 min of the separation, 100% of solvent B was used followed by 70%/30% (solvent A/solvent B) for the remaining 30 min. A Pbp standard was synthesized (University of Wisconsin-Madison Biotechnology Center) and added to *E. coli* JM105 protein extracts to determine the elution time.

Acid Challenge of E. coli *JM105 Strains*

Acid tolerance was assessed in Trypticase soy broth (TSB) (Becton Dickinson and Co., Cockeysville, MD) adjusted to pH 3.0 with HCl (12 *N*) and filter sterilized as previously described *(13)*. Cultures in log-phase (A_{600} = 0.4 ± 0.1) were used to inoculate 250-mL flasks containing 100 mL of acidified TSB to achieve a final concentration of approx 10^5 CFU/mL. After inoculation, the flasks were incubated at 37°C with shaking (150 rpm) and aliquots were removed at appropriate intervals and plated in duplicate on TSA plates using a Model D Spiral plater (Spiral Biotech, Norwood, MA). The plates were incubated overnight at 37°C, and the percent survivors calculated using the CFU/mL immediately after inoculation as 100%. A maximum decrease of 4-$\log_{10}$ CFU/mL could be detected, because the limit of detection for this method was 20 CFU/mL *(14)*.

Acid Challenge of Z. mobilis *Strains*

The acid tolerance of log-phase cells was determined in filter-sterilized TSB adjusted to pH 3.0 with 12 *N* HCl, TSB adjusted to pH 3.5 with glacial acetic acid, glycine-HCl buffer (pH 3.0), and sodium acetate-acetic acid buffer (pH 3.5). Log-phase cultures were used to inoculate 100 mL of acidified medium or buffer in 250-mL Erlenmeyer flasks to yield a final concentration of approx 10^5 CFU/mL. The inoculated flasks were then incubated at 30°C with shaking (75 rpm) and aliquots removed at appropriate intervals and plated in duplicate on RM agar (pH 5.5) using a Model D Spiral plater. The plates were incubated for 3 d at 30°C. The percent survivors were calculated as described previously for *E. coli*.

Minimum Growth pH

Five microliters of an 18-h culture of *E. coli* JM105 was used to inoculate triplicate tubes containing 5 mL of LB broth (including ampicillin for

the pJB99-containing strain and tetracycline for the pZB-186 containing strain) adjusted with HCl (12 *N*) to pH 4.0 to 6.0 (0.5 pH unit increments) and incubated with shaking (150 rpm) at 37°C for 2 d. For *Z. mobilis* cultures, 100 µL of a 24-h culture of the parent and plasmid-containing strains was used to inoculate 10 mL of RM broth adjusted to pH 3.5 to 5.5 (0.5 pH unit increments) using HCl (12 *N*) or acetic acid and incubated without shaking at 30°C for 3 d.

Statistical Analyses

The data reported are the average values from at least three trials. Statistical significance was determined using the *t*-test (Sigmaplot; Hallogram Publishing, Aurora, CO).

Results and Discussion

Construction of pJB99-2 and Pbp Expression

A portion of *cbpA* was cloned into the shuttle vector pJB99 (Fig. 1A) and transformed into *Z. mobilis* CP4. Because previous work in our laboratory found that the use of *tetA* as a selective marker decreases acid tolerance in *E. coli* whereas *bla* and growth in media containing ampicillin had no impact on acid tolerance (unpublished observation), the *tetA* gene was removed from pZB-186 and replaced with *bla* as the selective resistance marker for pJB99. Verification of the constructed plasmids was conducted by agarose gel electrophoresis following restriction endonuclease digestion with *Eco*RI and *Pst*I (Fig. 1B). The expected fragments for each digested plasmid were: pJB99 (3307 and 1704 bp) and pJB99-2 (3307, 1690, 616, 77 bp). The 77-bp fragment generated from pJB99-2 was not detectable with the electrophoresis conditions employed.

Confirmation of expression of Pbp in pJB99-2-containing strains of *E. coli* JM105 and *Z. mobilis* CP4 was conducted by HPLC (Fig. 2). Synthesized Pbp added to JM105 extracts eluted at 28.4 min (Fig 2A). Pbp expression was demonstrated in strains harboring pJB99-2 and was inducible with IPTG in JM105 (Fig. 2B) and constitutively produced in *Z. mobilis* (Fig. 2D). Protein extracts from CP4 (Fig. 2C) and CP4 containing the control plasmid (pJB99, data not shown) had no detectable peaks at an elution time of 28.4 min. Likewise, there was no peptide detected in JM105 and JM105 containing the control plasmid (pJB99) (data not shown). These data confirm Pbp expression in both JM105 and CP4-containing pJB99-2.

Acid Tolerance of Z. mobilis

Because acetic acid is considered one of the main causes of cell death in batch fermentations, acid tolerance using acetic acid as the acidulate in both nutrient-rich (TSB) and nutrient-free conditions was investigated. In addition, to determine if *Z. mobilis* cells survive differently in a strong acid, survival was also evaluated in HCl-acidified TSB and glycine-HCl

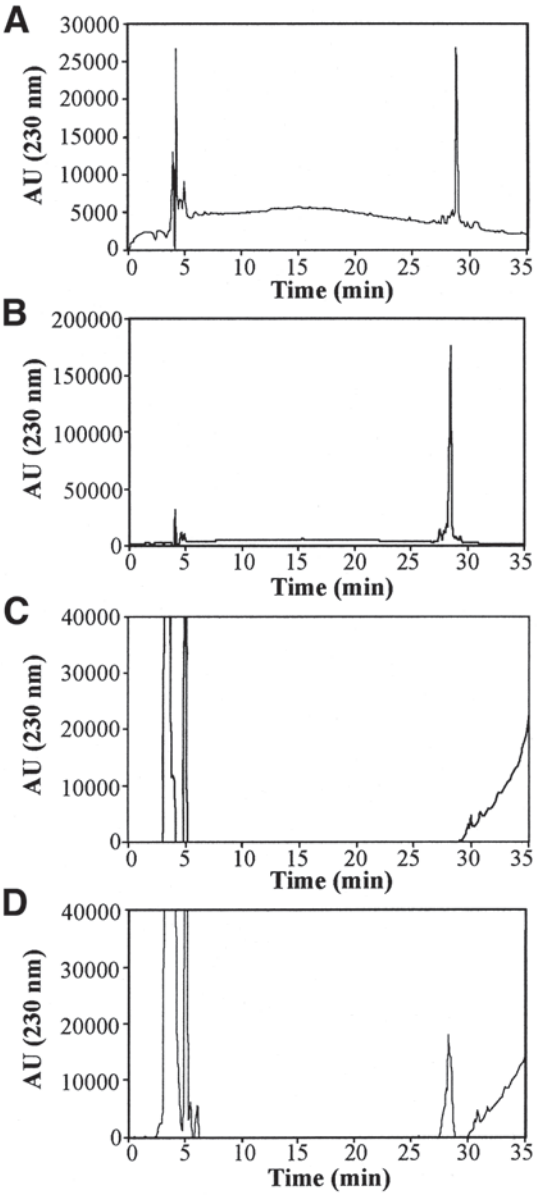

Fig. 2. Detection of Pbp by HPLC **(A)** in wild-type *E. coli* JM105 protein extracts with synthesized Pbp, **(B)** JM105 with pJB99-2, and **(D)** *Z. mobilis* CP4 with pJB99-2, and absence of a peak in the parent CP4 **(C)**.

buffer. Because CP4 strain is naturally tolerant of 100 µg/mL of ampicillin *(15)*, plasmid-containing strains were grown in the presence of 200 µg ampicillin/mL. Results from acid challenge experiments found that CP4 grown in the presence of ampicillin (100 µg ampicillin/mL) were significantly more acid tolerant ($p < 0.05$) than the parent strain grown without ampicillin in all acid challenge conditions (Table 2). Growth with a greater concen-

Table 2
Survival of Log-Phase *Z. mobilis* Strains After 1 h of Acid Challenge in Each Respective Medium

	% Survivors ± SE			
Strain	TSB (acetic acid[a])	Sodium acetate-acetic acid buffer[a]	TSB (HCl[b])	Glycine-HCl buffer[b]
CP4	0.17 ± 0.01	1.2 ± 0.1	5.4 ± 0.3	21.1 ± 0.8
CP4 grown in Amp[c]	13.4 ± 0.3	4.5 ± 0.4	20.6 ± 0.3	37.1 ± 2.1
CP4 with pJB99[d]	23.0 ± 1.1	22.2 ± 1.2	51.0 ± 0.4	60.4 ± 2.7
CP4 with pJB99-2[d]	70.6 ± 0.6	72.0 ± 1.3	67.6 ± 0.5	85.7 ± 1.5
CP4 with pZB-186[e]	nd[f]	nd	1.8 ± 0.2	nd

[a]pH 3.5.
[b]pH 3.0.
[c]Grown in 100 μg/mL ampicillin.
[d]Grown in 200 μg/mL ampicillin .
[e]Grown in 20 μg/mL tetracycline.
[f]Not determined.

tration of ampicillin also increased acid tolerance. CP4 with pJB99 grown in 200 μg/mL was significantly more acid tolerant ($p < 0.01$) than CP4 grown in 100 μg ampicillin/mL. Growth in the presence of ampicillin resulted in greater protection from HCl than acetic acid ($p < 0.05$) and was more pronounced in glycine-HCl buffer than TSB-HCl ($p < 0.01$). In contrast, the presence and absence of nutrients had little to no effect on tolerance to acetic acid. The "ampicillin effect" is likely a consequence of the cellular changes that are triggered or selected for by ampicillin. However, the genome of *Z. mobilis* strain ZM4 [CP4] ATCC 31821 has been sequenced *(16)* and reveals an ORF predicted to encode a β-lactamase (GI 56542573). Thus, if the strain used in this study produces β-lactamase, the exact mechanism by which ampicillin enhances acid tolerance is unclear and requires additional study.

The expression of Pbp significantly increased acid tolerance in all acid challenge conditions and CP4 with pJB99-2 was also significantly more acid tolerant ($p < 0.05$) than cells containing the control plasmid and grown in 200 μg ampicillin/mL (Table 2). The protection afforded by Pbp was not consistently affected by the type of acid or the presence or absence of nutrients in the challenge medium although the greatest number of survivors was recovered from challenges conducted in glycine-HCl buffer. We hypothesize that Pbp contributes to cytoplasmic buffering by sequestering protons. The amino acid composition of Pbp along with the end-amino group theoretically allows the peptide to occupy 9 or more protons when the internal pH of the cell is below the lowest pKa (R-group) of 6.0 *(17)*. The percent of amino acids in Pbp capable of occupying one or more protons (33.4%) is greater than the composition of typical proteins (13.6%) (Table 3) *(18)*. Additionally, acid tolerance was associated with production

Table 3
Percent of Amino Acids With Positively Charged R Groups in Typical Proteins and Pbp

Amino acid	pKa[a] (R group)	Amino acid composition (%) Typical protein[b]	β-lactamase[c]	Pbp
Arginine	12.0	5.7	6.6	12.5
Lysine	10.5	5.7	3.8	16.7
Histidine	6.0	2.2	2.5	4.2
Amino acids capable of occupying ≥1 proton		13.6	12.9	33.4

[a]Reported pKa of R groups of free amino acids *(17)*.
[b]Frequency of occurrence and pKa of amino acid residues from 1021 unrelated proteins of known sequence *(18)*.
[c]β-lactamase is provided for comparative purposes *(21)*.

of Pbp but not β-lactamase (12.9% basic amino acids), which had no influence on acid tolerance in *E. coli* (unpublished data). The ability of amino acids or proteins to bind protons in the cytoplasm may play a role in pH homeostasis although the mechanisms of pH homeostasis in *Z. mobilis* have not been fully elucidated. CP4 with pZB-186 was significantly more sensitive to acid challenge ($p < 0.001$) than all other strains in TSB-HCl, which indicates that pZB-186 renders cells sensitive to acid stress. These results demonstrate that growth in the presence of ampicillin and the production of Pbp both increase acid tolerance in *Z. mobilis*.

Minimum Growth pH of Z. mobilis

Although the survival of *Z. mobilis* at low pH was influenced by Pbp production and growth in the presence of ampicillin, the minimum growth pH was not affected. CP4 strains produced turbidity ($A_{600} > 0.8$) in the pH range of 4.0 to 5.5 and slight turbidity ($A_{600} = 0.03 \pm 0.005$) at pH 3.5. Similarly, the growth of *E. coli* JM105 and JM105 strains with pJB99 or pJB99-2 (data not shown) grew in the pH range of 4.5 to 6.0. However, the minimum pH for growth of JM105 harboring pZB-186 was 5.0. It is noteworthy that during growth studies with *Z. mobilis*, the final pH of the medium decreased when the starting pH was greater than 5.0 but increased when the starting pH was 3.5 to 4.5 (data not shown). Belaich and Senez *(19)* showed that half of the carbon in *Z. mobilis* is derived from glucose whereas the other half comes from one or more amino acids. Therefore, amino acids can serve not only as a nitrogen source but also as a carbon source for *Z. mobilis (4)*. Some genera of lactic acid fermenting bacteria (*Lactococci*, *Streptococcus*, and *Lactobacilli*) utilize deiminase pathways to metabolize amino acids to survive in acidic conditions *(20)*. A survey of the *Z. mobilis* ZM4 [CP4] annotated genome reveals ORF's predicted to encode for peptidylarginine deiminase

(GI 56543839) and ornithine carbamoyltransferase (GI 56542879), which represent potential candidates for the first two enzymes in an arginine deiminase pathway. It is possible that a deiminase pathway exists in *Z. mobilis* that contributes to pH homeostasis during acid stress.

The increase in acid tolerance observed in *Z. mobilis* expressing Pbp demonstrates that the production of a basic peptide can supplement an organism's ability to counter the detrimental effects of acid stress. The increased acid tolerance observed when *Z. mobilis* was grown in the presence of ampicillin warrants further investigation to determine the mechanism of protection and evaluate cross protection to other stresses. These results further our understanding of acid tolerance in *Z. mobilis* and metabolic bottlenecks that must be addressed in order to achieve the maximum potential of this bacterium in industrial fermentations.

Acknowledgments

We thank Adam Borger, Jeffrey Byrd, Mark Finkelstein, Thomas Jeffries, and Amy Wong for helpful discussions and review of this manuscript, and Barbara Cochrane for technical assistance in the preparation of the manuscript. We are grateful to Mark Finkelstein for providing *Z. mobilis* strains and pZB-186.

David Baumler was supported in part by an Edward J. and Katherine L. Schantz fellowship. The work was supported by the National Renewable Energy Laboratory, Golden, CO and the College of Agricultural and Life Sciences, University of Wisconsin, Madison.

References

1. Dien, B. S., Cotta, M. A., and Jeffries, T. W. (2003) *Appl. Microbiol. Biotechnol*. **63,** 258–266.
2. Jeffries, T. W. and Jin, Y. S. (2004) *Appl. Microbiol. Biotechnol*. **63,** 495–509.
3. Picataggio, S. K., Zhang, M., and Finkelstein, M. (1994), in *Enzymatic Conversion of Biomass For Fuels Production* (Himmel, M. E., Baker, J. O., and Overend, R. P., eds.), ACS Symp. Ser. 566, 342–362.
4. Swings, J. and DeLey, J. (1977) *Bacteriol. Rev*. **41,** 1–46.
5. Stokes, H. W., Picataggio, S. K., and Eveleigh, D. E. (1983), in *Advances in Solar Energy* (Böer, K. W. and Duffie, J. A., eds.), American Solar Energy Society, New York, NY, pp. 113–132.
6. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. (1995) *Science.* **267,** 240–243.
7. Mohagheghi, A., Evans, K., Chou, Y. C., and Zhang, M. (2002) *Appl. Biochem. Biotechnol*. **98–100,** 885–898.
8. Joachimsthal, E. L., Haggett, K. D., and Rogers, P. L. (1999) *Appl. Biochem. Biotechnol*. **77,** 147–157.
9. Kim, I. S., Barrow, K. D., and Rogers, P. L. (2000) *Appl. Biochem. Biotechnol*. **84–86,** 357–370.
10. Lawford, H. G. and Rousseau, J. D. (1999) *Appl. Biochem. Biotechnol*. **77–79,** 235–249.
11. Tucker, D. L., Tucker, N., and Conway, T. (2002) *J. Bacteriol*. **184,** 6551–6558.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual, 2nd ed*., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

13. Arnold, K. W. and Kaspar, C. W. (1995) *Appl. Environ. Microbiol*. **61,** 2037–2039.
14. Byrd, J. J., Cheville, A. M., Bose, J. L., and Kaspar, C. W. (1999) *Appl. Environ. Microbiol*. **65,** 2396–2401.
15. Afendra, A. S. and Drainas, C. (1987) *J. Gen. Microbiol*. **133,** 127–134.
16. Seo, J. S., Chong, H., Park, H. S., Yoon, K. O., Jung, C., Kim, J. J., Hong, J. H., et al. (2005) *Nature Biotech*. **23(1),** 63–68.
17. Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1993), in *Principles of Biochemistry, 2nd ed*., Worth Publishers, New York, NY, p. 113.
18. Creighton, T. E. (1993) *Proteins: Structures and Molecular Properties, 2nd ed*., W. H. Freeman and Co., New York, NY.
19. Belaich, J. P. and Senez, J. C. (1965) *J. Bacteriol*. **89,** 1195–1200.
20. Foster, J. W. (2000), in *Bacterial Stress Responses* (Storz, G., and Hengge-Aronis, R., eds.), American Society for Microbiology Press, Washington, D.C. pp. 99–115.
21. Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. USA* **75(8),** 3737–3741.