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Cloning of the *Zymomonas mobilis* Structural Gene Encoding Alcohol Dehydrogenase I (*adhA*): Sequence Comparison and Expression in *Escherichia coli*†

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Zymomonas mobilis **ferments sugars to produce ethanol with two biochemically distinct isoenzymes of alcohol dehydrogenase. The *adhA* gene encoding alcohol dehydrogenase I has now been sequenced and compared with the *adhB* gene, which encodes the second isoenzyme. The deduced amino acid sequences for these gene products exhibited no apparent homology. Alcohol dehydrogenase I contained 337 amino acids, with a subunit molecular weight of 36,096. Based on comparisons of primary amino acid sequences, this enzyme belongs to the family of zinc alcohol dehydrogenases which have been described primarily in eucaryotes. Nearly all of the 22 strictly conserved amino acids in this group were also conserved in *Z. mobilis* alcohol dehydrogenase I. Alcohol dehydrogenase I is an abundant protein, although *adhA* lacked many of the features previously reported in four other highly expressed genes from *Z. mobilis*. Codon usage in *adhA* is not highly biased and includes many codons which were unused by *pdc*, *adhB*, *gap*, and *pgk*. The ribosomal binding region of *adhA* lacked the canonical Shine-Dalgarno sequence found in the other highly expressed genes from *Z. mobilis*. Although these features may facilitate the expression of high enzyme levels, they do not appear to be essential for the expression of *Z. mobilis adhA*.**

The gram-negative bacterium *Zymomonas mobilis* is obligately fermentative and dependent upon substrate-level phosphorylation for ATP generation (33). Ethanol production serves as the dominant route for NADH oxidation, consuming 95% of pyruvate produced by glycolysis. The ethanologenic pathway in *Z. mobilis*, like that of *Saccharomyces cerevisiae*, consists of two essential activities, pyruvate decarboxylase and alcohol dehydrogenase (ADH). These two activities and the enzymes of glycolysis comprise 30 to 50% of the soluble protein in *Z. mobilis* (28).

Two isoenzymes of ADH present in *Z. mobilis* appear to be of nearly equal importance during fermentation (17, 25, 34). Such occurrences of ADH isoenzymes for fermentation are widespread among eucaryotes (13, 35). Typically, these isoenzymes are very similar in sequence and are presumed to share a common ancestry (16). However, the isoenzymes in *Z. mobilis* are unrelated, based on biochemical properties and a partial peptide sequence (4, 25). Zinc is bound to the active center of ADHI (11, 17, 25, 34), analogous to the fermentative ADH of *S. cerevisiae* (35). ADHII binds iron instead of zinc (25) and does not exhibit sequence similarity with the N terminus of ADHI (7, 25).

The relative contribution of *Z. mobilis* isoenzymes to cellular ADH activity is dependent upon the availability of iron and zinc (22), the presence of alcohol (11, 25), and the phase of growth (17, 27). The combined ADH activities do not appear to be present in large excess over metabolic needs, as evidenced by the accumulation of acetaldehyde during fermentation (33). ADHI is inhibited by ethanol (11), and it has been proposed that it functions primarily during the early stages of fermentation (33).

The effects of metal ion availability on *Z. mobilis* ADH isoenzymes have been studied in some detail (22) and appear to be coordinated. The level of ADHI increased in response to a reduction of ADHII activity. The gene encoding ADHII (*adhB*) from *Z. mobilis* has been cloned, sequenced, and overexpressed in recombinant *Z. mobilis* (7, 21). The presence of multiple copies of the *adhB* promoter region (and truncated gene) reduced the expression of chromosomal *adhB* but had no effect on the level of ADHI (*adhA* [21]). ADHII was overexpressed 7- to 14-fold in recombinants containing the complete gene, but these changes did not affect ADHI levels. However, the expression of β-galactosidase in *adhB-lacZ* operon fusions was reduced under conditions in which ADHI activity was high (21).

This study describes the sequencing of *adhA* and compares the translated amino acid sequence of this protein to *Z. mobilis* ADHII and other alcohol dehydrogenases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and vectors used in this study have been described previously (5, 27). *Z. mobilis* CP4 was grown at 30°C in complex medium (22) containing 2% glucose. *Escherichia coli* TC4 was grown in Luria broth without added carbohydrate (19) or on medium solidified with 1.5% agar.

Aldehyde indicator plates were prepared with 2% ethanol as described previously (7). Where indicated, 0.5% butanol was used instead of ethanol. Indicator plates containing ampicillin (50 mg/liter) were used for the direct selection of transformants expressing alcohol dehydrogenase activity.

DNA manipulations. Standard methods were used for the purification of DNA, plasmid constructions, Southern blot analysis, and transformation of *E. coli* (23). Digestions with restriction enzymes were carried out as recommended by the manufacturer. The probe for Southern blot analysis was prepared by the random primer method from a 1.25-kilobase-pair (kbp) fragment within the *adhA* gene (*Hin*dIII-to-*Eco*RI fragment).

Construction of gene library. A library of *Z. mobilis*

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chromosomal DNA was constructed as described previously (7), except that 5- to 10-kbp fragments generated by partial digestion with *Sau*3A were purified by agarose gel electrophoresis. These were inserted into the *Bam*HI site of pUC18. Plasmids were prepared by the alkaline lysis method (23) from the pooled colonies on the original transformation plates and served as an amplified library.

Assay of alcohol dehydrogenase activity. Cells were prepared and assayed as described previously (7). Enzyme activities are expressed as international units per milligram of total cell protein.

Gel electrophoresis. Soluble proteins were examined by using the Phast gel electrophoresis system (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). French press extracts (22) were compared on native gels (7.5% acrylamide, pH 8.8), denaturing gels (12.5% acrylamide gels containing 0.55% sodium dodecyl sulfate), and isoelectric focusing gels (pH 3 to 9). Proteins were stained with Coomassie blue. Alcohol dehydrogenase activity was visualized in isoelectric focusing gels and native gels by using phenazine methosulfate and nitrotetrazolium blue (9). Protein standards were obtained from Pharmacia.

DNA sequencing and analysis. The gene encoding ADHI from *Z. mobilis* was sequenced by the dideoxy method by using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio). Double-stranded plasmid DNA was prepared for use as a template. The 1.25-kbp insert contained in pLOI134 and pLOI135 was used for the construction of ordered deletions by using the Erase-a-Base system (Promega Biotec, Madison, Wis.), which allowed sequencing of both strands (Fig. 1). Resulting sequences were analyzed with GenePro sequence analysis software (Hoefer Scientific Instruments, San Francisco, Calif.) and the Sequence Analysis Software Package (University of Wisconsin, Genetics Computer Group, Madison, Wis.).

Purification of alcohol dehydrogenase I. ADHI was purified from *Z. mobilis* CP4 grown to stationary phase in complex medium containing 5% glucose. Harvested cells were washed in KOH-MES (morpholineethanesulfonic acid) buffer (pH 6.5) containing 30 mM NaCl and 2 mM $MgCl_2$ and disrupted with a French press at 20,000 lb/in^2. After an initial protamine sulfate treatment (8 mg/g of cell pellet), the supernatant was dialyzed overnight against the above buffer to which 0.1% β-mercaptoethanol was also added. ADHI was purified on a Cibacron-blue Sepharose column by affinity elution with NADH (34). The eluate was subsequently concentrated, desalted, and further purified on a QAE Zetaprep disk (Pharmacia LKB Biotechnology, Inc.), as described previously (22).

Immunological methods. The preparation of rabbit antibody against *Z. mobilis* ADHII has been previously described (22). Antibody against ADHI was prepared in a similar manner by using purified ADHI. Ouchterlony immunodiffusion tests were performed by using 1% agarose in physiological buffered saline. Cells were grown overnight and harvested. Pellets equivalent to 0.25 mg of cell protein were washed, suspended in 100 μl of cold 20 mM K-MES buffer (pH 6.5) and disrupted by vigorous mixing in the presence of 1.0 mg of lysozyme and 0.2 ml of chloroform. After 10 min of incubation at 4°C, the suspension was centrifuged at 10,000 × *g* for 2 min and the supernatant was used to fill the wells.

RESULTS

Cloning the gene encoding ADHI. The amplified plasmid library containing *Z. mobilis* CP4 DNA was transformed into

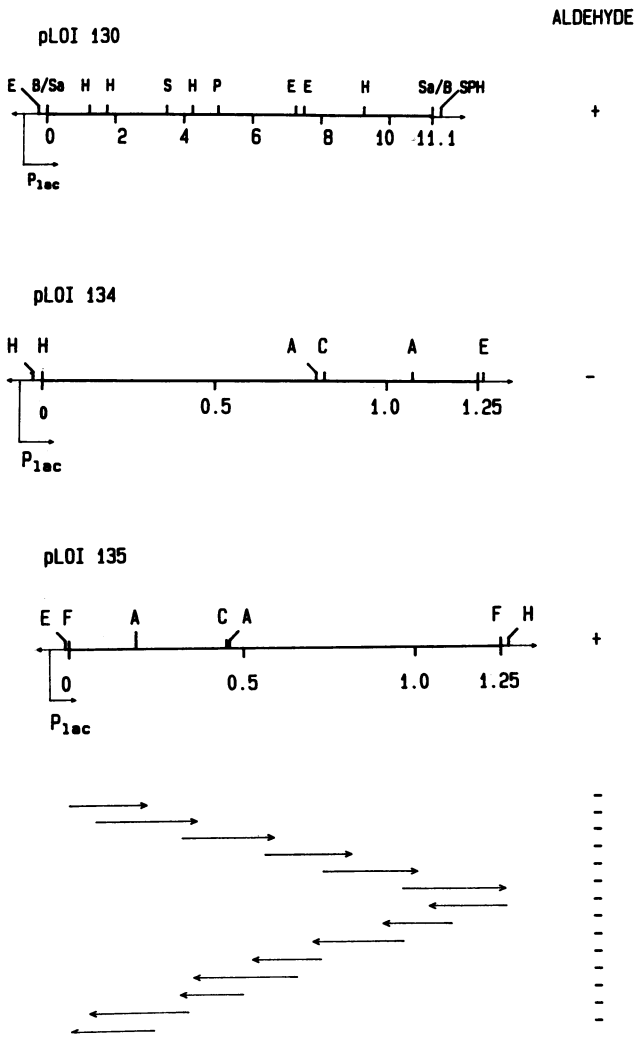

FIG. 1. Restriction maps of DNA fragments and subclones containing the *Z. mobilis adhA* gene and sequencing strategy. ADH activity of recombinant *E. coli* containing each respective plasmid is indicated on the right as positive (+) or negative (−), based on aldehyde indicator plates containing ethanol. The direction of transcription from the *lac* promoter (P_{lac}) is shown. Numbers on the map refer to size in kilobase pairs. Abbreviations: A, *Hae*II; B, *Bam*HI; C, *Hin*cII; E, *Eco*RI; F, site of blunt fusion; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sa, *Sau*3A.

E. coli TC4. Clones were selected for resistance to ampicillin on aldehyde indicator plates containing ethanol. Approximately 150 clones containing ADH activity were observed as bright red colonies among 20,000 ampicillin-resistant transformants. These clones would be expected to include both ADH isoenzymes, since ADHI and ADHII isoenzymes catalyze the reversible oxidation of ethanol. However, only ADHI oxidizes butanol as a substrate (34). Approximately half of the ethanol-positive clones were positive on aldehyde plates containing butanol, indicating the presence of *adhA*.

DNA was prepared from 48 butanol-positive clones and compared after digestion with *Pst*I. Most clones which were strongly positive contained an identical fragment of DNA, and one of these, pLOI130, was selected for further study. This plasmid contained an 11.1-kbp DNA fragment which was mapped with a variety of restriction endonucleases (Fig. 1). Large segments of DNA were deleted by digesting with

TABLE 1. Expression of *Z. mobilis* gene encoding ADHI in *E. coli* TC4

Plasmid	Activity (IU/mg of protein)[a]	
	Ethanol	Butanol
pUC18	0.2	0.1
pLOI130	7.8	2.3
pLOI134	0.3	0.2
pLOI135	28.1	9.3

[a] Activity expressed per milligram of total cell protein.

*Pst*I, *Sal*I, *Eco*RI, or *Hin*dIII, followed by self-ligation. All but the *Eco*RI subclone retained ADHI activity. The *Hin*dIII subclone was designated pLOI133 and contained only 1.25 kbp of *Z. mobilis* DNA.

The *adhA* fragment from pLOI133 was purified from an agarose gel after digestion with *Eco*RI and *Hin*dIII. Two constructs were made with this purified fragment. The fragment was ligated into pUC19 in the opposite orientation with respect to the *lac* promoter to produce pLOI134, a construct which was inactive on aldehyde indicator plates. In a second construct, missing bases were filled at the ends of the *Eco*RI-to-*Hin*dIII fragment by the Klenow fragment of *E. coli* DNA polymerase, followed by insertion into the *Sma*I site of pUC18 to produce pLOI135. The *Z. mobilis* DNA fragment in pLOI135 retained the same orientation as the original clone, pLOI130, with respect to the *lac* promoter and formed red colonies (positive) on aldehyde indicator plates.

The presence of the gene encoding ADHI on pLOI135 was further confirmed by Ouchterlony diffusion analysis (not shown). A diffuse precipitin band formed between TC4(pLOI135) and ADHI antiserum. This diffuse band from TC4(pLOI135) exhibited partial identity with the single band from *Z. mobilis*. No bands were observed with ADHII antiserum. No precipitin bands were observed with protein from the control, TC4(pUC18), using either antisera.

The 1.25-kbp fragment in pLOI133, pLOI134, and pLOI135 approximates the minimal size required to encode ADHI, a protein of 40,000 M_W (25). Deletions from either end of this fragment resulted in a loss of activity (Fig. 1). Southern blots with fragments from this clone indicated the *adhA* gene is present as a single copy on the *Z. mobilis* chromosome (not shown).

Expression of *Z. mobilis* gene encoding ADHI in *E. coli*. The expression of ADHI in *E. coli* was confirmed by examining substrate specificity (Table 1). *Z. mobilis* ADHI is known to oxidize butanol at approximately one-third the rate of ethanol oxidation, while ADHII oxidizes only ethanol (17). Both ethanol and butanol oxidizing activity were expressed in TC4(pLOI130) and TC4(pLOI135), consistent with the presence of ADHI activity. Over threefold higher activity was observed with the 1.25-kbp DNA fragment (pLOI135) than in the original clone, pLOI130, containing 11.1 kbp of *Z. mobilis* DNA. ADHI can be calculated to represent 12% of the total cellular protein and 17% of the soluble protein in TC4(pLOI135), assuming a specific activity of 240 IU/mg of pure enzyme (25). Little additional ADH activity above that of the control containing pUC18 was observed in TC4(pLOI134), a plasmid in which the *Z. mobilis* DNA fragment is in the opposite orientation with respect to the *lac* promoter.

The expression of ADHI in TC4(pLOI135) was further confirmed on zymograms, native gels stained for ADH activity (Fig. 2A). Two bands, which corresponded to ADHI and ADHII (lane 3), were observed in protein extracts from *Z. mobilis* CP4. The more anodally migrating band corresponds to ADHII (34). The slower band is ADHI and migrated to the same position as the dominant ADH activity band in extracts from TC4(pLOI135) (lane 2). Two minor ADH activity bands which migrated more slowly were observed in TC4(pLOI135). These also appear to be derived from pLOI135, since no ADH bands were observed in control extracts of TC4(pUC18) (lane 1). The two minor ADH bands are presumed to be of higher molecular weight and may represent multimers with more than four subunits.

On denaturing gels (Fig. 2B), a prominent protein band which was absent in TC4(pUC18) (lane 1) was observed in extracts from TC4(pLOI135) (lane 2). This prominent band corresponds to a M_W of approximately 40,000, the reported subunit size of ADHI (25). ADHI is the most abundant protein in TC4(pLOI135), as expected from estimates based on specific activity. A prominent band was also present in this region in extracts of *Z. mobilis* CP4 (lane 3).

Isoelectric focusing gels resolved three new bands in

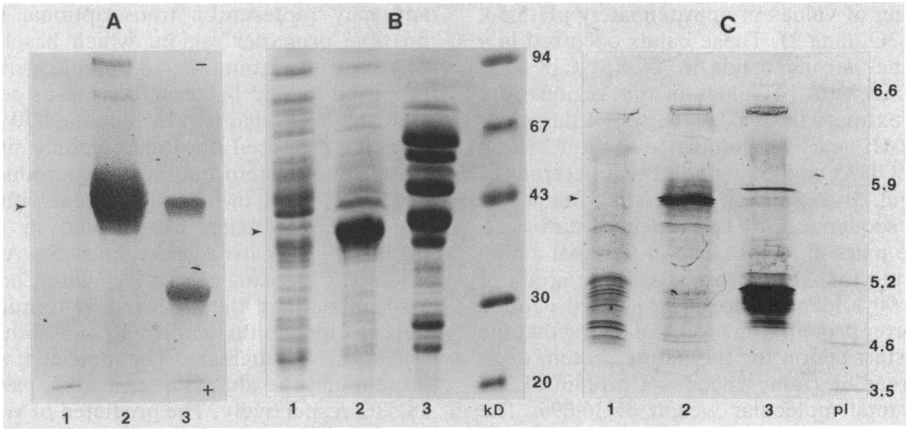

FIG. 2. Polyacrylamide gel electrophoresis showing expression of the *Z. mobilis adhA* gene in *E. coli*. The ADHI region is marked by arrows on the left side of each figure. ADHII is also marked on the zymogram. The isoelectric points (pI) and molecular masses for standards are indicated at the right. (A) Native polyacrylamide gel stained for ADH activity. (B) Denaturing polyacrylamide gel stained with Coomassie blue. (C) Isoelectric focusing gel stained with Coomassie blue (pH 3 to 9). Lanes were loaded with approximately 10 μg of protein each. Lanes: 1, *E. coli* TC4(pUC18); 2, *E. coli* TC4(pLOI135); 3, *Z. mobilis* CP4.

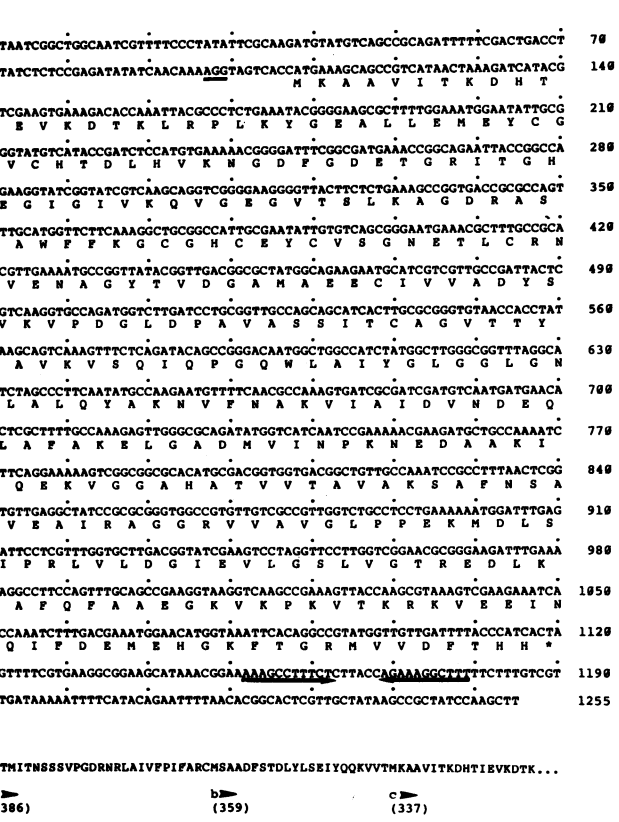

FIG. 3. Nucleotide and amino acid sequence of *Z. mobilis* ADHI and fusion proteins (GenBank accession no. M32100). (A) Sequence and translation of the open reading frame encoding native ADHI enzyme. The proposed ribosomal binding site is underlined. A palindromic sequence which may serve as transcriptional terminator is marked with double arrows at the end of the coding region. (B) Translation of the open reading frames encoding the N terminus of two potential start sites for ADHI in *E. coli* and for the fusion protein which includes the start codon from the *lac* α-peptide. The N-terminal methionines are indicated by arrows and the number of amino acid residues contained in each respective protein. Abbreviations: a, start codon of the α-peptide; b, alternate start codon at *Z. mobilis* methionine-42; c, start for native *adhA* in *Z. mobilis*.

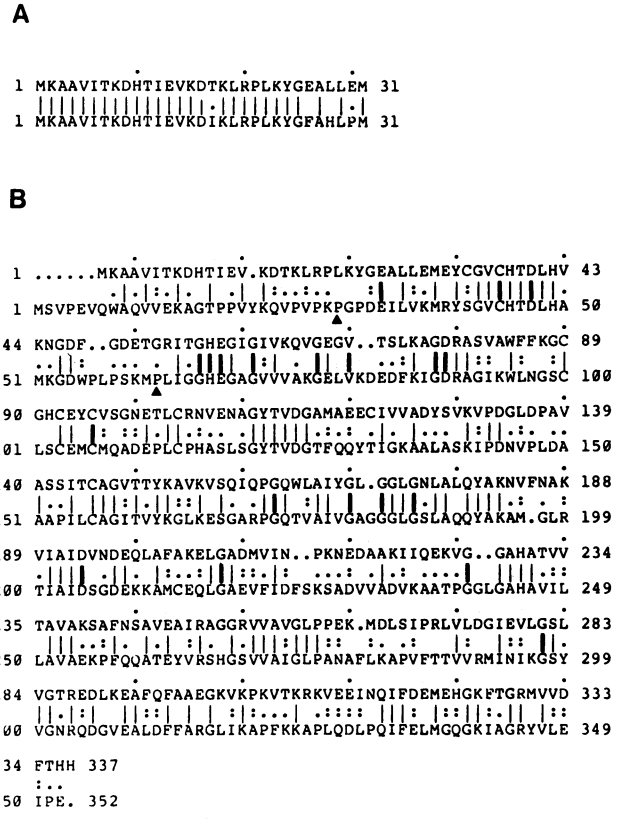

FIG. 4. Comparison of amino acid homology between alcohol dehydrogenases. Identity (|) and degree of similarity (.) or (:) are indicated. (A) Comparison between the N-terminal amino acid sequence deduced from the nucleotide sequence and the sequence determined by N-terminal analysis (25). (B) Comparison between the *Z. mobilis* gene and that of *A. nidulans*. ▮, Identity between strictly conserved residues of zinc-containing, long-chain alcohol dehydrogenases and *Z. mobilis* ADHI; ▲, positions of the two conserved prolines absent from *Z. mobilis* ADHI.

TC4(pLOI135), having pI values of approximately pH 5.50, 5.70, and 5.75 (Fig. 2C, lane 2). These bands occurred in a region devoid of other strong bands in TC4(pUC18), the control (lane 1). Bands with pI values in this region were observed in protein extracts from *Z. mobilis* CP4 (lane 3).

Sequence of the ADHI gene. The sequence of the 1.25-kbp DNA fragment in pLOI135 containing *adhA* was determined in both directions (Fig. 3). A single open reading frame was identified within this sequence, with two potential start sites, 1,011 and 1,077 base pairs in length. The N-terminal amino acid sequence reported for ADHI from a different strain of *Z. mobilis* (25) was 90% identical to the predicted peptide sequence for the shorter protein (Fig. 4A), indicating that the second ATG is the start codon for the native protein in *Z. mobilis*. This open reading frame encodes a protein of 337 amino acids with a total molecular weight of 36,096. The predicted amino acid composition from the sequence is in excellent agreement with that determined by Wills et al. (34).

The native coding region of *adhA* is preceded by the sequence AGG, a probable ribosomal binding site (31, 32) located 10 base pairs upstream from the start codon (Fig. 3A). The open reading frame is followed by tandem, in-frame termination codons which are separated by 21 base pairs. A palindromic sequence capable of forming a stem and loop structure begins immediately after the second stop codon and may represent a transcriptional termination site. A possible promoter region, which has homology with promoter regions from other *Z. mobilis* genes (14) and with the proposed enteric bacterial consensus sequence (29) for the −10 and −35 regions, was identified 70 base pairs upstream from the proposed ribosomal binding site.

A fusion protein may also be produced in *E. coli* from pLOI135, due to in-frame alignment with the *lacZ* α-peptide. Three new proteins were noted in extracts from TC4 (pLOI135) on native gels stained for ADH activity and on isoelectric focusing gels stained with Coomassie blue. Figure 3B summarizes the deduced N-terminal amino acid sequences for the fusion protein and both start sites in the *Z. mobilis* DNA fragment. The molecular weight of the fusion protein and the alternate start in *Z. mobilis* are 41,565 and 38,586, respectively. The predicted pI values based upon the deduced primary sequences are identical for both the fusion protein and for the native *Z. mobilis* ADHI, pH 5.9, while that of the alternate *Z. mobilis* start site is pH 5.5. Since ADHI is multimeric, the multiple isoelectric species may reflect different ratios of monomer types.

TABLE 2. Comparison of codon usage in *Z. mobilis*

Amino acid	Codon	Frequency[a]		Amino acid	Codon	Frequency	
		adhA	Combined[b]			*adhA*	Combined
Phe	TTT	10	9	Ala	GCT	4	138
	TTC	5	44		GCC	7	51
					GCA	7	40
Leu	TTA	6	4		GCG	13	14
	TTG	17	25				
	CTT	3	32	Tyr	TAT	3	26
	CTC	5	28		TAC	4	12
	CTA	5	0				
	CTG	6	65	His	CAT	2	18
					CAC	2	21
Ile	ATT	10	21				
	ATC	10	57	Gln	CAA	3	2
	ATA	5	0		CAG	10	27
Met	ATG	15	45	Asn	AAT	9	26
					AAC	3	52
Val	GTT	4	94				
	GTC	5	43	Lys	AAA	18	66
	GTA	9	1		AAG	25	45
	GTG	6	9				
				Asp	GAT	1	57
Ser	TCT	6	19		GAC	1	47
	TCC	5	23				
	TCA	17	5	Glu	GAA	2	86
	TCG	19	6		GAG	0	6
	AGT	2	4				
	AGC	5	21	Cys	TGT	1	3
					TGC	3	15
Pro	CCT	5	14				
	CCC	2	4	Trp	TGG	14	10
	CCA	12	6				
	CCG	12	49	Arg	CGT	0	33
					CGC	3	22
Thr	ACT	3	10		CGA	6	0
	ACC	3	64		CGG	12	1
	ACA	2	1		AGA	4	0
	ACG	8	21		AGG	9	0
Gly	GGT	1	97				
	GGC	3	38				
	GGA	1	2				
	GGG	6	0				

[a] Total number of times the codon was used.
[b] Sum of codons used in *Z. mobilis gap*, *pgk*, *adhB*, and *pdc*.

Codon usage. The pattern of codon usage in the *adhA* gene is less biased than that of other glycolytic and alcohologenic enzymes from *Z. mobilis* (Table 2). Six codons not used in the other *Z. mobilis* genes (CTA, ATA, CGA, AGA, AGG, and GGG) occur frequently in *adhA*. A single codon is clearly dominant for 16 of the 20 amino acids in *gap*, *pgk*, *adhB*, and *pdc*, but only 5 of these codons are used frequently in *adhA*.

Conservation of primary amino acid sequence among genes encoding alcohol dehydrogenase. The characteristics of zinc-containing long-chain alcohol dehydrogenases have been reviewed by Jornvall et al. (16). Comparison of the deduced amino acid sequence of *adhA* revealed a high degree of amino acid conservation with this type of alcohol dehydrogenase (Table 3). Two other types of alcohol dehydrogenases, nonmetallo short-chain dimeric alcohol dehydrogenases (*Drosophila melanogaster*) and tetrameric iron-containing alcohol dehydrogenases (*Z. mobilis* ADHII and *Clostridium acetobutylicum* ADHI) exhibited low identity with ADHI from *Z. mobilis*. Comparisons were also made between the

TABLE 3. Comparisons of amino acid sequence between *Z. mobilis* ADHI and ADH enzymes from other organisms

Organism	Comparison (%)		Reference
	Similarity	Identity	
B. stearothermophilus N terminus	64.3	45.2	2
A. nidulans ADH3	58.3	40.2	24
Schizosaccharomyces pombe	59.4	37.9	30
S. cerevisiae ADH3	57.1	37.2	35
Human liver class I gamma subunit	52.0	34.1	13
Horse liver E chain	50.2	30.9	15
Maize ADH1	49.1	26.2	8
Alcaligenes eutrophus	51.1	25.4	14
C. acetobutylicum adh1	44.2	19.6	36
Z. mobilis ADHII	44.0	19.5	7
D. melanogaster Adh-S	43.4	17.6	18

adhA amino acid sequence and two other dehydrogenases from bacteria, methanol dehydrogenase from *Methylobacterium organophilum* (20) and threonine dehydrogenase (*tdh*) from *E. coli*. This latter sequence (sequence file designator, ecokbltdh; R. L. Somerville, P. D. Ravnikar, and B. D. Ronson, unpublished observations) was identified during homology searches of the current Genbank database and contains 27% identity with *Z. mobilis* ADHI. The potential homology of this enzyme with zinc alcohol dehydrogenases has not been previously recognized. Methanol dehydrogenase showed little similarity to the *Z. mobilis* enzyme. Among the available sequences, the N-terminal peptide sequence of ADH from *Bacillus stearothermophilus* (45.2%) and the complete sequence of ADH3 from *Aspergillus nidulans* (40.2%) were most similar to that of *Z. mobilis* ADHI. It is interesting to note that the phosphoglycerate kinase enzymes from *Z. mobilis* and *A. nidulans* also exhibited the highest degree of amino acid identity among the available sequences (3).

Figure 4 compares the primary sequences of *Z. mobilis* and *A. nidulans*. Twenty-two residues are strictly conserved in zinc-containing long-chain alcohol dehydrogenases (16); these are marked by thick bars. All but two of these residues were conserved in ADHI, including residues forming the active-site zinc ligands, (cysteine-37 and histidine-60), acidic residues having defined binding interactions (aspartate-40, aspartate-193, and glutamate-61), and all glycine residues (glycine-169, glycine-172, and glycine-175) which are proposed to have special roles in the coenzyme-binding fold. The conservation of two proline residues in ADH enzymes from yeasts, fungi, higher plants, and mammals has been presumed to also reflect important functions for these hydrophobic residues in secondary structure (16). However, neither proline is essential, since both are absent from *Z. mobilis* ADHI. The positions of a further 13 residues have been deduced by Jornvall et al. (16) as being typical but not invariable in alcohol dehydrogenases. Eight of these consensus residues are conserved in ADHI: valine-66, lysine-130, lysine-203, glycine-253, valine-256, valine-258, glycine-259, and glycine-324.

DISCUSSION

Z. mobilis is unusual in having two different functional isoenzymes for ethanol production which appear to be both biochemically (7, 17, 25, 35) and genetically unrelated. Comparison of the deduced amino acid sequence with other

ADH enzymes indicates that *Z. mobilis* ADHI is similar to eucaryotic zinc-dependent alcohol dehydrogenases (about 35% identity) and appears unrelated to *Z. mobilis* ADHII. *Z. mobilis* ADHI appears most similar to the alcohol dehydrogenase from *B. stearothermophilus* (N terminus only [2]) and to ADHI from *A. nidulans* (24). Based upon similarities in primary amino acid sequence, *Z. mobilis adhA* fits within the group of eucaryotic genes proposed by Jornvall et al. (16) to have evolved from an ancient, long-chain alcohol dehydrogenase.

Z. mobilis adhA does not share many of the features found in the other highly expressed glycolytic and ethanologenic enzymes (13a). Codon usage patterns for genes from *Z. mobilis* and from *S. cerevisiae* (1, 12) which are expressed in large amounts are highly biased. The replacement of single codons in the yeast *pgk1* gene with codons for rare tRNA species has been shown to dramatically reduce expression (10). The highly expressed *gap* (6), *pgk* (3), *pdc* (5), and *adhB* (7) genes of *Z. mobilis* use only 48 of the potential 61 amino acid codons and exhibit preferential use of a single codon for many amino acids. The *adhA* gene uses 59 codons and does not exhibit a strongly preferential use of single codons for most amino acids.

The ribosomal binding regions of other highly expressed *Z. mobilis* genes have many features in common (13a). The four such genes which have been previously described contain the sequence GAG in the Shine-Dalgarno region and match a minimum of five of the seven bases of the *E. coli* 16S RNA complement in this region. The *adhA* gene did not contain this consensus sequence and matched the *E. coli* sequence in only four positions. Other features of the translational control sequences common to highly expressed genes in *E. coli* (31, 32) and in *Z. mobilis* (13a), such as an A+T-rich region between the Shine-Dalgarno and ATG start codon followed by an alanine codon, were absent from the corresponding regions of the *adhA* sequence.

Despite the lack of expected features found in other *Z. mobilis* genes which are expressed at high levels, abundant ADHI protein is produced from the single chromosomal copy of *adhA*. The amounts of individual glycolytic enzymes in protein extracts can be calculated from measurements of native levels and activities for pure enzymes (25, 26, 28). In exponentially growing cells of *Z. mobilis*, approximately 1.4, 0.6, 3.3, and 0.9% of soluble cell protein is glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate decarboxylase, and alcohol dehydrogenase II, respectively (21). ADHI represents 0.6% of soluble protein under identical growth conditions, suggesting that neither highly biased codon usage nor a highly conserved ribosomal binding site is essential for high-level expression. Although these features may contribute to high-level expression, they are not present in all glycolytic and ethanologenic enzymes in *Z. mobilis*.

The four cloned *Z. mobilis* genes have been expressed from the *lac* promoter in *E. coli* as derivatives of pUC18, and all were expressed at very high levels (3, 5–7). Expression of ADHI was comparable to or better than that of the four other genes in *E. coli*, despite the lack of translational features commonly attributed to highly expressed genes.

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