

Expression of an L-Alanine Dehydrogenase Gene in *Zymomonas mobilis* and Excretion of L-Alanine

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An approach to broaden the product range of the ethanologenic, gram-negative bacterium *Zymomonas mobilis* by means of genetic engineering is presented. Gene *alaD* for L-alanine dehydrogenase (EC 1.4.1.1) from *Bacillus sphaericus* was cloned and introduced into *Z. mobilis*. Under the control of the strong promoter of the pyruvate decarboxylase (*pdc*) gene, the enzyme was expressed up to a specific activity of nearly $1 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ in recombinant cells. As a result of this high L-alanine dehydrogenase activity, growing cells excreted up to 10 mmol of alanine per 280 mmol of glucose utilized into a mineral salts medium. By the addition of 85 mM NH₄⁺ to the medium, growth of the recombinant cells stopped, and up to 41 mmol of alanine was secreted. As alanine dehydrogenase competed with pyruvate decarboxylase (PDC) (EC 4.1.1.1) for the same substrate (pyruvate), PDC activity was reduced by starvation for the essential PDC cofactor thiamine PP₁. A thiamine auxotrophy mutant of *Z. mobilis* which carried the *alaD* gene was starved for 40 h in glucose-supplemented mineral salts medium and then shifted to mineral salts medium with 85 mM NH₄⁺ and 280 mmol of glucose. The recombinants excreted up to 84 mmol of alanine (7.5 g/liter) over 25 h. Alanine excretion proceeded at an initial velocity of $238 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ [dry weight]⁻¹. Despite this high activity, the excretion rate seemed to be a limiting factor, as the intracellular concentration of alanine was as high as 260 mM at the beginning of the excretion phase and decreased to 80 to 90 mM over 24 h.

The gram-negative bacterium *Zymomonas mobilis* is a strong ethanol producer because of high ethanol yields (up to 95% of the theoretical maximal value) and great productivity (five to six times as fast as yeasts) (29, 35). These favorable traits, however, have not yet been used to form new products. As a prerequisite for the enlargement of the product spectrum, it is necessary to express heterologous genes in *Z. mobilis*. Recently, expression shuttle vectors for *Z. mobilis* were constructed (6, 7, 22, 28) and used to broaden the substrate range of *Z. mobilis* (18, 31).

The aim of the present study was to investigate L-alanine formation in *Z. mobilis* by the introduction and expression of the gene (*alaD*) for the enzyme L-alanine dehydrogenase (AlaDh) (EC 1.4.1.1) from *Bacillus sphaericus* IFO3525. The aim was to redirect a portion of the carbon flux between glucose and ethanol at the intermediate pyruvate to L-alanine (Fig. 1). AlaDh catalyzes the reversible deamination of L-alanine to pyruvate and ammonia with NAD⁺ as a cofactor. The enzyme is found in vegetative cells and spores of various bacilli and in other bacteria in which it plays a role in both ammonium assimilation and the generation of energy through the tricarboxylic acid cycle during sporulation (12, 24, 38). The enzyme of *B. sphaericus* IFO3525 has been purified to homogeneity (27). Recently, the corresponding gene (*alaD*) was cloned and its DNA sequence was determined (20). The present study describes the cloning and expression of the *alaD* gene from *B. sphaericus* in *Z. mobilis*, with the subsequent excretion of L-alanine by the recombinant cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are shown in

Table 1. Strains of *Z. mobilis* were cultivated anaerobically in rich medium with 2% glucose as described before (4). Selective media were supplemented with 30 mg of tetracycline or 20 mg of tetracycline plus 25 mg of nalidixic acid per liter for counterselection against *Escherichia coli* donor strains in filter matings. Alanine formation by recombinant *Z. mobilis* strains was carried out anaerobically at 30°C in mineral salts medium (ZMM) as described by Fein et al. (10) or in complementation medium (PM) containing 85 mM NH₄-acetate and 5% glucose (280 mM). The pH of this medium was adjusted to 7.0 with 5 M KOH. *E. coli* strains and *B. sphaericus* IFO3525 (DSM5019) were cultivated aerobically in LB medium consisting of 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of sodium chloride per liter. Selective media were supplemented with 100 mg of ampicillin or 25 mg of tetracycline per liter.

Recombinant DNA techniques. Preparation, restriction analysis, ligation, and agarose gel electrophoresis of plasmid DNA were carried out as described by Maniatis et al. (23). Cloning of the *alaD* gene from *B. sphaericus* IFO3525 was done as follows. Chromosomal DNA was prepared as described by Byun et al. (6) and completely digested with *EcoRI* plus *HindIII*. Chromosomal fragments of about 2.5 to 3.5 kb were isolated from 1.0% agarose gels and ligated into vector pUC19 linearized with *EcoRI-HindIII*. For identification of *alaD*⁺ clones, *E. coli* ED8767 was transformed with the ligation mixture and selection was done on LB-ampicillin plates. Plasmid preparations from 10 ampicillin-resistant clones each were transferred to a nylon membrane (Biohyne A; Pall, Dreieich, Federal Republic of Germany [FRG]) and hybridized against a ³²P-labeled 27-mer oligonucleotide (synthesized by Pharmacia LKB, Freiburg, FRG) which was homologous to the N terminus of the *alaD* gene (5'-GCAGGAGTTGTATCCTTAACGCATGCT-3') (20) by the method of Zeff and Geliebter (40).

Enzyme assays. Cells from the late exponential growth

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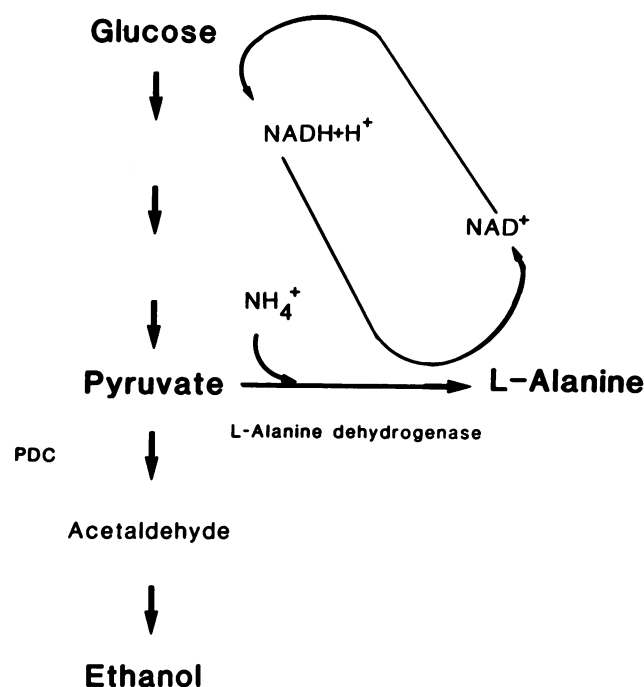


FIG. 1. Scheme of redirection of the carbon flux to alanine formation in *Z. mobilis*.

phase were harvested by centrifugation, washed once in ZMM, and resuspended in the appropriate test buffer. The bacteria were disrupted by sonification five times for 30 s each time at 35 to 40 W in an ice bath with a Sonifier B 15 (Branson Sonic Power Co., Danbury, Conn.). Cell debris was centrifuged at $38,000 \times g$, and the supernatant was used

as a cell extract. Protein content was measured as described by Bradford (3) with bovine serum albumin as a standard.

AlaDh activity was determined in the direction of oxidative deamination at 37°C as described by Williamson (39).

Pyruvate decarboxylase (PDC) (EC 4.1.1.1.) activity was determined at 30°C as described by Ullrich (36).

Specific enzyme activities are expressed as units (micro-moles \cdot minute $^{-1} \cdot$ milligram of protein $^{-1}$).

Analysis of fermentation products and glucose consumption. Culture samples were analyzed after centrifugation. The amounts of ethanol and acetate were estimated by gas chromatography as described by Finn et al. (11). Alanine and other amino acids were determined by reversed-phase high-pressure liquid chromatography after precolumn derivatization with *o*-phthalaldehyde on an HP 1090 chromatograph (Hewlett-Packard, Waldbronn, FRG). D-Glucose concentrations were determined spectrophotometrically with hexokinase and glucose-6-phosphate dehydrogenase in accordance with the manufacturer's instructions (Boehringer, Mannheim, FRG), and DL-lactic acid concentrations were determined enzymatically with D- and L-lactic acid dehydrogenases and glutamic acid dehydrogenase as described previously (26).

Measurements of intracellular amino acids. Cells of a 200- μ l culture aliquot were separated from the medium by silicone oil centrifugation with 65 μ l of silicone oil (PN 200; Bayer, Leverkusen, FRG) as the separation layer and 30 μ l of 20% HClO₄ as the acid fixation layer (19). After homogenization of the sedimented cells in the acid layer, the perchloric extracts were neutralized with a solution containing 5 M KOH and 1 M triethanolamine and chilled on ice to precipitate the KClO₄ formed during neutralization. The precipitated salt together with the denatured protein and residual oil was centrifuged, and the supernatant was used for amino acid analysis.

TABLE 1. Strains and plasmids used in the present work

Strain or plasmid	Relevant characteristics and markers ^a	Source or reference
Strains		
<i>E. coli</i> K-12		
ED8767	<i>thi met hsdS recA</i>	25
S17-1	<i>thi pro hsdR::RP4</i>	33
ECG18	<i>thi thr leu pro/pRK2013 aphA</i>	21
<i>B. sphæricus</i> IFO3525 (DSM5019)		Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, FRG
<i>Z. mobilis</i>		
Cu1Rif2	ATCC 10988 Rif ^r	C. Drinas (1)
CP4	ATCC 31821	14
CP4thi	CP4 thiamine auxotroph	12a
Plasmids		
pUC19	2.7 kb, <i>bla</i>	37
pPTZ4	6.7 kb, <i>bla tet</i>	28
pSUP104	9.5 kb, <i>cat tet</i>	32
pZY70	5.8 kb, <i>bla alaD</i>	This work
pZY71	5.6 kb, <i>bla alaD</i>	This work
pZY72	8.6 kb, <i>bla tet alaD</i>	This work
pZY73	12.3 kb, <i>tet alaD</i>	This work
pZY74	12.3 kb, <i>alaD</i> (reverse)	This work

^a *bla*, Ampicillin resistance; *tet*, tetracycline resistance; *aphA*, kanamycin resistance.

Calculation of intracellular amino acid concentrations. For the determination of intra- and extracellular water volumes of *Z. mobilis*, cells from a 500- μ l culture aliquot were incubated with $^3\text{H}_2\text{O}$ and [^{14}C]taurine (Amersham, Braunschweig, FRG) and separated from the surrounding medium by silicone oil centrifugation. The total water-permeable space and the extracellular space were determined from the distribution of $^3\text{H}_2\text{O}$ and [^{14}C]taurine (30). The intracellular volume of *Z. mobilis* cells ranged from 1.8 to 2.2 $\mu\text{l} \cdot \text{mg}$ (dry weight) $^{-1}$, depending on the culture conditions.

The total amount of amino acids found in the cell extracts was corrected for the fraction of the medium that contaminated the extracts because of cosedimentation during centrifugation. The intracellular amino acid concentrations were calculated by dividing the corrected amount of amino acids in the extracts by the actual cytoplasmic volume measured as described above.

RESULTS

Subcloning of the *B. sphaericus alaD* gene. Recently, it was shown that the gene for AlaDh from *B. sphaericus* IFO3525 is located on a 3.1-kb *EcoRI-HindIII* DNA fragment (19a, 20). Therefore, the *alaD* gene was cloned from a chromosomal DNA preparation of *B. sphaericus* IFO3525 by digestion of the DNA with *EcoRI* and *HindIII*, and fragments of 2.5 to 3.5 kb were isolated after agarose electrophoresis. For enrichment for the desired fragment, an additional digestion with *Bam*HI, *Pst*I, and *Sal*I was done, as it is known that these restriction enzymes do not cut the *alaD* fragment (20). By using an oligonucleotide probe (27-mer) complementary to the 5' coding region of the *alaD* gene (20), we investigated about 300 clones for the presence of the *alaD* gene. Two clones showed a positive reaction with the labeled DNA probe. The restriction profiles of the plasmids were in accordance with the published map (20), and both clones showed AlaDh activity. One clone was examined further, and its recombinant plasmid was termed pZY70 (Fig. 2). The detailed restriction map of pZY70 was based on data published earlier (20).

For shortening of the DNA fragment with the *alaD* gene, a limited exonuclease III treatment (16) from both ends of the *HindIII-EcoRI* fragment was done. A clone which had about 100 bp removed from each end was treated with S1 nuclease and Klenow polymerase (23) and blunt-end ligated into the *Sma*I site of vector pUC19 (37) to yield pZY71. For the expression of AlaDh in *Z. mobilis*, a 1.95-kb *Kpn*I fragment of pZY71 was prepared and ligated into the *Kpn*I-linearized vector pPTZ4 (28). The plasmid carrying the *alaD* gene in phase with the *pdh* promoter was termed pZY72. For propagation in vector pSUP104 (32), which has a broad host range, pZY72 DNA was cleaved with *EcoRI-Sca*I, an *EcoRI* linker was added to the *Sca*I blunt end, and the 2.3-kb fragment was inserted into the *EcoRI*-linearized vector pSUP104. The recombinant plasmid carrying the *alaD* gene in phase with the *cat* promoter of pSUP104 was termed pZY73; that carrying the gene in counter-phase was termed pZY74. An illustration of the steps is given in Fig. 2.

Expression of *alaD* in *E. coli* and *Z. mobilis* strains. Plasmid pZY72 could be transferred by triparental mating (ED8767/pZY72, ECG18/pRK2013, and Cu1Rif2) into *Z. mobilis* Cu1Rif2, but several attempts to transfer this plasmid into *Z. mobilis* CP4 and its derivative CP4thi failed. Although vector pZY72 had the advantage of being a small (8.64-kb) shuttle vector between *E. coli* and *Z. mobilis*, its narrow host range and its apparent instability in Cu1Rif2 (loss or recombination

with residing cryptic plasmids) were drawbacks for subsequent studies. Vectors pZY73 and pZY74, derived from the broad-host-range vector pSUP104, however, were successfully conjugated (with the helper strain S17-1) into *Z. mobilis* (conjugation frequency, ca. $10^{-5} \cdot \text{donor}^{-1}$). Both plasmids were stable without antibiotic pressure for more than 60 generations. By retransformation into *E. coli* ED8767 and subsequent restriction analysis, the integrity of the recombinant plasmids in *Z. mobilis* could be confirmed. AlaDh activities in cell extracts of various recombinant strains were measured. Rather high enzyme activities were found (Table 2). It was estimated that, on the basis of the maximal activity of purified AlaDh from *B. sphaericus* (27), about 0.6% of the total soluble protein was constituted by the AlaDh protein in *Z. mobilis* CP4/pZY73; this value was about 7% in the case of *E. coli* S17-1/pZY73. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with extracts from the parental strains and the recombinant strains revealed an extra protein band at about 38 kDa in the recombinant strains. This size corresponds well to the size of the AlaDh subunits from the purified enzyme of *B. sphaericus* (27).

Alanine formation and excretion. When these different strains of *Z. mobilis* were grown in ZMM with 5% glucose and 15 mM ammonium at pH 5.5, wild-type strains (Cu1Rif2 and CP4) did not excrete alanine, while recombinant strains excreted up to 10 mM alanine. As the AlaDh of *B. sphaericus* has a pH optimum for amination of 8.5 to 9.0 and a K_m for NH_4^+ of 28 mM (27), the conditions for optimal enzyme activity and for optimal growth of *Z. mobilis* are different. To overcome this problem, we developed a two-step procedure. In the first phase, recombinant cells were grown in ZMM to an optical density at 600 nm of about 2 (late exponential growth phase); in the second phase, they were washed and transferred to PM, which had a pH of 7.0 and included 85 mM NH_4^+ as well as 50 g of glucose $\cdot \text{liter}^{-1}$. The intermediate pH value was chosen to reconcile as far as possible the pH optimum of a series of Entner-Doudoroff pathway enzymes of *Z. mobilis* (pH 6.0) (2) with that of AlaDh. Under these conditions, L-alanine excretion could be increased to 41 mM (Table 3).

Although alanine had gained a considerable share (7 to 8%) of the carbon balance, ethanol was still the major fermentation end product. For enhancement of alanine formation, a mutant with reduced or deficient PDC activity would have been of interest. As PDC-deficient mutants of *Z. mobilis* were not available, a thiamine-deficient mutant of strain CP4 was used to generate a phenotype of transient PDC deficiency by starving the auxotrophic cells for the cofactor of PDC. When cells of CP4thi were transferred to a thiamine-deficient medium, PDC activity dropped from about 5 U $\cdot \text{mg}^{-1}$ to less than 1 U $\cdot \text{mg}^{-1}$ within 40 h. After cells of CP4thi/pZY73 were starved in a thiamine-free medium, they excreted more than twice as much alanine as did cells of CP4/pZY73 (Table 3). As the time course of alanine production showed, the highest alanine productivity occurred during the first 6 h (Fig. 3). This result can be explained by the fact that AlaDh activity decreased in parallel in the same range.

A change in the carbon flux from ethanol fermentation to alanine formation would demand that L-alanine be excreted very efficiently. While ethanol can pass through cell membranes quite well, nothing is known of the secretion of L-alanine. To obtain more information on the transport of L-alanine, we measured the cytosolic and external alanine concentrations in several recombinant *Z. mobilis* strains (Table 4). The intracellular alanine concentration in thia-

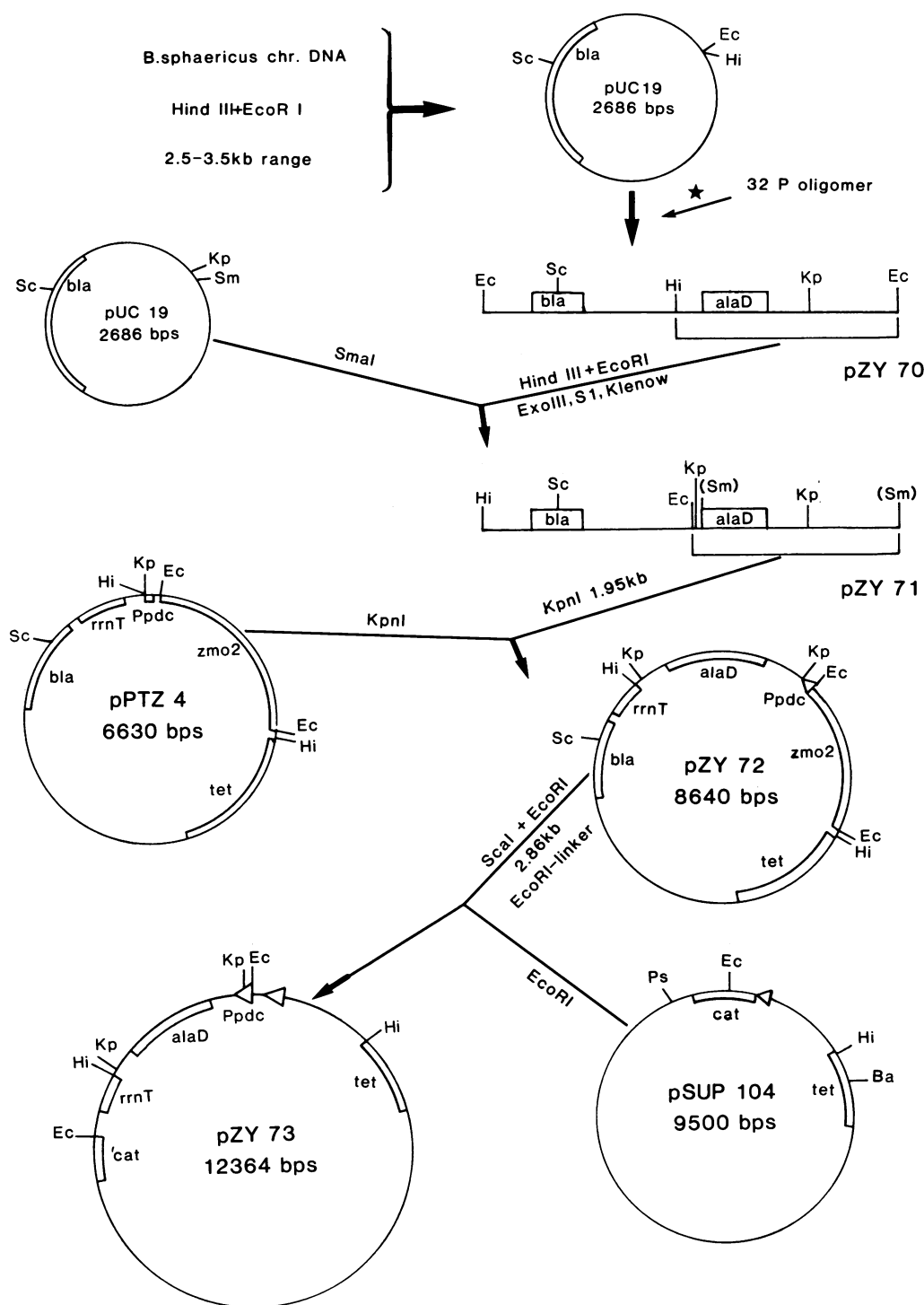


FIG. 2. Construction of expression vectors carrying the *alaD* gene. Starting from newly isolated *B. sphaericus* chromosomal (chr.) DNA, the *alaD* gene was subcloned into different vectors as described in Materials and Methods. Gene symbols are as follows: bla, ampicillin resistance; cat, chloramphenicol resistance; 'cat, truncated *cat* gene; tet, tetracycline resistance; *alaD*, alanine dehydrogenase; *rrnT*, transcriptional terminator of the *rrnB* operon; *zmo2*, cryptic plasmid pZMO2 of *Z. mobilis* ATCC 10988; *Ppdc*, promoter of the *pdc* gene from *Z. mobilis*. Other abbreviations are as follows: *Exo* III, exonuclease III; *S1*, nuclease S1; *Klenow*, subunit of DNA polymerase; 32 P oligomer, oligonucleotide (27-mer) used as a DNA probe for hybridization to the 5'-terminal end of the *alaD* gene. Restriction sites: Ba, *Bam*HI; Ec, *Eco*RI; Hi, *Hind*III; Kp, *Kpn*I; Ps, *Pst*I; Sc, *Sca*I; Sm, *Sma*I; (Sm), *Sma*I site removed by nonhomologous blunt-end ligation.

TABLE 2. Specific AlaDh activities of various recombinant strains^a

Strain	Plasmid	Sp act (U · mg ⁻¹)
<i>E. coli</i> ED8767	None	0.0
	pZY70	0.90
	pZY72	0.04
	pZY73	11.2
	pZY74	0.11
<i>Z. mobilis</i>	None	0.0
	CP4	0.0
	Cu1Rif2	0.3
	pZY72	0.99
	pZY73	0.96
	CP4thi	0.96

^a Extracts were prepared as described in Materials and Methods. Measurements were done in duplicate and are the means of at least two independent assays.

mine-starved cells of recombinant CP4thi/pZY73 increased more than 50-fold in comparison with that of the parental strain without the *alaD* plasmid. The gradient of internal and external alanine in the *alaD*⁺ strains was high at the beginning and leveled off during the incubation to reach an equilibrium. The alanine excretion rate of strain CP4thi/pZY73 was extremely high in the initial phase (up to 238 nmol · min⁻¹ · mg [dry weight]⁻¹). It is important to note that the drastic difference in the internal alanine concentration in the initial phase between the thiamine-deficient and non-deficient recombinant strains was not reflected in a corresponding difference in the efflux rate, indicating a saturation behavior. As a control for nonspecific permeability or lysis, the internal and external concentrations of glutamate were determined. The internal glutamate concentration remained more or less unchanged and decreased only after 22 h of incubation, when the internal volume of the cells also started to drop, probably because of lysis. The specific cytoplasmic volume of the bacterial cells provided an additional monitor for the intactness of the cells in PM. As this value was above 1.8 µl · mg (dry weight)⁻¹ during effective alanine efflux, the cells did not become leaky during the alanine production phase. Furthermore, we determined the

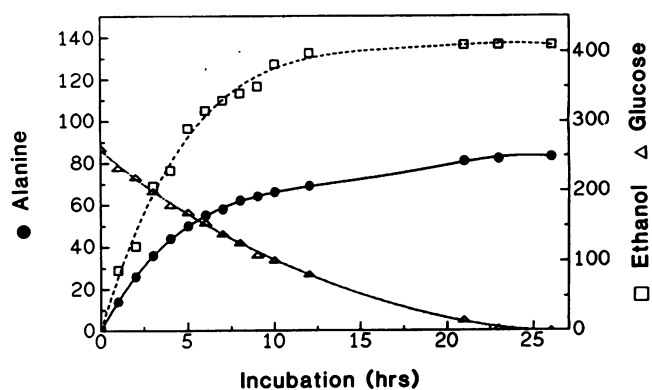


FIG. 3. Time course of alanine formation with CP4thi/pZY73 in PM. Strain CP4thi/pZY73 was pregrown in ZMM, washed twice in ZMM (buffered at pH 6.0), and incubated for 40 h at 30°C in the presence of excess glucose. At time zero, the culture was inoculated into PM (see Materials and Methods). Glucose, ethanol, and alanine concentrations were determined as described in Materials and Methods and are given as millimolar.

chemical gradient of potassium, which also remained significant (high internal concentration) during this time period.

DISCUSSION

As a prerequisite for alanine production with *Z. mobilis*, an appropriate gene had to be introduced and expressed in this bacterium. This was achieved by cloning the *alaD* gene from *B. sphaericus* and expressing it under the control of the strong *pdC* promoter of *Z. mobilis* (28). The expression of the enzyme reached 1 U · mg⁻¹ with plasmid pZY73. Cells of *B. sphaericus* IFO3525 had been reported to express the highest AlaDh activity (0.41 U · mg⁻¹) of 29 bacterial strains tested (27). Thus, the expression in *Z. mobilis* was more than twice as high as that in this natural AlaDh producer and may reflect a gene dosage effect. In recombinant *E. coli* cells with the same plasmid (pZY73), up to 11 U · mg⁻¹ was observed. The difference in expression between *E. coli* and *Z. mobilis* can be explained by the better usage in pZY73 of the *cat* promoter that is located 5' upstream of the *alaD* gene and that enhances gene expression in *E. coli*,

TABLE 3. Alanine excretion in recombinant *Z. mobilis* cells^a

Strain	Medium	Duration of incubation (h)	Concn (mM) of:			% of C source
			Glucose	Ethanol	Alanine	
CP4a	ZMM	15.5	266	505	0	0
Cu1Rif2/pZY72	ZMM	29	264	480	1.4	0.3
	PM	120	243	476	17	3.5
CP4/pZY73	ZMM	15.5	264	450	9	2
	PM	24	245	459	41	8.3
CP4thi/pZY73	ZMM	15.5	290	430	9	2
	PM	26	262	410	84	16

^a Cells were grown in ZMM (see Materials and Methods) with 5% glucose at 30°C to an optical density at 600 nm of 2 to 3, washed, and transferred to PM at 37°C. % of C source is the molar ratio of alanine formed to glucose consumed. Because of different error margins of the individual assays for residual glucose, ethanol formed, and alanine formed (see Materials and Methods), the carbon balance is not accurate in every case. Other end products were acetate and lactate (both below 2 mM each). Each value represents the mean of two independent measurements. For thiamine starvation, CP4thi/pZY73 was transferred from ZMM to ZMM buffered at pH 6.0 and incubated for 40 h at 30°C before being inoculated into PM.

TABLE 4. Internal (In) and external (Ex) concentrations of alanine (Ala) and glutamate (Glu) in different strains of *Z. mobilis* over time

Strain	Time (h)	CV ^a	Concn (mM) of:		In/Ex ^b	Concn (mM) of:		In/Ex ^b
			Ala In	Ala Ex		Glu In	Glu Ex	
CP4/pSUP104	0	2.2	4.5	0.1	45	3.2	0.1	32
	2	2.0	6.5	0.1	65	3.2	0.1	32
	4	2.0	6.7	0.3	22	2.0	0.1	20
	6	2.0	6.6	0.4	17	2.4	0.1	24
	8	1.6	6.6	0.4	17	2.1	0.2	11
	22	0.5	6.5	0.4	16	1.0	0.3	3
CP4/pZY73	0	2.5	30.1	0.7	43	3.0	0.1	30
	2	2.3	18.2	3.1	6	3.5	0.2	18
	4	2.3	30.0	8.9	3	5.5	0.3	18
	6	2.0	40.2	15.4	3	5.5	0.3	18
	8	1.4	42.8	25.8	2	4.5	0.3	15
	22	1.0	0.9	47.3	0	1.0	0.3	3
CP4thi/pZY73	0	2.2	266.0	3.7	72	3.7	0.1	37
	2	2.2	255.0	22.4	11	3.3	0.4	8
	4	2.2	206.4	32.9	6	2.9	0.4	7
	6	2.2	228.0	35.5	7	2.5	0.5	5
	8	2.2	146.6	42.8	3	3.0	0.5	6
	22	2.2	137.5	60.4	2	3.8	0.5	8
	26	2.1	129.0	61.9	2	1.5	0.6	3
	30	2.1	88.2	64.4	1	1.0	0.6	2
	48	1.8	84.0	64.8	1	1.6	0.6	3

^a CV, Cell volume (microliters · milligram [dry weight]⁻¹).^b In/Ex, Ratio of internal to external concentrations.

while with pZY74 (opposite direction of *alaD* with respect to the *cat* promoter), only 0.11 U of AlaDh was observed. The *cat* promoter, however, while being recognized, is less active in *Z. mobilis* (28). Expression of heterologous genes could also be variable in different host strains because of a suboptimal arrangement of the ribosome-binding site (13) or different codon usage (15). In the case of AlaDh, the nucleotide-binding site consists of four glycine residues (20) which are encoded preferentially by the GGU or GGA codon. *Z. mobilis*, however, nearly lacks the GGA codon (8), possibly resulting in stalled ribosomes on the *alaD* mRNA in recombinant *Z. mobilis* cells and in a less efficient translation of the messenger. Oligonucleotide-directed mutagenesis of this region to exchange the minor codons with the *Z. mobilis* major codons (15, 34) could be a way to improve enzyme activity.

Growing cells of CP4/pZY73 excreted 7 to 8% of the end-product carbon as alanine, while ethanol was still the major fermentation product. This result can be rationalized by a comparison of the kinetic parameters of PDC and AlaDh. PDC activity in *Z. mobilis* was found to be in the range of 3 to 5 U · mg⁻¹, while AlaDh activity was found to be about 1 U · mg⁻¹. The *K_m*s for the common substrate pyruvate, however, are 0.4 mM for PDC (5) and 1.7 mM for AlaDh (27). With in vivo concentrations of pyruvate near 1 mM (2) during the growth of *Z. mobilis* on glucose, these parameters are in favor of the ethanologenic pathway.

During the first 6 h of incubation of CP4thi/pZY73 in PM, about 24% of the carbon flow from glucose to pyruvate was directed into alanine formation, subtracting a substantial portion of carbon from the ethanologenic pathway. Prolonged incubation, however, resulted in the loss of AlaDh activity. For a total redirection of the carbon flow from ethanologenic to "homoalanine" fermentation, the AlaDh activity should be stabilized and/or increased to equal the in vivo activities of other enzymes of the Entner-Doudoroff pathway or even the concurrent PDC activity of 3 to 5

U · mg⁻¹ (5) to dispose of both the pyruvate and the NADH + H⁺ equivalents. Further improvements in AlaDh expression could be achieved by optimizing the distance between the *pdc* promoter and the translation start of *alaD*. Concomitantly, the concurrent PDC activity could be diminished by use of PDC-deficient mutants of *Z. mobilis*.

The mode of L-alanine excretion needs to be studied in more detail. Our results demonstrate that (i) during the productive phase cells are physically intact, (ii) alanine efflux is specific, as another amino acid (glutamate) was not excreted, and (iii) the secretion of alanine appears to be saturable. These results are not in agreement with the simple diffusion of alanine. Instead, they provide evidence for the presence of a carrier system for the secretion of this amino acid in *Z. mobilis*. Active efflux carrier systems in *Corynebacterium glutamicum* for other amino acids, namely, glutamate (17) and isoleucine (9), have recently been described.

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