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Differential Expression of *gap* and *pgk* Genes within the *gap* Operon of *Zymomonas mobilis*†

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In *Zymomonas mobilis*, the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAP) and phosphoglycerate kinase (PGK) are encoded in an operon that is transcribed from tandem promoters. The promoter-proximal *gap* gene is expressed at six- to ninefold higher levels than the *pgk* gene from chromosomal genes and from multiple copies of plasmid-borne genes. Two dominant transcripts were identified. The smaller, most abundant transcript contained primarily the *gap* message, whereas the larger, less abundant message contained both genes. The ratio of message levels for *gap* and *pgk* was calculated to be 5:1 and is sufficient to account for the observed differences in levels of GAP and PGK. The differences in message abundance are proposed to result from either transcriptional attenuation or preferential degradation of the 3′ region encoding *pgk*. Increases in gene dosage were accompanied by one-third the expected increase in enzymatic activity on the basis of estimates of copy number, consistent with the presence of a limiting, positive regulatory factor. However, GAP and PGK expressions were not reduced from the chromosome in recombinants that contained multiple copies of the *gap* operon with inactive genes.

Glyceraldehyde-3-phosphate dehydrogenase (GAP) and phosphoglycerate kinase (PGK) catalyze the synthesis of a high-energy phosphate bond and its transfer to ADP during glycolysis. This enzyme system and pyruvate kinase represent primary routes for ATP synthesis in *Zymomonas mobilis* (16, 24). The genes encoding GAP and PGK have been cloned and sequenced (7, 9). These two genes were found to be adjacent in several independent library clones containing *Z. mobilis* DNA, separated by 221 base pairs (bp) of DNA. Primer extension analysis identified tandem promoter regions for the *gap* gene (9). No promoter regions were found immediately upstream of the *pgk* gene by primer extension analysis, and the two genes were proposed to constitute a single operon (7). The *pgk* gene is followed by an unusual 7-bp sequence (CCTGCA) that is repeated 52 times and could serve as a transcriptional terminator.

The GAP and PGK proteins are approximately equal in size and catalyze sequential reactions in glycolysis. Approximately equal activities are needed. However, the catalytic rate of PGK is four times that of GAP (18), and four times more GAP than PGK is required. The separation of *gap* and *pgk* genes in *Escherichia coli* (1) and *Saccharomyces cerevisiae* (11) provides a simple means to independently control the synthesis of these proteins that is not available in *Z. mobilis*. On the basis of activities reported previously (13, 18), GAP can be calculated to be four- to eightfold more abundant than PGK in disrupted-cell preparations. The two genes have similar patterns of codon usage and canonical ribosomal-binding sites for efficient translation. Such differential expression would not be expected from adjacent genes in an operon in the absence of additional regulatory features.

In this study, we have begun to address possible reasons for the differential expression of the *gap* and *pgk* genes in *Z. mobilis*. We have investigated the possibility that additional copies of the *gap* gene are present on the chromosome by Southern hybridization analysis, confirmed by Northern (RNA) hybridization analysis that a large message which contains both the *gap* and *pgk* genes is present, identified a shorter, more abundant message containing the *gap* gene, and investigated the effects of multiple copies of the operon and promoter regions in recombinant strains on the activities of GAP, PGK, and other glycolytic enzymes.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms and plasmids used are shown in Table 1. Recombinants of *Z. mobilis* CP4 were maintained and grown as described previously (8, 13) in complex medium containing 100 g of glucose and 120 mg of chloramphenicol per liter. Cultures were incubated in a water bath maintained at 30°C and agitated with a magnetic stirrer (60 rpm). Growth was monitored spectrophotometrically at 550 nm with a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Genetic methods. Methods for plasmid purification, digestion with restriction enzymes, ligation, transformation of *E. coli*, and conjugation of plasmids into *Z. mobilis* CP4 have been described elsewhere (7, 8). DNA fragments were purified on agarose gels, electroeluted, and labeled with [^{32}P]phosphate by the random primer method, using a kit purchased from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.).

Southern hybridization. Genomic DNA was isolated from *Z. mobilis* CP4 (5) and digested with a series of restriction enzymes. Digested DNA was separated by electrophoresis in a 0.8% agarose gel and blotted onto a GeneScreen Plus filter (Dupont, NEN Research Products, Boston, Mass.). DNA concentrations were estimated by absorbance; approximately 2 μg of digested DNA was loaded in each lane. Filters were hybridized with internal fragments from *Z. mobilis gap* and *pgk* genes according to the recommendations of the manufacturer. A 0.45-kbp fragment (*Eco*RV to *Hin*cII) from pLOI312 (9) was used as a probe for *gap*, and a 0.51-kbp fragment (*Pst*I to *Eco*RI) from pLOI322 (7) was used as a probe for *pgk*.

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TABLE 1. Strains and plasmids used

Bacterial strain or plasmid	Relevant genotype	Source or reference
Strains		
Z. mobilis CP4	Prototroph	18
E. coli TC4	*tra recA*	8
Plasmids		
pLOI193 (shuttle vector)	*tet cat mob*	6
pLOI310	*gap pgk cat mob*	9
pLOI312	*gap bla*	9
pLOI322	*pgk bla*	7
pLOI323	*gap pgk cat mob*	7
pLOI437	*gap* promoter, *tet cat mob*	This study
pLOI440	*pgk cat mob gap*$^-$	This study
pLOI446	*cat mob gap*$^-$ *pgk*$^-$	This study
pLOI448	*gap cat mob pgk*$^-$	This study

Northern hybridization. RNA was isolated from cells in exponential phase by pouring the culture onto frozen buffer, harvesting by centrifugation, and extraction in boiling phenol as described by Bialkowska-Hobrzanska et al. (4). Northern blot analysis was carried out with RNA samples separated on a 1% agarose-formaldehyde gel (15) by using GeneScreen Plus filters.

Plasmid copy number. The copy number of the vector, pLOI193, was previously estimated to be 22 per chromosome equivalent (6). The gene encoding chloramphenicol acetyltransferase is expressed from a synthetic, enteric consensus promoter in this plasmid (6, 20) and is presumed to be unregulated. Relative plasmid copy numbers among recombinants were estimated by measuring chloramphenicol acetyltransferase activity. Cells were harvested and disrupted as described previously (6). Activity was measured as described by Shaw (23).

Assays of glycolytic and ethanologenic enzyme activities. Enzyme activities were determined in cells harvested during exponential growth phase in medium containing 10% glucose (optical density at 550 nm of 0.5) unless specified otherwise. Cell suspensions were prepared and assayed for GAP and PGK as described previously (13) but using a potassium morpholineethanesulfonic acid (MES) buffer (50 mM, pH 6.5) containing 30 mM KCl, 3 mM $MgCl_2$, and 10 mM mercaptoethanol. Alcohol dehydrogenase activities were assayed as described by Mackenzie et al. (14) in fresh preparations. Other glycolytic enzymes were assayed in cell pellets that had been stored at −20°C. Glucokinase and glucose-6-phosphate dehydrogenase activities were assayed as described by Scopes et al. (22). Pyruvate decarboxylase activity was assayed as described by Neale et al. (17). Remaining *Z. mobilis* glycolytic enzyme activities were assayed as described by Pawluk et al. (19). Activities are expressed as international units per milligram of total cell protein and represent an average of three or more determinations.

Protein gel electrophoresis. French press extracts were prepared as previously described (14). Proteins were examined in isoelectric focusing gels (pH 3 to 9), using the Phast Gel System (Pharmacia, Inc., Piscataway, N.J.). Separations and staining conditions were essentially those recommended by the manufacturer. Protein standards were obtained from Pharmacia.

RESULTS

Southern hybridization analysis. The possibility that additional copies of the *gap* gene were present on the *Z. mobilis* chromosome which contributed to the higher level of GAP than of PGK was examined by Southern blot hydridization (Fig. 1). Digestions with six restriction enzymes all yielded fragments that were consistent with the presence of a single copy of *gap* and *pgk* on the chromosome. Both the *gap* and *pgk* probes hybridized to the same DNA fragments except in the *Pst*I digestion, which is known to cleave between the two probes (7).

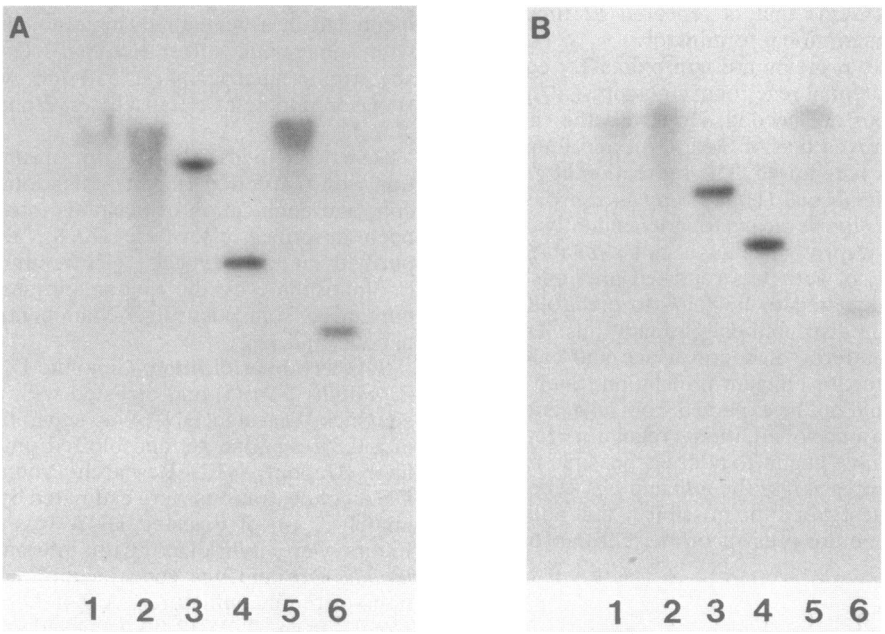

FIG. 1. Southern hybridization of *gap* (A) and *pgk* (B) fragments to a chromosomal digest of *Z. mobilis* CP4. Lanes: 1, *Bam*HI; 2, *Kpn*I; 3, *Pst*I; 4, *Eco*RI; 5, *Sal*I; 6, *Hin*dIII.

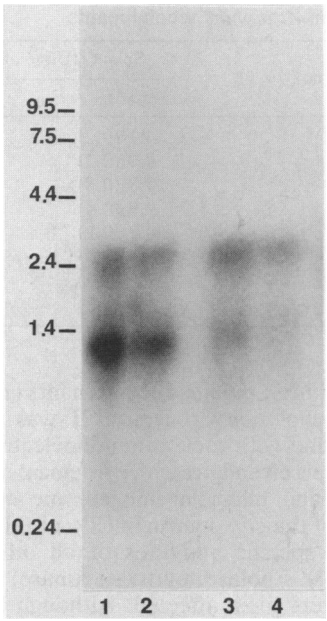

FIG. 2. Northern hybridization of *Z. mobilis* CP4 RNA. A 0.24- to 9.5-kbp RNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used to estimate size. RNA sizes are indicated in kilobases on the left. Lanes 1 and 3 contained 10 μg of RNA; lanes 2 and 4 contained half this amount. Lanes 1 and 2 were probed for *gap*; lanes 3 and 4 were probed for *pgk*.

Northern blots of mRNA from CP4. RNA extracts from CP4 lacking plasmid were separated by molecular weight and probed for both *gap* and *pgk* (Fig. 2). Two probes were used: for *gap*, a 636-bp *Xba*I-to-*Eco*RV fragment that includes the promoter through codon 93; for *pgk*, a 261-bp *Hin*dIII fragment that spans codons 309 to 396 in the carboxy terminus. Probes for each enzyme hybridized with various intensities to two bands of identical sizes. The dominant *gap* band was 1.2 kbp long, corresponding to the predicted size of the promoter-proximal *gap* gene with a portion of the intercistronic sequence. The second, larger transcript had an approximate size of 2.6 kbp, sufficiently large to encode both the *gap* and *pgk* genes. In contrast, the *pgk* probe hybidized more strongly the higher-molecular-weight mRNA band, although a second band of lower molecular weight also bound probe. Since no upstream promoter region was detected previously by primer extension analysis using a primer corresponding to codons 2 through 6 of *pgk* (7), it is likely that this smaller *pgk* transcript contains an incomplete *pgk* gene.

The radioactive bands were removed, and bound radioactivity was measured with a scintillation counter to estimate the relative abundance of the two transcripts. The smaller transcript bound fourfold more *gap* probe than did the larger transcript. Since the *gap* message is presumed to be present in both by virtue of binding, this probe predicts a ratio of five copies of *gap* message for each copy of *pgk* present in the larger message. The *pgk* probe bound the large message containing both genes and a smaller message of approximately 1.2 kbp in size that is presumed to contain an incomplete *pgk* message. Assuming translation to be equally efficient in the two messages, this 5:1 ratio of *gap* message to *pgk* message is nearly sufficient to cause the observed differences in the levels of GAP and PGK during exponential phase.

Construction of shuttle vectors containing *gap* and *pgk*. To examine the expression of plasmid-borne *gap* and *pgk* genes, a series of shuttle plasmids was constructed in the vector, pLOI193 (Fig. 3). The original library isolate, pLOI310, contained the complete operon, including the promoter region and terminator region, within the *Bam*HI site of the tetracycline gene in pLOI193. The *pgk* gene in pLOI310 was inactivated by taking advantage of a unique restriction site. A frameshift was produced by digestion with *Bgl*II, blunting of protruding ends with the Klenow fragment from *E. coli* DNA polymerase, and religation (pLOI448). The *gap* gene was inactivated by an analogous procedure at a *Kpn*I site to produce pLOI440. The *pgk* gene in pLOI440 was inactivated by using *Bgl*II to produce pLOI446, in which neither *Z. mobilis* gene is functional. A shuttle plasmid, designated pLOI437, containing the promoter and 93 codons of *gap* was also constructed for comparison by inserting the *gap-pgk* promoter from pLOI323 contained within a *Eco*RI to *Eco*RV fragment (1.0 kbp) into the *Not*I site of pLOI193 after the addition of appropriate linkers. All shuttle constructs except pLOI437 included the promoter and terminator regions.

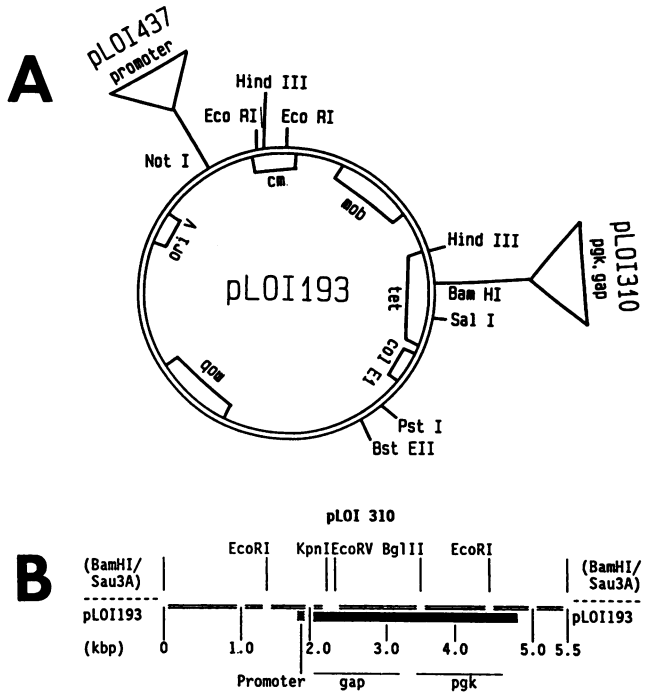

FIG. 3. Restriction maps of plasmid constructs containing *Z. mobilis gap* and *pgk*. (A) Map of pLOI193 (shuttle vector) showing the sites of insertion of the DNA fragments containing the complete *gap* operon (pLOI310) and of the promoter fragment with a truncated *gap* gene (pLOI437). (B) Map of the *gap* operon. Selected restriction endonuclease sites are labeled. Enzymes in parentheses mark sites that were lost during construction. Symbols: ═, *Z. mobilis* DNA; – – –, vector; ▬, *gap* operon; ——, *gap* or *pgk* gene.

Recombinants from the conjugation of unmodified vector or vector containing only the promoter were at least an order magnitude more frequent than recombinants from conjugations that transferred the longer constructs with the entire *gap* operon. All recombinants grew at equivalent rates and to similar final densities in rich medium containing 10% glucose.

Plasmid copy number. Expression of the *cat* gene, encoding chloramphenicol acetyltransferase, was used as a relative measure of copy number among the different recombi-

TABLE 2. Plasmid copy number and expression of GAP and PGK in *Z. mobilis* recombinants

Plasmid	Description	Copy no.	Sp act (IU/mg of protein [SD])	
			GAP	PGK
pLOI193	Vector, exponential phase	22	4.1 (0.2)	1.8 (0.3)
pLOI193	Vector, stationary phase		0.9 (0.2)	0.9 (0.1)
pLOI310	*gap* and *pgk*, exponential phase	9	11.8 (0.8)	8.2 (1.2)
pLOI310	*gap* and *pgk*, stationary phase		2.8 (0.4)	4.6 (0.4)
pLOI440	*pgk* with inactive *gap*, exponential phase	17	4.3 (0.2)	6.8 (0.4)
pLOI448	*gap* with inactive *pgk*, exponential phase	8	8.4 (0.6)	2.6 (0.4)
pLOI446	Inactive *gap* and *pgk*, exponential phase	9	2.2 (0.4)	1.9 (0.2)
pLOI437	Promoter + N terminus of *gap*, exponential phase	22	4.4 (0.2)	2.2 (0.2)

nants (Table 2). The copy number of pLOI437 containing the promoter fragment was identical to that of pLOI193 in CP4, 22 copies per chromosome equivalent. A lower copy number, eight to nine, was estimated for recombinants containing three of the four *gap* operon constructs. The reason for the intermediate level of plasmid copies in the construct with *gap* inactivated is unknown.

Effects of multiple gene copies on GAP and PGK activities. The presence of 9 to 17 additional copies of *gap* and *pgk* in CP4 recombinants was accompanied by less than a fourfold increase in specific activity (Table 2). Minimal values for the amounts of GAP and PGK can be estimated on the basis of the specific activities reported in Table 2 by using the activities reported for pure enzymes, 205 and 800 IU/mg of protein, respectively (19). Assuming that cytoplasmic proteins represent 70% of total cellular protein, GAP can be calculated to represent 2.9% of soluble proteins in CP4 (pLOI193) and 8.3% of soluble proteins in CP4(pLOI310). Similarly, PGK represents 0.3% of soluble protein in CP4(pLOI193) and 1.4% of soluble proteins in CP4 (pLOI310). Thus, approximately six- to ninefold more GAP than PGK appeared to be present in cell extracts from CP4 (pLOI193) and CP4(pLOI310).

A similar difference in expression of *gap* and *pgk* was retained in CP4(pLOI310) with the complete operon, CP4 (pLOI437) with the promoter region and truncated *gap* gene, and CP4(pLOI446) with the complete operon and frameshift mutations in both genes. Although the specific activities of GAP and PGK were lower in stationary-phase cells, differential expression of GAP and PGK was retained. No polar effects of frameshift mutations were observed, and inactivation of either *gap* or *pgk* alone did not affect expression of the remaining gene (pLOI440 and pLOI448, respectively). The presence of such constructs did not alter expression from the chromosomal genes.

Isoelectric focusing gels were used to examine the relative amounts of GAP and PGK independent of enzyme activity (Fig. 4). Two prominent bands were observed in CP4 (pLOI310) (lanes 4 and 5) that were less intense in CP4(pLOI193) (lanes 2 and 3). The identity of these bands was confirmed by comparison with bands from extracts from recombinants expressing only one activity (not shown). The band corresponding to GAP was much more intense than the band corresponding to PGK in extracts from exponential-phase cells (lane 3), indicating the presence of higher levels of GAP. Isoelectric points for PGK and GAP were estimated as 5.5 and 4.9, respectively.

Although GAP and PGK activities declined during stationary phase (Table 2), the intensities of bands corresponding to these proteins remained high in CP4(pLOI310), indicating that these proteins are relatively resistant to proteolysis (Fig. 4). Similar results were observed with overexpressed alcohol dehydrogenase II in *Z. mobilis* recombinants (13). Abundant but inactive alcohol dehydrogenase II was identified on acrylamide gels and with rocket immunoelectrophoresis.

Effects of *gap* operon and promoter fragment on the levels of other glycolytic and ethanologenic enzyme activities. The overexpression of the *gap* operon in CP4(pLOI310) caused a decrease in the specific activities of all other glycolytic enzymes (Table 3). Enolase, pyruvate kinase, and pyruvate decarboxylase were most affected. Although differences in activity for some enzymes were not statistically significant, the overall trend appeared clear. Enzyme activities in CP4(pLOI437) containing the promoter fragment without a functional *gap* or *pgk* gene were more similar to those in CP4(pLOI193), consistent with the effect being caused by overexpression of GAP or PGK. The promoter fragment did not cause a measurable change in GAP or PGK activity expressed from the single-chromosomal genes.

DISCUSSION

GAP and PGK function sequentially to synthesize ATP during glycolysis, and presumably equal catalytic activities are needed. Typically, the turnover number (K_{cat}) of PGK (21) is fourfold that of GAP (10), consistent with the production of lower levels of PGK than of GAP (13, 18). In other

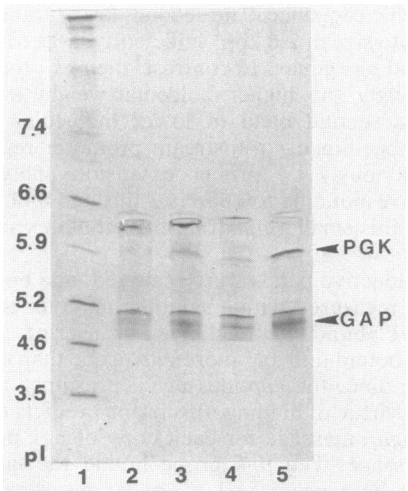

FIG. 4. Isoelectric focusing gels of cell extracts. Each lane contained approximately 6 μg of protein. Isoelectric points (pI) of standards are indicated on the left; bands corresponding to GAP and PGK are marked on the right. Extracts were prepared from exponential-phase (optical density at 550 nm of 0.5) and stationary-phase (48-h cultures) cells. Lanes: 1, markers; 2, CP4(pLOI193), exponential phase; 3, CP4(pLOI193), stationary phase; 4, CP4(pLOI310), exponential phase; 5, CP4(pLOI310), stationary phase.

TABLE 3. Activities of glycolytic and ethanologenic enzymes in recombinant *Z. mobilis* grown in complex medium with 10% glucose

Enzyme	Relative activity (% of pLOI193 [SD])	
	pLOI310	pLOI437
Glucokinase	83 (5)	102 (12)
Glucose-6-phosphate dehydrogenase	76 (4)	95 (9)
GAP	290 (32)	107 (6)
PGK	442 (32)	118 (15)
Phosphoglyceromutase	86 (4)	92 (10)
Enolase	58 (20)	83 (18)
Pyruvate kinase	65 (8)	88 (11)
Pyruvate decarboxylase	69 (7)	83 (7)
Alcohol dehydrogenase I	93 (20)	114 (4)
Alcohol dehydrogenase II	86 (8)	125 (5)

microorganisms, the *gap* and *pgk* genes are transcribed separately, allowing independent control of expression (1, 11).

In *Z. mobilis*, the combination of *gap* and *pgk* within an operon appears to include additional regulatory elements to reduce the level of PGK production. This regulatory information appears to be included within the operon, since similar differential expression was observed in recombinants containing single chromosomal copies of *gap* and *pgk* and those containing multiple copies of these genes. Six- to ninefold more GAP than PGK was expressed, as determined by measurements of enzyme activity. Higher levels of protein are generally produced from genes closest to the promoter, but the abundance of PGK was unexpectedly low.

The differential expression of *gap* and *pgk* appears to be controlled at the mRNA level. Although the *gap* operon was present as a single copy on the *Z. mobilis* chromosome, two different mRNAs encoding *gap* were observed. The smaller, more abundant mRNA is presumed to encode *gap* alone. Only the larger message was of sufficient size to encode both complete genes. The presence of both genes on the larger, polycistronic message was confirmed by Northern blot analysis. The ratio of *gap* message to *pgk* message was estimated from Northern blots as approximately 5:1. Considering the accuracy of such measurements, this ratio is in good agreement with the observed differences in GAP and PGK levels.

Our data are consistent with either of two mechanisms for the differential expression of *gap* and *pgk* in *Z. mobilis*: attenuation of transcription (12) or preferential mRNA degradation at the 3′ end specifying PGK (3). An intervening region of 221 bp separates the *gap* and *pgk* genes. This region is particularly rich in palindromic sequences and is capable of forming numerous stem and loop structures. No region with the features of a Rho-dependent termination site could be identified (2). It is interesting that similar high-level expression of the promoter-proximal gene has been reported for the photosynthetic genes of *Rhodopseudomonas capsulata* (3, 25). This phenomenon was originally hypothesized to result from transcriptional attenuation (25) but later was shown to result from the preferential degradation of the 3′ portion the polycistronic mRNA (3). More detailed analysis of mRNA structure, synthesis, and turnover are needed to resolve these hypotheses in *Z. mobilis*.

Additional regulatory signals may be associated with the *gap* and *pgk* genes. *Z. mobilis* may have some form of control that prevents the massive overexpression of the encoded glycolytic enzymes, a condition that would be expected to be adverse for an organism with minimal energy-generating ability. Overexpression of GAP and PGK in recombinant *Z. mobilis* was one-third that which would be predicted from the increase in gene dosage, consistent with the existence of a limiting positive factor. However, no significant reduction in GAP or PGK expression was observed from chromosomal genes in recombinants containing the complete operon with frameshift mutations in *gap* and *pgk*.

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