JOURNAL OF BACTERIOLOGY, May 1992, p. 2816–2823
0021-9193/92/092816-08$02.00/0

Vol. 174, No. 9

The *Zymomonas mobilis glf*, *zwf*, *edd*, and *glk* Genes Form an Operon: Localization of the Promoter and Identification of a Conserved Sequence in the Regulatory Region

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Received 9 December 1991/Accepted 19 February 1992

The *Zymomonas mobilis* genes that encode the glucose-facilitated diffusion transporter (*glf*), glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconate dehydratase (*edd*), and glucokinase (*glk*) are clustered on the genome. The data presented here firmly establish that the *glf*, *zwf*, *edd*, and *glk* genes form an operon, in that order. The four genes of the operon are cotranscribed on a 6.14-kb mRNA. The site of transcriptional initiation for the polycistronic message was mapped by primer extension and nuclease S1 protection analysis. The *glf* operon promoter region showed significant homology to other highly expressed *Z. mobilis* promoters, but not to consensus promoters from other bacteria. The highly expressed *Z. mobilis* promoter set contains two independent, overlapping, conserved sequences that extend from approximately bp −100 to +15 with respect to the transcriptional start sites. Expression of the *glf* operon was shown to be subject to carbon source-dependent regulation. The mRNA level was threefold higher in cells grown on fructose than in cells grown on glucose. This increase was not the result of differential mRNA processing when cells were grown on the different carbon sources, nor was it the result of differential transcript stability. Degradation of the 6.14-kb *glf* operon mRNA was biphasic, with initial half-lives of 11.5 min in fructose-grown cells and 12.0 min in glucose-grown cells. Thus, the higher level of *glf* operon mRNA in fructose-grown cells is the result of an increased rate of transcription. The importance of increasing *glf* expression in cells growing on fructose is discussed.

Zymomonas mobilis has a dedicated fermentative metabolism that produces ethanol and carbon dioxide as the sole fermentation products (49). *Z. mobilis* is the only organism that uses the Entner-Doudoroff pathway anaerobically and, as a consequence of its inherent inefficiency, obtains only a single mole of ATP per mole of glucose fermented (34). Therefore, the organism is forced to maintain rapid carbon flux to obtain sufficient energy for growth. This is facilitated by high-level expression of the glycolytic and alcohologenic enzymes (approximately 50% of the total soluble protein [2]). Despite the need for rapid carbon flux, *Z. mobilis* must keep the levels of toxic metabolic intermediates low while providing sufficient pools of precursor metabolites for biosynthetic pathways. The physiology and biochemistry of the organism are dictated by these constraints on metabolism.

Recently, it was shown that the *Z. mobilis* genes that encode the glucose-facilitated diffusion transporter (*glf*), glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconate dehydratase (*edd*), and glucokinase (*glk*) are tightly linked (5). Two genes of the operon, *zwf* and *edd*, overlap by 8 bp, suggesting that their expression might be translationally coupled. Such an arrangement of the genes that govern glucose uptake and the first three steps of the Entner-Doudoroff glycolytic pathway provides the organism with a mechanism for carefully regulating the levels of the enzymes that control carbon flux to 2-keto-3-deoxy-6-phosphogluconate, a compound that has been shown to be toxic in *Escherichia coli* (24).

The metabolic requirements for rapid carbon flux and high levels of the glycolytic enzymes present *Z. mobilis* with a special problem: how to elevate expression of the respective genes far above that of the majority of other cell functions. The high level of glycolytic enzyme synthesis is strikingly obvious on two-dimensional protein gels (3). In stark contrast to similar snapshots of *E. coli*, where some 600 proteins are present at levels that vary by approximately 1 order of magnitude (48), only 25 to 30 *Z. mobilis* proteins appear to compose the bulk of protein in the cell. Thirteen of the most abundant proteins were identified as glycolytic enzymes. Some 200 or 300 other spots on the *Z. mobilis* gels are present in amounts that appear to be several orders of magnitude lower. This is a clear indication that in *Z. mobilis* there are two distinct classes of genes and proteins with respect to the amounts required.

The underlying mechanisms that serve to coordinate high-level, balanced expression of the genes of central metabolism are only beginning to be understood (5, 10). The current study focuses on the transcriptional expression of four *Z. mobilis* glycolytic genes: *glf*, *zwf*, *edd*, and *glk*. In this paper we show that these four genes are arranged in an operon and are cotranscribed on a 6.14-kb mRNA. A characterization of the promoter that drives transcription of the polycistronic mRNA and identification of highly conserved sequences within the regulatory region are also presented. The possibility that the primary 6.14-kb transcript is processed by endonucleolytic cleavage is explored in the accompanying paper (30).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Z. mobilis* CP4 was grown at 30°C in complex medium containing 100 g of carbohydrate per liter as described previously (37). *E. coli* strains were routinely grown in Luria broth without added carbohydrate (31) at 37°C. Media were solidified by

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adding agar (1.5%). Antibiotic-resistant transformants were selected adding ampicillin (50 mg/liter) or chloramphenicol (120 mg/liter).

DNA methods. Transformation and recombinant DNA techniques were carried out by standard methods (42). Small-scale plasmid isolations were prepared by a modification of the method of Birnboim and Doly (7) as described previously (42). Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturers.

DNA sequence analysis. DNA was sequenced by the dideoxy method (43) with a Sequenase kit. The University of Wisconsin Genetic Computer Group sequence analysis software package version 6.1 was used for some data analysis (18). Promoter searches were performed as described previously (35, 36). The sequence of the *glf* operon has been published (5) and can be accessed from GenBank under accession number M37982.

RNA isolation and analysis. Initially, RNA isolations were conducted as described previously (13). RNA prepared in this way was used for the experiments shown in Fig. 1 and 3. For the experiments shown in Fig. 2 and 4, an improved RNA extraction procedure was used. For this method, RNA was isolated by a modification of the procedure of Mackie (33). Basically, 5 ml of a log-phase *Z. mobilis* culture was pipetted into 2.5 ml of 0.3 M sodium acetate (pH 4.0)–30 mM EDTA–3% sodium dodecyl sulfate at 100°C in a boiling-water bath and then extracted twice with phenol (equilibrated with 50 mM sodium acetate at pH 4.0) at 60°C and once with chloroform at room temperature (27). RNA isolated by the improved extraction method consistently provided less smearing on Northern RNA blots. Northern analysis was carried out as described previously (12) with DNA hybridization probes that were labeled with a random-primed labeling kit. RNA half-life determinations after the addition of rifampin were as described previously (27). Slopes of mRNA decay curves were obtained by linear regression. Primer extension analysis of the transcriptional initiation site was accomplished by published methods (13). The hybridization temperature was 23°C. The S1 nuclease protection protocol was as described previously (10). 5′-end labeling of the S1 probe was done with polynucleotide kinase. The amount of radioactive probe that hybridized to the filters was quantitated by using the AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, Calif.).

Enzymes and chemicals. Restriction enzymes and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The random-primed DNA labeling kit was ordered from U.S. Biochemical Corp. (Cleveland, Ohio). Radioactive compounds were purchased from DuPont NEN Products (Boston, Mass.). Biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Cotranscription of the *glf*, *zwf*, *edd*, and *glk* genes. The clustering of the four genes that are responsible for glucose transport and the first three steps in *Z. mobilis* central metabolism suggested that these genes might form an operon (5). This was tested by Northern hybridization analysis (Fig. 1). Total RNA was harvested from a glucose-grown culture of *Z. mobilis* in the middle of the logarithmic phase. Four gene-specific probes, consisting of restriction fragments that contained only DNA from within the respective reading frames, were used for hybridization (Fig. 1). The same filter (and hence the same RNA sample) was probed and analyzed, stripped of the probe, probed again, and analyzed for each of the four gene-specific probes. All four of the probes hybridized to a 6.14-kb band on the Northern filter, the largest transcript detected with any of the probes. This transcript is of sufficient length to encode all four genes.

The hybridization pattern was different for each of the four gene-specific probes. The *glf*-specific probe hybridized to a second band at 1.7 kb. The *zwf*-specific probe hybridized to additional bands at 4.5 and 3.2 kb. The *edd*-specific probe hybridized to bands at 4.5, 3.2, and 2.1 kb. A virtually identical pattern was observed with the *glk*-specific probe. The possibility that these smaller transcripts were generated by endonucleolytic cleavage of the primary 6.14-kb transcript is explored in the accompanying paper (30).

Mapping of the *glf* operon promoter. The 5′ end of the 6.14-kb transcript was mapped by primer extension analysis and S1 nuclease protection (Fig. 2). A 21-base oligonucleotide spanning bp 129 through 149 (with respect to the transcriptional start site) was used for primer extension. The major primer extension product was mapped to an adenine that corresponds to base 59 of the sequence reported by Barnell et al. (5). Thus, the transcriptional initiation site for the operon is 126 bases upstream of the *glf* start codon. A minor primer extension product mapped just 3 bases downstream of the major product at base 62 of the published sequence (5). These two bands were the only primer extension products detected, even after several days of exposure. The transcriptional start site was confirmed by S1 nuclease protection. The DNA probe used for this experiment was a 798-bp *Sma*I-to-*Eco*RV fragment that was labeled at the 5′ end (5, 30). The longest of the three S1 protected fragments is indicated by the double arrow in Fig. 2. This protected fragment is 243 bases long and corresponds precisely to the adenine that was determined by primer extension analysis to be the transcriptional start site. The sequence upstream of *glf* contains an open reading frame that is transcribed in the same direction as the *glf* operon and ends just 379 bp upstream of the *glf* start codon. This upstream reading frame encodes DNA ligase (*lig*) and most probably delineates the farthest point upstream that might contain the *glf* operon promoter (46). Therefore, it is likely that the mapped 5′ end of the operon mRNA represents the true site for transcriptional initiation of the *glf* operon and that the promoter lies in this intergenic region.

Identification of conserved sequences in the *glf* operon regulatory region. The sequence upstream of the *Z. mobilis* *glf* operon transcriptional start site is shown in Tables 1 and 2. The transcriptional initiation sites for several other highly expressed *Z. mobilis* genes that code for glycolytic enzymes, including *adhB* (alcohol dehydrogenase II [15]), *eda* (2-keto-3-deoxy-6-phosphogluconate aldolase [10]), the *gap-pgk* operon (glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase [11, 14]), and *pdc* (pyruvate decarboxylase [13]) were determined previously. In the past, numerous attempts to identify a common sequence element upstream of the transcriptional initiation sites that might be responsible for efficient transcription have met with little success (10, 14, 27). To conduct a more comprehensive search for the predicted regulatory sequence(s), we used the computer programs designed by O'Neill and coworkers (35, 36). A computer comparison of the putative *glf* operon promoter with other promoter regions of *Z. mobilis* glycolytic genes revealed two independent sequences with a striking degree of conservation.

The first of the two overlapping, conserved sequences that lie upstream of the transcriptional initiation sites spans an extensive region from approximately bp −100 to +15 with

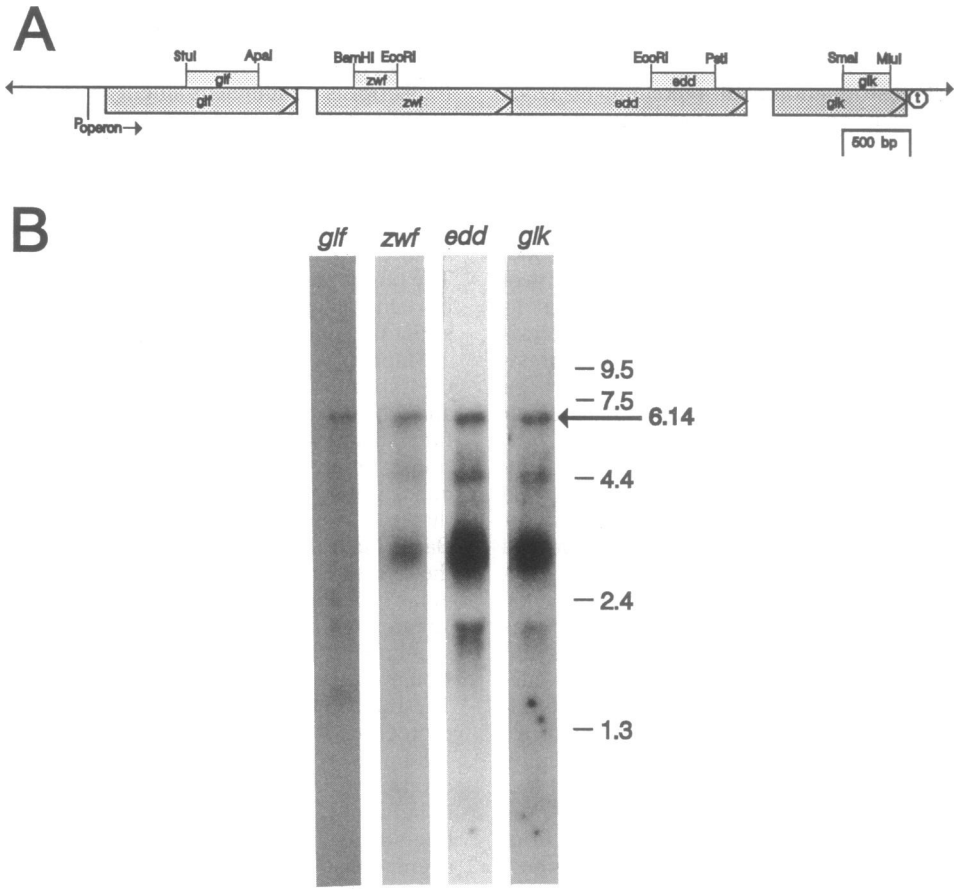

FIG. 1. Northern blot analysis confirming cotranscription of the *Z. mobilis glf*, *zwf*, *edd*, and *glk* genes. (A) Restriction map showing the four gene-specific DNA hybridization probes used in this study. (B) Northern blot analysis of *Z. mobilis* total RNA hybridized with the four gene-specific probes. RNA standards (in kilobases) are shown on the right. The 6.14-kb transcript is indicated by the arrow.

respect to the 5′ ends of the mRNAs (Table 1). A computer-generated consensus for these sequences showed significant homology among all of the promoter regions. A consensus derived from this alignment showed, over 115 positions, 65.2% homology to the *glf* and $adhBp_1$ promoters, 64.3% homology to the $adhBp_2$ promoter, 63.3% homology to the *eda* promoter, 62.6% homology to the *pgi* promoter, 55.7% homology to the *eno* promoter, 54.8% homology to the *pdc* promoter, and 49.5% homology to the *gap* promoter. By comparison, a similar computer comparison of 39 of the best-characterized 17-base *E. coli* promoters showed that no two of these promoters exhibit greater that 60% homology within the bp −50 to +8 regions (35). The most highly conserved sequence element within the *Z. mobilis* promoter regions is TAGANNT. The location of this sequence with respect to bp +1 varies from −3 to −11 bp. A second, 41-base conserved sequence was also identified by computer analysis (Table 2). This second conserved sequence was independent of the first conserved sequence. A consensus sequence derived from this alignment showed significant homology to all of the highly expressed *Z. mobilis* promoters. The *glf* operon sequence is 68.3% identical to the consensus, whereas the similarity to the consensus for the other promoters is 70.7% for $adhBp_1$ and *pdc*, 68.3% for *gap*, 63.4% for $adhBp_2$, 61.0% for *eno*, 58.5% for *eda*, and 48.8% for *pgi*.

The consensus sequences of the highly expressed *Z. mobilis* promoter regions do not share significant identity with the *E. coli* σ^{70} consensus sequence (28). Likewise, the *Z. mobilis* consensus sequences are not similar to any of the *Pseudomonas* promoter consensus sequences compiled by Deretic et al. (17). Searches of GenBank with the FASTA program (Genetics Computer Group) and either of the two extended *Z. mobilis* consensus sequences as the query sequence resulted in significant matches to the regulatory regions of highly expressed *Z. mobilis* genes, but matches to catalogued sequences from other organisms were not significant. Comparison of the two consensus sequences with the sequences of four *Z. mobilis* genes that are expressed at low levels, *adhA* (29), *sacA* (26), *phoC* (39), and *lig* (46), failed to show any significant matches.

Carbon source-dependent regulation of the *glf* operon. The activities of glucose-6-phosphate dehydrogenase and glucokinase were found to be 2.3- and 1.4-fold higher, respectively, in cells grown on fructose than in cells grown on glucose. For logarithmic-phase cultures, glucose-6-phosphate dehydrogenase activities were 1.02 (standard deviation, 0.13) μmol/min/mg of protein in fructose cultures and 0.44 (standard deviation, 0.06) μmol/min/mg of protein in glucose cultures. Glucokinase activities were 1.07 (standard deviation, 0.06) μmol/min/mg of protein on fructose and 0.79 (standard deviation, 0.04) μmol/min/mg of protein on glucose. Previous experiments have shown a similar stimulation of phosphoglucose isomerase (27) and fructokinase (50)

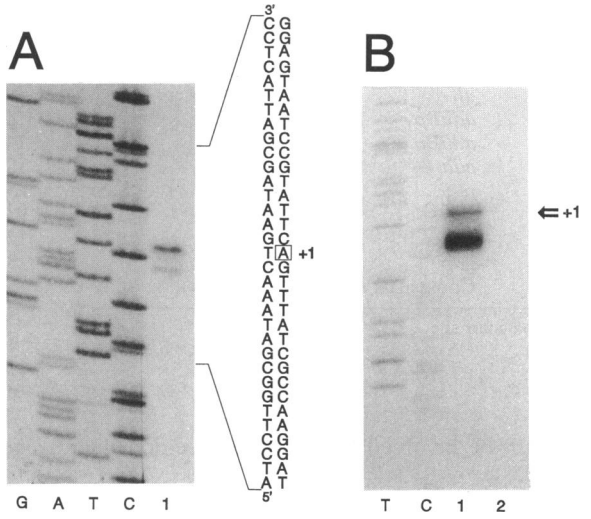

FIG. 2. 5'-end mapping of the *glf* operon mRNA. (A) Primer extension analysis with a 21-base oligonucleotide corresponding to nucleotides 129 through 149 with respect to the transcriptional start site (5). The sequence ladder (labeled G, A, T, C) was generated by using the same oligonucleotide that was used for primer extension. The primer extension reaction was run in lane 1. The sequence of the transcriptional initiation site is detailed on the right, and the 5' end is indicated (+1). (B) S1 nuclease protection to confirm the primer extension result. Details of the experiment are provided in Results. The T and C lanes of the same sequence ladder used in panel A are labeled. S1 nuclease protection with total *Z. mobilis* RNA is shown in lane 1. A control with *Saccharomyces cerevisiae* tRNA is shown in lane 2.

activities in cells grown on fructose. However, other enzymes of central metabolism, such as glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase, alcohol dehydrogenase, and pyruvate decarboxylase, do not show carbon source-dependent regulation (27). During the course of this study it was found that enolase activity is also the same in media containing fructose or glucose.

Recently it was shown that the increase in phosphoglucose isomerase and fructokinase activities in *Z. mobilis* cells grown on fructose relative to those of cells grown on glucose was the result of transcriptional regulation (27, 50). In the present study, the levels of the *glf* operon mRNA in fructose-grown cells were greater than those in glucose-grown cells (Fig. 3). Similar results were obtained with each of the four gene-specific hybridization probes corresponding to the polycistronic mRNA. In each of the panels shown in Fig. 3, the ratio of counts per minute for the full-length 6.14-kb transcript in cells grown on fructose to that in cells grown on glucose was approximately 3:1, as measured by radioanalytic imaging. Similar results were obtained with total RNA isolated from three separate cultures for both carbon sources. The pattern of the smaller, gene-specific mRNAs that hybridized to the four probes appeared to be the same when cells were grown on either carbon source. This rules out the possibility that the increase in enzyme activities is the result of differential mRNA processing on the two carbon sources. It is interesting that the increase in enzyme activity did not parallel the more substantial increase in mRNA levels and that on fructose medium the level of glucokinase induction was less than that of glucose-6-phosphate dehydrogenase. In related studies, it was observed that increased phosphoglucose isomerase and fructokinase activities on

TABLE 1. *Z. mobilis* extended promoter sequence comparison[a]

Sequence	Gene	% Conserved	Reference
GGATAAGTAAAAAAAATAATGGTAGATGATGGACGGCAAATATTGTTCCAATAATGATAAGGAGGTTCCAAAATCGGTTCAATAGGAGTAATCCGTATTTCAGTTTATCGGCAAGGAT	*glf*	65.2	5
CTTTTTTCATAATTTGCATAAGTCTTGATGTAAAAAATACATATTTAGAAAGAACAAGCAGCCTTGCTCATCACCGCTGTGGCGAGTAGAAAAAATCTCGGCTTTCAGAAAAAAGAGG	*adhBp*$_1$	65.2	14
TTTCAGAAAAAAGAGGCCGCTTCGTTAAACAGACTATAAATGTGCTGGAATAAAGCGAACCCCTTGATCTGATAAAACTGATAGACATATTGCTTTTGGCGTGCCCGATTGCTGAA	*adhBp*$_2$	64.3	14
CGAGAACATGCCAGAGACCATCGCGCACACAAGATTTACGATGCCGCAGCGTAAAAAAACCTTGATTTTTTGGTCGATGGGGTAGATGCAATTCAACGAACCCCTAAGGGAAATAAA	*eda*	63.5	10
TCTGATTGAAGTCAATCGATAAAATCGATCAAGATAGAAATAGCTGTTTCTACTTAATTAATCTTTTCAGTTCTTGTTATTCGTCCTACTATCTCTTCACTTTGACGAAAACGAA	*pgi*	62.6	25
TAGAATCGGATGAGAAGCGATAAGCGTTTCACCATAAGCAATATATATTCAATTGCAACAGTGGAATTGCCTTATGCGTCAAGGAAGGATAGATCATTGACGGACTGAGTTCAAAAAGA	*eno*	55.7	9
TTTTCGCTTCGGCAGAAGAGGTTCATCATGAACAAAAATTCGGGCATTTTTTAAAAATGCCTATAGCTAAATCCGGAACGACACTTTAGAGGTTTCTGGGTCATCCTGATTCAGACA	*pdc*	54.8	12
TTTTTGCCCGATCAGCCTCAATCGACAATTTTACGGCGTTTCGATCGAAGCAGGGACGACAATTGGCTGGGAACGGTATACTGGAATAAATGGTCTTCGTTATGGTATTGATGTTTT	*gap*	49.5	13
TTTTATCAWAAGARANACAARTACTTAATCAACAAAAAAATWGCTGCAWTRAAAAAAAACGANTTKYTCAGNTCGANTGRNTAGANNTAATNCTTCTNNGCTTTMAGANAAAGAAA	Consensus		

[a] A highly conserved sequence element is underlined. W indicates A or T; R indicates A or G; N indicates A, G, T, or C; K indicates G or T; Y indicates C or T; S indicates C or G; M indicates A or C (18). Asterisks indicate the transcriptional start sites.

TABLE 2. *Z. mobilis* short promoter sequence comparison

Position[a]	Sequence[b]	Gene	% Conserved	Reference
−64	CAAATATTGTTCCAATAATGATAAGGAGGTTCCAAAATCGG	*glf*	68.3	5
−66	TAAAAAATACATATTTAGAAAGAACAAGCAGCCTTGCTCAT	$adhBp_1$	70.7	14
−66	AGACTATAAATGTGCTGGAATAAAGCGAACCCCTTGATCTG	$adhBp_2$	63.4	14
−43	AAAAAACTTGATTTTTTGGTCGATGGGGTAGATGCAATTCA	*eda*	58.5	10
−78	CATAAAATCCATCAAGATAGAATAGCTGTTTCTACTTAATT	*pgi*	48.8	25
−63	TAAGCAATATATTCATTGCAACAGTGGAATTCCTTATGCGT	*eno*	61.0	9
−64	CAAAAATTCGGCATTTTTAAAAATGCCTATAGCTAAATCCG	*pdc*	70.7	12
−66	ACAATTTTACGCGTTTCGATCGAAGCAGGGACGACAATTGG	*gap*	68.3	13
	WAAAWATTACAYTTTTTGAAARAAGSRGRTTCCTWAATCGG	Consensus		

[a] The position of the first base in the conserved sequence with respect to the transcriptional start site.
[b] W indicates A or T; R indicates A or G; Y indicates C or T; and S indicates C or G (18).

fructose do not parallel the more substantial increases in *pgi* and *frk* message levels (27, 50).

To determine whether the increased levels of *glf* operon mRNAs in fructose cultures were the result of differential transcript stability, the half-life of the primary transcript was determined in glucose- and fructose-grown cultures with the *zwf*-specific hybridization probe (Fig. 4). The decays of the primary transcript in both cultures followed similar patterns and were biphasic. The measured half-life (during the initial phase of decay) of the 6.14-kb transcript in glucose-grown cells was 12.0 ± 0.4 min. The half-life of the transcript in fructose-grown cells was measured as 11.5 ± 0.8 min. These rates increased approximately 2.5-fold during the second phase of decay, which began at 18 min after rifampin was added. Thus, differential mRNA stability does not appear to play a role in regulating the carbon source-dependent changes of the *glf* operon mRNA levels. Rather, the increase of *glf* operon mRNA observed in fructose cultures is the result of an increased rate of transcription. A search for sequences common only to the *glf* operon promoter region and the *pgi* promoter region failed to reveal any sequences that might be involved in carbon source-dependent regulation (27).

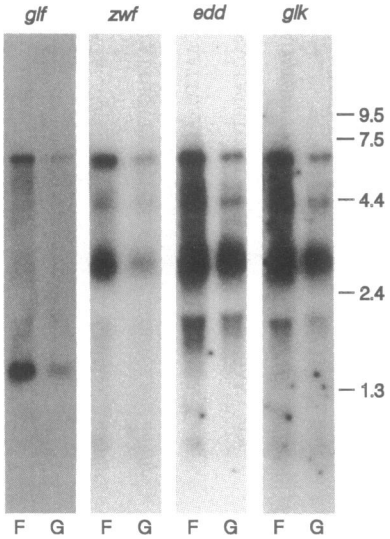

FIG. 3. Northern blot analysis of the carbon source-dependent regulation of *glf* operon transcription. Cells were grown to the midlogarithmic phase on fructose (F) or glucose (G), and total RNA was extracted. Each of the four gene-specific probes (Fig. 1A) was used for hybridization. RNA standards (in kilobases) are provided on the right.

DISCUSSION

The results of this study clearly demonstrate that the four clustered *Z. mobilis* genes specifying the enzymes responsible for glucose transport and the first three steps of central metabolism form an operon. The promoter region that drives transcription of the operon was mapped by primer extension analysis and S1 protection. Computer comparisons of the sequence upstream of the transcriptional start site with the analogous regions in several other highly expressed *Z. mobilis* genes indicated a high degree of conservation. Increased expression of the operon in cells grown on fructose relative to that in cells grown on glucose was shown to be the result of an increased transcription rate.

Organization of the genes in an operon provides *Z. mobilis* with a way to coregulate metabolically related functions. The arrangement of glycolytic genes in operons is not unprecedented. In *E. coli*, *pgk* (encoding phosphoglycerate kinase) and *gapB* (encoding glyceraldehyde phosphate dehydrogenase) are clustered with the *fda* gene that encodes fructose-1,6-diphosphate aldolase (1). In *Z. mobilis*, the *gap* and *pgk* genes that encode two consecutive steps of glycolysis are arranged in an operon (11). The genes that encode glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase (*zwf* and *edd*, respectively) in *Z. mobilis*

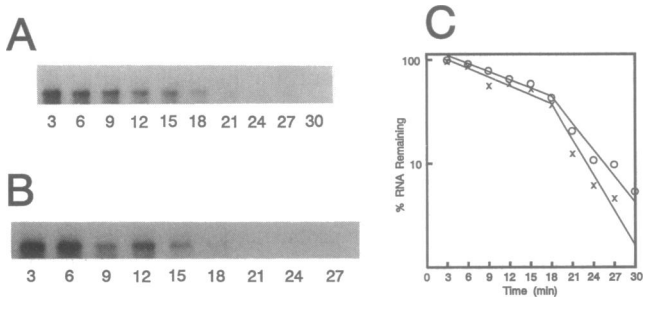

FIG. 4. Stability of the *glf* operon mRNA. (A and B) Total RNA was harvested from log-phase cultures after inhibition of transcription with rifampin at the indicated times (minutes). The gels were loaded with 2.5 μg of RNA per lane. Cells were grown on glucose (A) or fructose (B). (C) Semilogarithmic plot of the data shown in A and B, quantitated by scanning of the filters with the Ambis system. The half-lives of the *glf* operon mRNA were 12.0 ± 0.4 min in glucose-grown cells and 11.5 ± 0.8 min in fructose-grown cells.

overlap (5). This fact made cotranscription of these two genes a virtual certainty. It remains to be seen whether the *glf* promoter is the only site of transcriptional initiation or whether there are additional promoters within the operon. However, the newly identified consensus sequences are not found elsewhere within the *glf* operon. Each of the other glycolytic genes that have been studied in *Z. mobilis* is monocistronic, including *eda* (10), *adhA* (29), *adhB* (15), *pdc* (13), *pgi* (27), *eno* (9), and *frk* (50).

The organization of the key genes of the Entner-Doudoroff pathway in *Z. mobilis* can now be compared with that in other organisms. The *edd* and *eda* genes are tightly linked to the *zwf* gene in *E. coli* (24) and *Pseudomonas aeruginosa* (38). The order of these genes in *E. coli* is *zwf-edd-eda* (16); the *glk* gene is unlinked (4). Despite tight linkage in *E. coli*, the *zwf* gene is expressed independently of the Entner-Doudoroff genes (41). The *E. coli edd* and *eda* genes are separated by only 36 bases and form an operon (23). In *P. aeruginosa* the gene order is *zwf-eda-edd-glk* (47). The molecular details of how these genes are expressed in *P. aeruginosa* remain to be worked out. Interestingly, the *eda* gene is not linked to the *zwf-edd* region in *Z. mobilis* (10).

As much as 50% of the cytoplasmic protein in *Z. mobilis* consists of glycolytic enzymes (2). Glucokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydratase comprise at least 4.4% of the total soluble protein in *Z. mobilis* (44, 45). The stability of the *glf* operon mRNA (>11 min) might contribute to the high level of expression of the genes of the *glf* operon.

The transcript mapping experiments shown in Fig. 2 clearly indicated a single site for transcriptional initiation of the primary operon transcript. The putative promoter lies between the first gene of the operon and an upstream gene encoding DNA ligase (46). The sequence of this region shows significant similarity to other *Z. mobilis* promoter sequences (Tables 1 and 2) but does not show significant similarity to *Pseudomonas* (17) or *E. coli* (28) consensus promoter sequences. Numerous previous attempts to identify a promoter consensus sequence for *Z. mobilis* were unsuccessful. Considering the common property of high-level expression of the *Z. mobilis* glycolytic genes, our long-standing inability to find an obvious consensus promoter sequence was difficult to explain (10, 14). It was not until more extensive computer search protocols were utilized that the *Z. mobilis* promoter region conservation was recognized (35, 36). It is worth noting that our previous promoter searches had been based on the preconceived notion that *Z. mobilis* promoters would somehow resemble *E. coli* promoters, at least in having −35 and −10 regions (28). It is obvious now that this preconception clouded our vision. There are in fact two overlapping, independent, conserved sequences that cover extended distances upstream of the transcription start sites of the highly expressed *Z. mobilis* genes. This level of similarity within a set of promoter regions is atypical (35). The unusually high degree of similarity for the *Z. mobilis* promoter set might indicate a sequence that facilitates high transcription rates. It is also tempting to speculate that the glycolytic gene set is transcribed from a separate class of promoters that allow distinction between the need for high-level expression of the glycolytic enzymes (50% of the cytoplasmic protein [2]) and the need for 100-fold lower levels of the majority of housekeeping enzymes. This possibility is supported by the finding that the regulatory regions of four genes that are expressed at low levels, *adhA* (29), *sacA* (26), *phoC* (39), and *lig* (46), do not show significant similarity to the two consensus sequences.

Previous studies have indicated the involvement of *trans*-acting, positive regulatory factors in expression of the *Z. mobilis adhB* and *gap-pgk* genes (22, 32). Future work on the *glf* operon promoter with a reporter gene fusion (*lacZ*) will focus on the sequences that are important for promoter function. These experiments, and similar studies with the *pgi* promoter (27), might also reveal regions common to the two promoters that are important for the observed carbon source-dependent increase in transcription. Such sequences have not yet been identified.

The elevation of glucokinase and glucose-6-phosphate dehydrogenase in cells growing on fructose as compared with that in cells growing on glucose is interesting. The reasons for a similar, carbon source-dependent increase in activities of the two enzymes that serve to direct fructose into central metabolism are clear. Phosphoglucose isomerase and fructokinase activities in *Z. mobilis* cells are adequate to support the flux observed when cells are growing on fructose (2, 27, 45, 49). However, when *Z. mobilis* is grown on glucose the levels of these two enzymes are lower than the level required for growth on fructose (20, 21, 27, 45). In the case of phosphoglucose isomerase, the increase in enzyme activity has been shown to be the result of an increased rate of transcription (27). The same is true for fructokinase (50). On the other hand, the levels of glucokinase, 6-phosphogluconate dehydratase, and glucose-6-phosphate dehydrogenase in glucose-grown cells are at levels sufficiently high to cope with the flux observed on either carbon source (2). Therefore, the reason for the need to increase expression of the *glf* operon when growing on fructose is obscure.

Increased transcription of *glf* and the remaining genes of the operon for growth on fructose could be explained if *Z. mobilis* depends upon a single transport system (*glf*) for both sugars. The reliance of *Z. mobilis* on a low-affinity, high velocity, nonconcentrative glucose transport system was demonstrated by DiMarco and Romano (19). The glucose facilitator was constitutively expressed in cells grown on glucose or fructose, and glucose transport was decreased modestly by competition with fructose. The rate of glucose transport, in general, was shown to be more than threefold higher than the rate of fructose transport. If there is only a single carbohydrate facilitator, it is apparent that the transport system has a low affinity for fructose. At present, the question of whether *Z. mobilis* possesses separate transport systems for glucose and fructose remains unanswered. If glucose and fructose do share the same transport system, the low affinity of the glucose transporter for fructose might necessitate increased *glf* expression to achieve the rate of fructose transport required to support the observed flux. In accordance with this model, the increase in expression of the other three genes of the operon would be viewed as fortuitous. Superfluous synthesis of these three enzymes and the consequent energy drain might be a factor contributing to the lower cell yields observed in cultures grown on fructose (49). Now that the *glf* gene has been cloned, the substrate range of the facilitator can be determined (5).

The order of the four genes of the operon does not follow their sequence in metabolism. The glucokinase gene is last in the operon, but it encodes the first enzyme of the pathway. This enzyme is present at a level that is just sufficient to support the overall flux of carbon through the pathway and is suspected to be the ultimate rate-limiting factor in fermentation (49). It is not at all unusual for the last gene in an operon to be expressed at the lowest level, and the position-

ing of the genes in the operon might play a very important role in adjusting the relative amounts of the enzymes to meet the physiological needs of the cell (6). Alternatively, the relatively low expression of glucokinase might simply be due to its reported instability rather than to its position in the operon (3).

With the need for rapid metabolism in *Z. mobilis*, it would not be at all surprising if the enzymes of the Entner-Doudoroff glycolytic pathway formed a multienzyme complex to facilitate channeling of metabolites, as has been suggested for the glycolytic enzymes of *E. coli* (25). Cotranscription of the genes would facilitate assembly of such glycolytic complexes in the cytoplasm. It is interesting that the glucose-facilitated diffusion protein is encoded by the operon. It might be that the glucokinase (and other associated enzymes of the complex) is capable of mediating an intimate association with the cytoplasmic face of the glucose transporter, resulting in vectorial phosphorylation and very efficient channeling of glucose into central metabolism. This seems reasonable, given that *Z. mobilis* is capable of extremely high carbon flux: >1 μmol/min/mg of protein (34, 37, 40, 49). Such an association of glucokinase with the glucose transporter might also help to explain how this kinase, which is specific for a single hexose (20, 21), does not possess an obvious glucose-binding domain (5). It might be relevant that direct interaction of kinases and carbohydrate transporters has been suggested as an underlying mechanism that explains the kinase-dependent, high-affinity, facilitated glucose transport in *Saccharomyces cerevisiae* (8).

ACKNOWLEDGMENTS

Computer time was provided courtesy of the University of Nebraska, Lincoln, Computer Resources Center. This work was supported by the Department of Energy (DE-FG02-90ER20002) and the Nebraska Energy Office (UNR-11).

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