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*J. Bacteriol.* 2005, 187(15):5179. DOI:  
10.1128/JB.187.15.5179-5188.2005.

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# Transcriptional Analysis of a Gene Cluster Involved in Glucose Tolerance in *Zymomonas mobilis*: Evidence for an Osmoregulated Promoter

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Received 14 September 2004/Accepted 16 February 2005

Exponentially growing cells of *Zymomonas mobilis* normally exhibit a lag period of up to 3 h when they are transferred from a liquid medium containing 2% glucose to a liquid medium containing 10% glucose. A mutant of *Z. mobilis* (CU1) exhibited a lag period of more than 20 h when it was grown under the same conditions, whereas it failed to grow on a solid medium containing 10% glucose. The glucose-defective phenotype of mutant CU1 was due to a spontaneous insertion in a putative gene (ORF4) identified as part of an operon (*glc*) which includes three additional putative genes (ORF1, ORF2, and ORF3) with no obvious involvement in the glucose tolerance mechanism. The common promoter controlling *glc* operon transcription, designated  $P_{glc}$ , was found to be osmoregulated and stimulated by the putative product of ORF4 in an autoregulated fashion, as indicated by expression of the *gfp* reporter gene. Additionally, reverse transcriptase PCR analysis showed that the gene cluster produces a single mRNA, which verified the operon organization of this transcription unit. Further transcriptional analysis demonstrated that *glc* operon expression is regulated by the concentration of glucose, which supported the hypothesis that this operon is directly involved in the uncharacterized glucose tolerance mechanism of *Z. mobilis*.

*Zymomonas mobilis* is a strictly fermentative gram-negative ethanologenic bacterium with industrial importance that produces ethanol from simple hexoses at high rates and yields (11). It also has an unusual tolerance to high concentrations of ethanol (up to 13%, wt/vol) and glucose (over 30% for most strains) (47, 48). Therefore, *Z. mobilis*, a typical saccharophilic organism, is ideal for studying glucose tolerance and osmoregulation mechanisms. Exponentially growing cells of *Z. mobilis* normally exhibit a lag period of up to 3 h when they are transferred from a liquid medium containing 0.11 M (2%) glucose to a liquid medium containing 0.55 M (10%) glucose. A mutant of *Z. mobilis* (CU1) (14) and a rifampin-resistant derivative of this strain (CU1Rif2) (1) exhibited a lag period of more than 20 h and were unable to grow on a solid medium containing 0.55 M glucose when they were grown under the same conditions.

In an effort to better understand this unusual glucose tolerance trait, we described in a previous paper isolation of a DNA fragment (4.5 kb) which complemented the glucose-defective phenotype of *Z. mobilis* mutant strains CU1 and CU1Rif2 (12). This fragment consists of four open reading frames (ORFs) coding for four putative polypeptides that are 167, 167, 145, and 220 amino acids long. These ORFs exhibit the typical *Z. mobilis* codon usage and have individual Shine-Dalgarno consensus sites under the control of a common  $-35$  and  $-10$  promoter element (Fig. 1). Interestingly, a protease-sensitive

diffusible factor in the medium of a wild-type culture grown in medium containing 10% glucose could correct the defect in the CU1 mutant and its derivative (12).

In the present work we focused on complete genetic analysis of this gene cluster of *Z. mobilis*. Our aim was to understand the role of the four ORFs in complementing the CU1 phenotype. In addition, the promoter activity and expression pattern of this gene cluster were investigated under various growth conditions with different glucose concentrations and osmotic pressures. In this effort the reporter gene *gfp* was used (4, 9), since it was recently shown to be suitable for transcriptional analysis in *Z. mobilis* (13). Furthermore, the nature of the CU1 mutation was characterized on the molecular level, which provided additional evidence for the autoregulation of the *glc* operon by the product of ORF4.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** *Z. mobilis* wild-type strains ATCC 10988 and CP4 (48) and mutants CU1 (14), CU1Rif2 (1), and CP4Rif<sup>r</sup> (33) were grown semianaerobically at 30°C on complete liquid or solid media as described previously (48). To avoid caramelization, carbohydrate solutions were sterilized separately as concentrated stock solutions and added aseptically to liquid media at the desired concentrations. Exponentially growing cells were used as inocula to obtain a starting liquid culture containing approximately  $10^7$  cells per ml. Growth was monitored turbidometrically at a wavelength of 600 nm. *Escherichia coli* strain DH5 $\alpha$  (21) was grown at 37°C in Luria broth (31). Solid media were obtained by adding 2% (wt/vol) agar. When antibiotics were needed for genetic selection or plasmid maintenance, they were added at the following concentrations. Chloramphenicol was used at concentrations of 20  $\mu\text{g ml}^{-1}$  and 100  $\mu\text{g ml}^{-1}$  and tetracycline was used at concentrations of 20  $\mu\text{g ml}^{-1}$  and 40  $\mu\text{g ml}^{-1}$  for *E. coli* and *Z. mobilis*, respectively. Kanamycin was used at a concentration of 50  $\mu\text{g ml}^{-1}$  for *E. coli*, and rifampin was used at a concentration of 20  $\mu\text{g ml}^{-1}$  for *Z. mobilis*. Plasmids used in this work are shown in Table 1.

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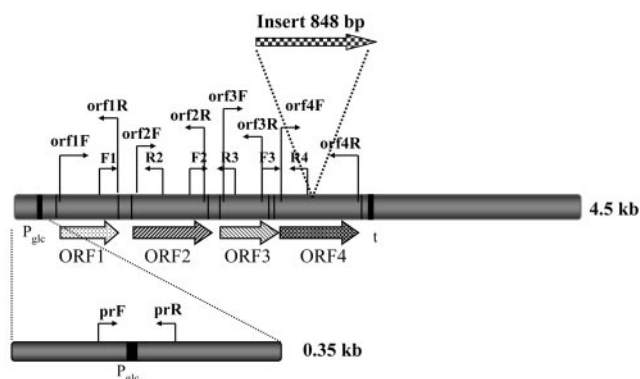


FIG. 1. Genetic map of the cluster that complements the glucose-defective phenotype of CU1Rif2. The transcript was detected by reverse transcription-PCR analysis by amplifying the regions between ORF1 and ORF2, between ORF1 and ORF3, between ORF2 and ORF3, and between ORF3 and ORF4. Primers R2, R3, and R4 were used for synthesis of first-strand cDNA by using reverse transcriptase. The sets of primers used to generate the four ORFs, the flanking regions between the ORFs, and the promoter region are indicated by arrows. The position of the 848-bp insert in the ORF4 region is also indicated. P, promoter; t, terminator.

**DNA methods and plasmid construction.** Preparation of plasmids from *E. coli*, restriction enzyme digestion, ligation, DNA electrophoresis, and Southern blot analysis were performed according to standard protocols (41). Plasmid DNA from *Z. mobilis* was isolated as previously described (43). Transformation of *E. coli* was carried out by chemical treatment (25). DNA was extracted from agarose gels by the GENECLAN II protocol (Bio 101, La Jolla, Calif.). DNA labeling and hybridization were performed by the digoxigenin nonradioactive labeling method (catalog no 1093657; Boehringer, Mannheim, Germany). Plasmid pAEG2 was constructed as shown in Fig. 2. The  $P_{pdc}$  promoter (36) was excised from plasmid pAEG1 (13) by KpnI digestion and replaced with the region of the putative promoter described above comprising a 0.23-kb fragment. This fragment was obtained by PCR amplification using the following primers carrying a KpnI extension at their 5' ends: forward primer 5'-GACGAAAGGGTACCTCCGT AACG-3' corresponding to bases 101 to 123 and reverse primer 5'-GAGTTAT CGGTACCTTGAATTGCCG-3' corresponding to bases 311 to 335. The amplified PCR product was treated with KpnI and ligated with KpnI-linearized pAEG1. The recombinant pAEG2 plasmid was isolated following transformation of *E. coli* DH5 $\alpha$  and screening for fluorescent colonies, which were visible under a UV lamp. Plasmid pHS119-g1, carrying the  $P_{glc}$  promoter subcloned upstream of the vector's *lacZ* gene (Fig. 3) (49), was constructed as follows. The  $P_{glc}$  promoter was obtained by PCR amplification using the following primers carrying a BamHI extension at their 5' ends: forward primer 5'-GTTTCCCGG ATCTCAGCC-3' corresponding to bases 28 to 45 and reverse primer 5'-CGC TATAGATCCGTTCTTTTC-3' corresponding to bases 381 to 402. The 0.37-kb amplification product was treated with BamHI and ligated with BamHI-linearized pHS119 (Fig. 3). The resulting recombinant pHS119-g1 plasmid was

isolated following transformation of *E. coli* DH5 $\alpha$  and screening for blue colonies. Plasmid pHS119-g2, carrying the promoter  $P_{glc}$  subcloned upstream of the ice nucleation gene, was constructed using the strategy described above, except that a different screening assay based on exhibition of ice nucleation activity was used (49, 50). Plasmids pHS119-g3 and pHS119-g4 were constructed as described above with the goal of detection of any promoter activity in the intergenic regions between ORF1 and ORF2 and between ORF2 and ORF3, respectively. To do this, the following primers were used: for the region between ORF1 and ORF2, forward primer 5'-GCAAGCCTTGATCCTGAGACC-3' corresponding to bases 854 to 875 and reverse primer 5'-CGAAACCGATCCGCAAACAG-3' corresponding to bases 1051 to 1071; and for the region between ORF2 and ORF3, forward primer 5'-GAAGGGATCCCTGCCCAAGC-3' corresponding to bases 1479 to 1498 and reverse primer 5'-GCAAATAGGGATCCCTATCC AG-3' corresponding to bases 1732 to 1753. Recombinant plasmids were selected by hybridization with appropriate probes and were tested for promoter activity by measuring either  $\beta$ -galactosidase or ice nucleation activity.

**Bacterial conjugation.** Conjugal transfer of recombinant plasmids in *Z. mobilis* CP4Rif<sup>r</sup> was performed by double-donor filter mating, as previously described (50), by using pRK2013 as a helper plasmid (18). All transconjugants were tested for their plasmid contents by plasmid isolation, back-transformation in *E. coli* when necessary, restriction analysis, and Southern blot hybridization using standard procedures (41).

**Glucokinase assay.** Cells from 200 ml of a liquid culture were harvested at the mid-exponential phase by centrifugation (6,000  $\times$  g, 10 min, 4°C), washed with enzyme buffer containing  $\beta$ -mercaptoethanol (14 mM), resuspended in 1 ml of the same buffer, and disrupted with a mini bead beater (Biospec Products, Bartlesville, Okla.) essentially as previously reported (24). The homogenate was centrifuged (10,000  $\times$  g, 5 min), and the supernatant was used as the crude cell extract. Glucokinase was assayed as described by Scopes et al. (42). The enzyme reaction was initiated by addition of cell extract, and enzyme activity was expressed in micromoles per minute per milligram of protein. Protein concentrations were determined by the method of Lowry et al. (28).

**Glucose uptake assay.** *Z. mobilis* cells were harvested at the mid-exponential phase as described above, washed with phosphate buffer (100 mM, pH 6.5), and resuspended in the same buffer essentially as described by Walsh et al. (52). Glucose uptake was measured with D-[U-<sup>14</sup>C]glucose (291 mCi/mmol; Amersham, Little Chalfont, Buckinghamshire, England) at concentrations ranging from 0.25 to 50 mM. *Z. mobilis* cells (50  $\mu$ l) and fivefold-concentrated radiolabeled glucose (12.5  $\mu$ l) were preincubated separately at 20°C, mixed to obtain the appropriate glucose concentration, and vortexed immediately. Uptake was stopped by addition of 10 ml cold (-2.5°C) phosphate buffer (100 mM, pH 7.5) containing 500 mM unlabeled glucose. Cells were immediately filtered and washed with 10 ml of the same buffer. The uptake rate was determined by determining the initial velocity by sampling every 10 s for a total of 60 s and was expressed in nanomoles of glucose taken up per minute per milligram of total protein.

**GFP fluorescence.** The fluorescence of green fluorescent protein (GFP)-expressing *E. coli* cells was assessed qualitatively by eye, using a UV lamp (366 nm; Ultra Violet Products Inc.). The amount of fluorescence emitted by *Z. mobilis* cells in liquid culture was quantified as described previously (10, 13) using a fluorimeter (Perkin-Elmer LS-3) set to excite the cells at 488 nm and to detect emission at 511 nm. Cells from a 30-ml liquid culture were harvested by centrifugation (6,000  $\times$  g, 10 min), washed with 10 mM Tris (pH 8), 600 mM NaCl, and resuspended in the same buffer, and this was followed by immediate measurement of fluorescence. Protein concentrations were determined by the method of Lowry et al. (28).

**RNA isolation, RT-PCR, and PCR DNA amplification.** Total RNA was isolated from cultured *Z. mobilis* ATCC 10988 and CU1Rif2 cells during the lag, exponential, or late exponential phase using a High Pure RNA isolation kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. RNA was quantified by photometric measurement (41). The reverse transcriptase PCR (RT-PCR) was performed using a RobuST I RT-PCR kit (FINNZYMES, Espoo, Finland). Total RNA (1  $\mu$ g) from samples was used as the template for all the RT-PCRs, and cDNA was synthesized using a thermostable reverse transcriptase (avian myeloblastosis virus reverse transcriptase; FINNZYMES, Espoo, Finland) according to the manufacturer's recommendations and the reverse primers shown in Table 2. Double-stranded DNA was synthesized by PCR using both reverse and forward primers (Table 2) selected on the basis of the sequence of the four ORFs (12). Reverse transcription was carried out at 55°C for 40 min, and this was followed by 30 cycles of PCR (20), as follows: denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C. RT-PCR products were examined by agarose gel electrophoresis and Southern blot hybridization. DNA contamination of the

TABLE 1. Plasmids used in the present work

Plasmid	Antibiotic resistance marker	Reference
pAE1	Cm <sup>r</sup>	13
pAEG1	Cm <sup>r</sup>	13
pAEG2	Cm <sup>r</sup>	This study
pLAFR5	Tc <sup>r</sup>	23
pLAFR5: $P_{pdc}$ -ORF4	Tc <sup>r</sup>	This study
pRK2013	Km <sup>r</sup>	18
pHS119	Tc <sup>r</sup>	49
pHS119-g1	Tc <sup>r</sup>	This study
pHS119-g2	Tc <sup>r</sup>	This study
pHS119-g3	Tc <sup>r</sup>	This study
pHS119-g4	Tc <sup>r</sup>	This study

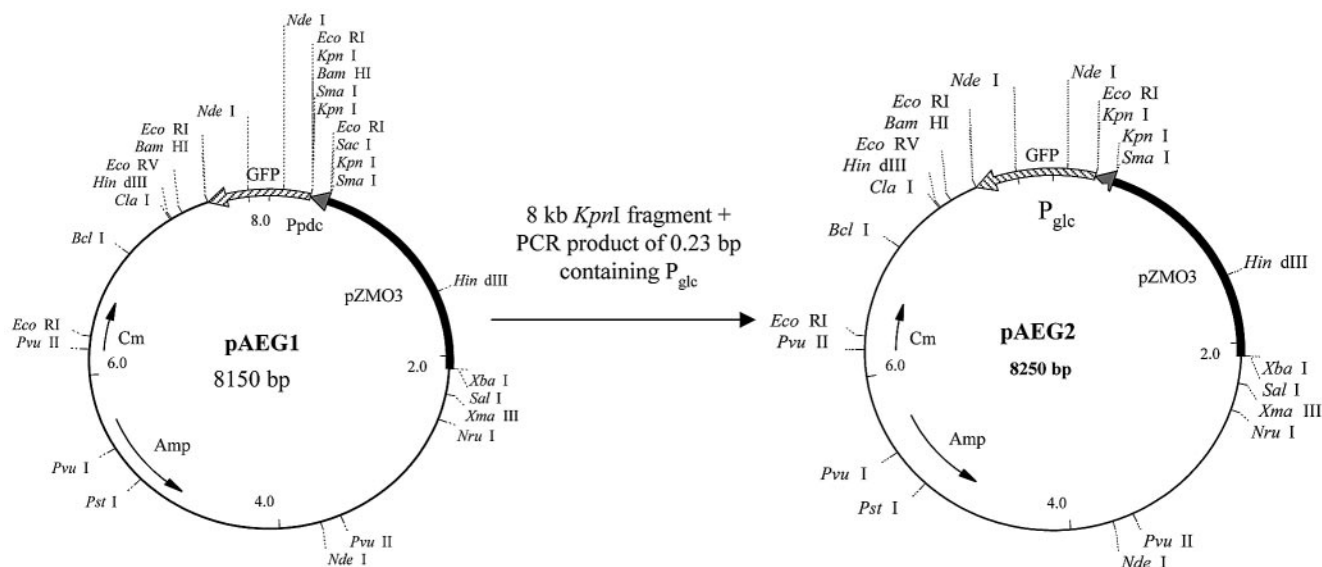


FIG. 2. Construction of plasmid pAEG2. The P<sub>pdC</sub> promoter was excised from plasmid pAEG1 by KpnI digestion and replaced with the putative P<sub>glc</sub> promoter region. The P<sub>glc</sub> fragment (0.23 kb) was obtained using primers corresponding to the cloned sequence.

mRNA was determined by PCR using *Taq* DNA polymerase without primer reverse transcription. DNA amplification was performed with a Perkin-Elmer thermal cycler using standard conditions (30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) and primers described above. All primers were obtained from MWG-Biotech AG, Germany.

**Computer analysis.** A computer search for homologies to known nucleotide or protein sequences was performed with the BLAST2 program at the EMBL-Heidelberg website. Analysis of the *Z. mobilis* sequences was aided by use of the IntelliGenetics PC/Gene software (Oxford Molecular).

**Nucleotide sequence accession number.** The nucleotide sequence of the insertion found in the ORF4 region in the *glc* operon (accession no. AJ009974) has been deposited in the EMBL database under accession no. AJ812655.

**RESULTS AND DISCUSSION**

**The region of the putative *glc* operon that contains -35 and -10 promoter elements exhibits real promoter activity.** As described previously and verified here by extensive BLAST analysis, the cluster of the four ORFs complementing the glucose-sensitive strain CU1Rif2 contains a single region having -35 and -10 *Z. mobilis* promoter elements only at bases 261 to 273 and 298 to 306, respectively (12) (Fig. 1), indicating that the four ORFs are controlled by a common promoter (desig-

nated P<sub>glc</sub>). The hypothesis described above was investigated experimentally by using *gfp* as a reporter gene. Plasmid pAEG2 was transferred to *Z. mobilis* CP4Rif<sup>r</sup> from *E. coli* donors by bacterial conjugation. CP4Rif<sup>r</sup>, a rifampin-resistant derivative of prototype *Z. mobilis* strain CP4 with the wild-type glucose tolerance phenotype (33), was used as a suitable recipient host of plasmid pAEG1 (13). The amount of fluorescence emitted by CP4Rif<sup>r</sup>/pAEG2 cells was measured using strain CP4Rif<sup>r</sup>/pAEG1 as a negative control (13). The CP4Rif<sup>r</sup>/pAEG2 isolate showed significantly greater amounts of fluorescence than strain CP4Rif<sup>r</sup>/pAEG1 but smaller amounts of fluorescence than strain CP4Rif<sup>r</sup>/pAEG2. Both *gfp*-carrying plasmids were stable in the *Z. mobilis* hosts and remained structurally intact for at least 100 cell cycles under nonselective conditions. The differences in promoter activity between a strong constitutive promoter (P<sub>pdC</sub>) and an inducible promoter (P<sub>glc</sub>), which could be easily measured by use of *gfp*, demonstrate the use-

TABLE 2. Sequences of the primers used

Primer	Sequence
F1	5'-TTG AGA CCG TGA TCG CTG GA-3'
R2	5'-AGG AAT TGG GTC GAA TTT GC-3'
F2	5'-CAG GCC GCA AAG AAG GGA TC-3'
R3	5'-ATC AAA GCC TGC GCT TCG GC-3'
F3	5'-AAT CGG GGA TGT TTC TG-3'
R4	5'-GCA ACG GCA ATA CGC TG-3'
PrF	5'-GTT TCC CGA ATT CTC GCC TG-3'
PrR	5'-CCA CGG TCA TAT GCG GCC TT-3'
orf1F	5'-GGC TCG GCG GCA TAT GAT GAA A-3'
orf1R	5'-GCG GTC AAC GGA TCC GAT TCT TG-3'
orf2F	5'-AGC CGA GGC TCA TAT GAC TGA AA-3'
orf2R	5'-AGC GAA TAC TGG GAT CCG GTT-3'
orf3F	5'-CAG ATC AAC ATA TGA CTC ATG CC-3'
orf3R	5'-CAA AAT ACG TGG ATC CTT GCC-3'
orf4F	5'-CGG GAG ATT AAA CAT ATG ACT ATG G-3'
orf4R	5'-TTC ATT CGG ATC CGC ATC-3'

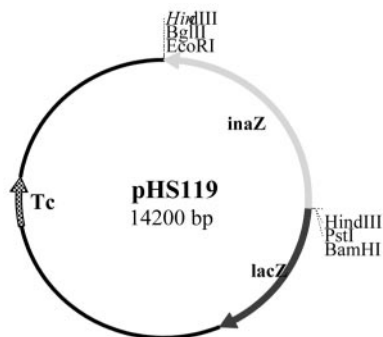


FIG. 3. Plasmid vector pHS119.



TABLE 3. Determination of fluorescence of *Z. mobilis* cells (optical density, 0.6) grown in complete medium containing 2% glucose<sup>a</sup>

Strain	Promoter	Total fluorescence/ mg protein	Corrected fluorescence/ mg protein
CP4Rif <sup>r</sup> /pAE1		0.24 ± 0.01	0.00
CP4Rif <sup>r</sup> /pAEG1	P <sub>pd<sub>c</sub></sub>	1.92 ± 0.10	1.68
CP4Rif <sup>r</sup> /pAEG2	P <sub>glc</sub>	0.88 ± 0.10	0.64

<sup>a</sup> Fluorescence was measured as described in Materials and Methods and is expressed per mg of total protein. Values were estimated from four independent measurements (means ± standard deviations) obtained in the late exponential phase. To properly assess the fluorescence due to GFP, the values obtained for CP4Rif<sup>r</sup>/pAE1 were subtracted from the values obtained for CP4Rif<sup>r</sup>/pAEG1 or CP4Rif<sup>r</sup>/pAEG2 (corrected fluorescence/mg protein).

fulness of this reporter gene in *Z. mobilis* genetic analysis. Additionally, the putative promoter and the intergenic regions between ORF1 and ORF2 and between ORF2 and ORF3 (Fig. 1) were subcloned as a BamHI fragment in plasmid pHS119 (Fig. 3), which contained two reporter genes (*inaZ* and *lacZ*) that had a common start but were in opposite orientations (49). Reporter gene activity (either ice nucleation or β-galactosidase) was obtained with the predicted promoter fragment only when it was cloned in the appropriate orientation. These results indicated that the amplified region carrying the -35 and -10 promoter elements exhibits real promoter activity. Finally, comparison of the P<sub>glc</sub> promoter with other known *Z. mobilis* promoters revealed that P<sub>glc</sub> contained the native consensus sequences (46) (Table 4). The greater spacing of P<sub>glc</sub> than of other native promoters is in an acceptable range and may account for the weak activity of this promoter.

**The four ORFs of the cluster are cotranscribed as an autonomous operon.** To study whether the four ORFs were included in the same transcription unit, a reverse transcription-PCR analysis was performed by using *Z. mobilis* DNA-free RNA and gene-specific primers located in the coding regions (Table 2). The same methodology was used for transcriptional analysis in several other cases, including analysis of an isoprenoid biosynthesis gene in *E. coli* (27), the *tol-oprL* gene cluster in *Pseudomonas aeruginosa* (15), a gene cluster involved in polysaccharide biosynthesis in *Enterococcus faecalis* (53), and the hydrogenase genes from cyanobacteria (3). The regions between ORF1 and ORF2, between ORF1 and ORF3, between ORF2 and ORF3, and between ORF3 and ORF4 (12) were amplified using total RNA as the template and the following sets of primers: for ORF1 and ORF2, primers F1 and

R2; for ORF1 and ORF3, primers F1 and R3; for ORF2 and ORF3, primers F2 and R3; and for ORF3 and ORF4, primers F3 and R4 (Fig. 4). Amplification products that were the predicted sizes (for ORF1 and ORF2, 400 bp; for ORF1 and ORF3, 800 bp; for ORF2 and ORF3, 210 bp; and for ORF3 and ORF4, 350 bp) were obtained in all cases (Fig. 4). These results, in agreement with the results described above, demonstrated that there is a single promoter and showed that the gene cluster complementing the glucose-defective phenotype of mutant CU1Rif2 is expressed as a single transcriptional unit constituting an operon, designated the *glc* operon.

**P<sub>glc</sub> promoter is osmoregulated.** The dependence of the P<sub>glc</sub> activity on the glucose concentration was investigated by measuring fluorescence due to expression of the *gfp* reporter placed under control of the P<sub>glc</sub> promoter. To do this, CP4Rif<sup>r</sup>/pAEG2 cells grown in 0.11 M glucose were harvested during the exponential phase and divided into two equal aliquots, which were used subsequently to infect two fresh cultures, one containing 0.11 M glucose and one containing 0.55 M glucose. Similar cultures of CP4Rif<sup>r</sup>/pAEG1 cells (with the *gfp* reporter under control of the P<sub>pd<sub>c</sub></sub> promoter) were used as controls. A significant increase in fluorescence was observed following a lag period of 2 h after transfer only in the CP4Rif<sup>r</sup>/pAEG2 cultures growing in 0.55 M glucose. The fluorescence was unchanged in the presence of 0.11 M glucose or in either CP4Rif<sup>r</sup>/pAEG1 culture (Fig. 5). These results indicate that the activity of P<sub>glc</sub> is regulated by the concentration of glucose in the medium. In order to examine whether P<sub>glc</sub> is induced specifically by increased glucose concentrations, we tested induction in the presence of various carbon sources. To do this, CP4Rif<sup>r</sup>/pAEG2 cells grown in medium containing 0.11 M glucose were harvested during the exponential phase and inoculated into the same volume of media containing elevated concentrations of various carbohydrates and NaCl. Fluorescence was measured 30 min and 3 h after inoculation. A significant increase in fluorescence was observed in all cases, as well as in the presence of 100 mM and 250 mM NaCl (Fig. 6). It should be pointed out that none of the carbohydrates used except glucose, fructose, and sucrose can be fermented by *Z. mobilis* cells. The induction pattern was identical when ATCC 10988 wild-type cells were used as a host for pAEG2, but in the mutant strain induction was very low or absent (Fig. 6). These results suggest that the P<sub>glc</sub> promoter is subject to induction specifically by increased sugar (hexose, disaccharide, or pentose) or salt concentrations, indicating that P<sub>glc</sub> activity is osmoregulated. This is the first report of an inducible promoter in *Z. mobilis* since all other promoters of this bacterium that have been studied thus far are expressed constitutively (5, 6, 7, 8, 34). However, as we reported previously (12), glucose is the only sugar that can delay at high concentrations the growth of CU1, implying that, in addition to the osmotic pressure, glucose generates an additional specific signal that may trigger growth under these conditions.

**Expression of the *glc* operon is affected by the concentration of glucose.** To investigate the effect of an increase in the glucose concentration in the medium on expression of the *glc* operon, *Z. mobilis* ATCC 10988 and CU1Rif2 cells were cultivated in 0.11 M and 0.55 M glucose, and specific operon mRNA transcript levels were determined by semiquantitative reverse transcriptase PCR by using a methodology similar to a

TABLE 4. Promoter sequences of *Z. mobilis*

Promoter	-35 region	Spacing (bp)	-10 region	Reference
P <sub>glc</sub>	GCTTTCCTGGCT	24	TATTGCATA	12
P <sub>eda</sub>	AAAAAACCCTGATT	15	TAGATGCAA	8
P2 (gap)	GGTATACTGGAAT	18	TATTGATGT	5
P1 (gap)	ACGACAATTGGCT	10	TACTGGAAT	5
P <sub>pd<sub>c</sub></sub>	AATGCCTATAGCT	18	TAGAGGTTT	6
P1 (adhB)	AGCAGCCTTGCTC	17	TAGAAAAAT	7
P2 (adhB)	GAACCCCTTGATC	18	TATTGCTTT	7
P <sub>phoC</sub>	ATTGCTTATTAT	18	TATTACAAT	34
Consensus	A—CTTG—		TA—T—T	8
	G A G		A A	

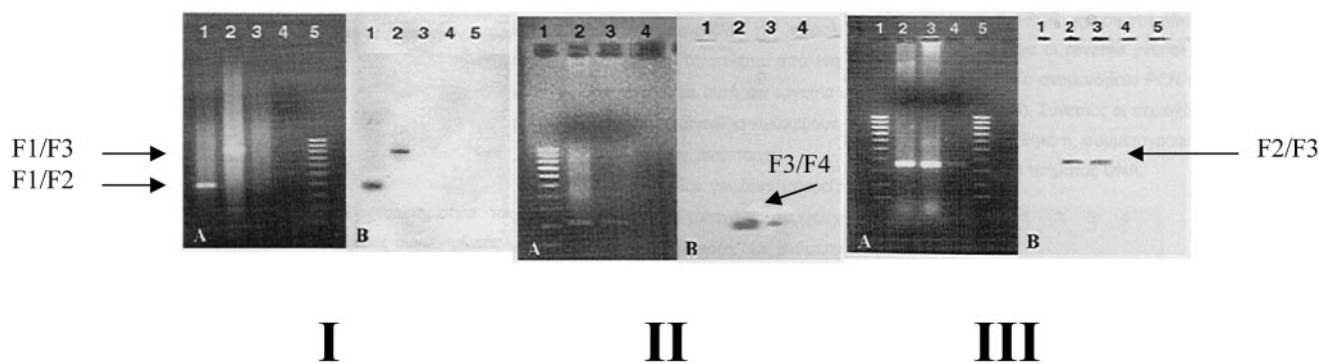


FIG. 4. Gel electrophoresis and Southern blot hybridization of the reverse transcriptase PCR products obtained by using total RNA from *Z. mobilis* cells and the following sets of primers: F1 and R2, F1 and R3, F2 and R3, and F3 and R4 (Fig. 1). (Gel IA) Lane 1, RT-PCR amplification product obtained by using primers F1 and R2; lane 2, RT-PCR amplification product obtained by using primers F1 and R3; lane 3, control reaction; lane 5, 100-bp DNA ladder. (Gel IIA) Lane 1, 100-bp DNA ladder; lane 2, RT-PCR amplification product obtained by using primers F2 and R3; lane 3, RT-PCR amplification product obtained by using primers F2 and R3; lane 4, control (reaction mixture without reverse transcriptase). (Gel IIIA) Lanes 1 and 5, 100-bp DNA ladder; lane 2, RT-PCR amplification product obtained by using primers F3 and R4; lane 3, RT-PCR amplification product obtained by using primers F3 and R4; lane 4, control reaction. (Gels IB, IIB, and IIIB) Southern hybridization blots obtained by using the 4.5-kb DNA fragment as a probe. The positions of expected amplified fragments are indicated by arrows.

methodology recently used in other studies (3, 29, 30, 35, 45). For RT-PCR, ATCC 10988 or CU1Rif2 cells were grown in 0.11 M or 0.55 M glucose and harvested at different times during exponential growth. Total RNA extracted from these cells and primers F2 and R3 (predicted size of the amplicon, 210 bp) were used for the RT-PCRs. *Z. mobilis* 16S rRNA expression was used as a control, as monitored by RT-PCR as described above with an appropriate set of primers corresponding to the *Z. mobilis* 16S rRNA sequence (26) (predicted size, 550 bp). The operon expression was found to be constant during growth of CU1Rif2 and ATCC 10988 cells in medium

containing 0.11 M glucose (Fig. 7B, lanes 1 to 6, and Fig. 7C, lanes 1 to 6, respectively). When ATCC 10988 cells were transferred to a medium containing 0.55 M glucose, the operon expression was repressed for the first 2 h of incubation (Fig. 7C, lanes 7 and 8). On the other hand, under the same conditions, the operon expression in CU1Rif2 cells was repressed for at least 22 h (Fig. 7B, lanes 7 and 8). The same amount of total RNA was analyzed in each case, as indicated by the constant 16S rRNA expression during growth of ATCC 10988 or CU1Rif2 cells in either medium (Fig. 7A). These results demonstrate that the operon expression is regulated by the

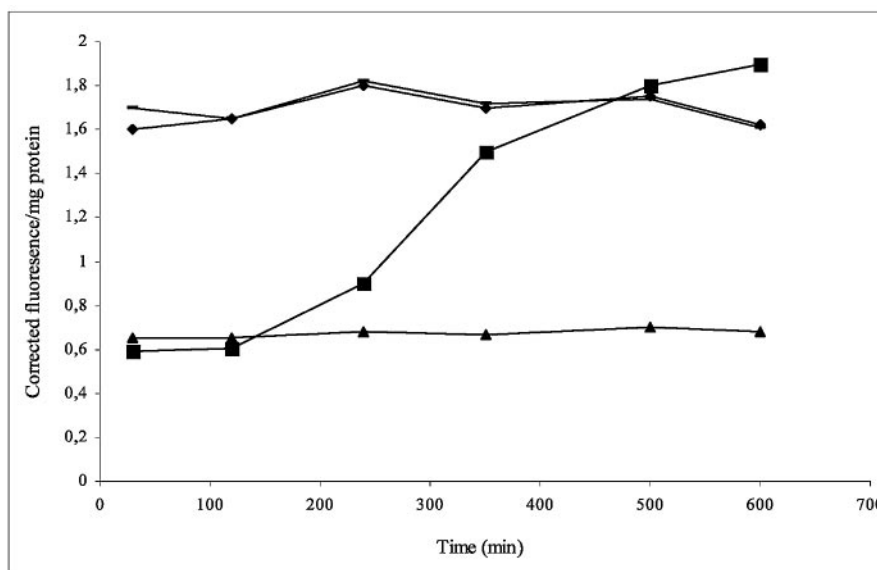


FIG. 5. Effect of glucose concentration on  $P_{pdc}$  and  $P_{glc}$  promoter activities during growth of CP4Rif<sup>r</sup>/pAEG1 and CP4Rif<sup>r</sup>/pAEG2 cells, respectively. Fluorescence was measured as described in Materials and Methods and was expressed per mg of total protein. Values were estimated from three replicates of four independent measurements during growth of cells. The standard error was less than 5%. To properly assess the fluorescence due to GFP, the values detected for CP4Rif<sup>r</sup>/pAE1 (negative control) were subtracted from the values obtained for CP4Rif<sup>r</sup>/pAEG1 or CP4Rif<sup>r</sup>/pAEG2 (Corrected fluorescence/mg protein). Symbols: ◆, CP4Rif<sup>r</sup>/pAEG1 in 2% glucose; ▲, CP4Rif<sup>r</sup>/pAEG2 in 2% glucose; ■, CP4Rif<sup>r</sup>/pAEG1 in 10% glucose; ●, CP4Rif<sup>r</sup>/pAEG2 in 10% glucose.

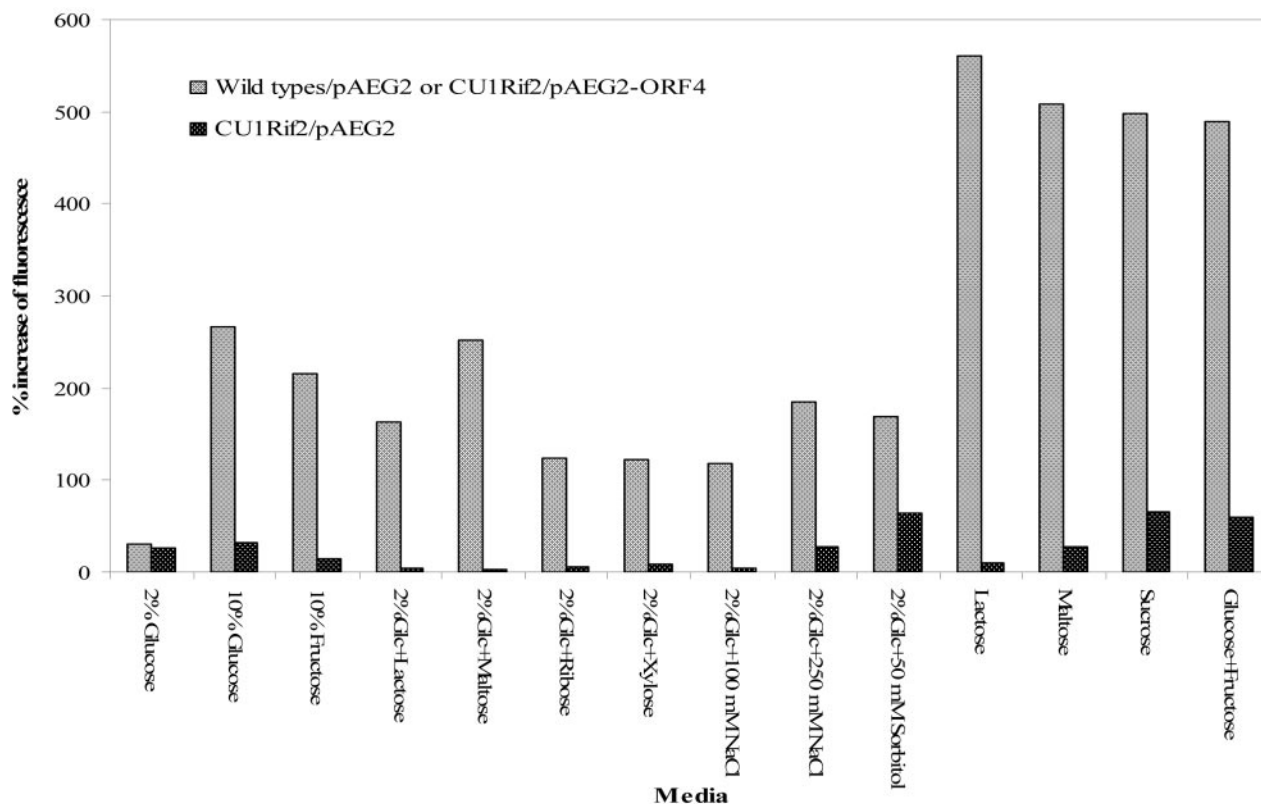


FIG. 6. Comparison of the increases in fluorescence for CP4Rif<sup>r</sup>/pAEPG2, ATCC 10988/pAEG2 (wild type), CU1Rif2-ORF4/pAEG2, and CU1Rif2/pAEG2 cells grown in various osmotic media. All concentrations of the sugars used were isomolecular to 0.55 M (10% glucose). The bars indicate the increases in fluorescence between 30 min and 3 h.

glucose concentration and parallel those described above for the  $P_{glc}$  activity. The duration of repression in the wild type and the mutant is equivalent to the lag period when cells are transferred to a medium with a higher glucose concentration. Interestingly, no other sugar tested in this study except glucose had a similar effect (i.e., appearance of a long lag period during repression). This result is consistent with our previous observation concerning the growth of the CU1 mutant in the presence of high concentrations of nonfermentable sugars and verifies the specificity of glucose for delaying the growth of *Z. mobilis* cells when it is provided at higher concentrations (12).

**ORF4 is solely responsible for the glucose-tolerant phenotype.** To investigate whether the whole cluster of four ORFs is responsible for the glucose-tolerant phenotype, each individual ORF was subcloned in the low-copy-number cosmid vector pLAFR5 (23) under the control of the  $P_{pdc}$  promoter and transferred by conjugation to CU1Rif2. Additionally, a fragment containing ORF1 to ORF3 together was also placed under the control of  $P_{pdc}$  and conjugally transferred to CU1Rif2 as described above. The presence of a strong constitutive promoter like  $P_{pdc}$  eliminated the possibility of deficient expression of the ORFs in the absence of the product of ORF4. All transconjugants were tested for growth on medium containing 0.55 M glucose. The results showed that only ORF4 alone could complement CU1Rif2 in both solid and liquid media containing 0.55 M glucose. Furthermore, CU1Rif2 cells exhibited a significantly shorter lag time in a medium contain-

ing 0.55 M glucose conditioned with CU1Rif2 ORF4 (results not shown), implying that ORF4 also controls formation of the diffusible factor responsible for undelayed growth on 0.55 M glucose, as described previously (12). The rest of the ORFs of this operon did not have an apparent role in complementing the CU1 phenotype.

**Evidence for interaction of the product of ORF4 with the  $P_{glc}$  promoter.** The observation that ORF4 alone could restore the glucose-defective phenotype of the mutant strain led us to test the activity of the  $P_{glc}$  promoter in CU1Rif2 in the presence of ORF4. Therefore, recombinant plasmid pLAFR5: $P_{pdc}$ -ORF4 was conjugally transferred in CU1Rif2/pAEG2, and fluorescence was measured as described above in the presence of various carbon sources, as well as salt. It was found that in transconjugant CU1Rif2/ORF4 isolates, the  $P_{glc}$  promoter was subject to induction, as shown in Fig. 6 for the wild-type strain. This result suggests that the product of ORF4 may interact with the  $P_{glc}$  promoter, either directly or via a transcription factor, indicating a mechanism of osmoregulated expression.

**Glucose uptake and glucokinase activity.** Glucose uptake and glucokinase activity were measured in cells grown on medium containing 0.11 M glucose and transferred for 3 h into a medium containing 0.55 M glucose. Previously (12), we reported decreases in glucose uptake and glucokinase activity in the mutant strain but not in the wild type. The *glc* operon could restore both glucose uptake and glucokinase activity in the mutant strain (12). We attempted to investigate whether

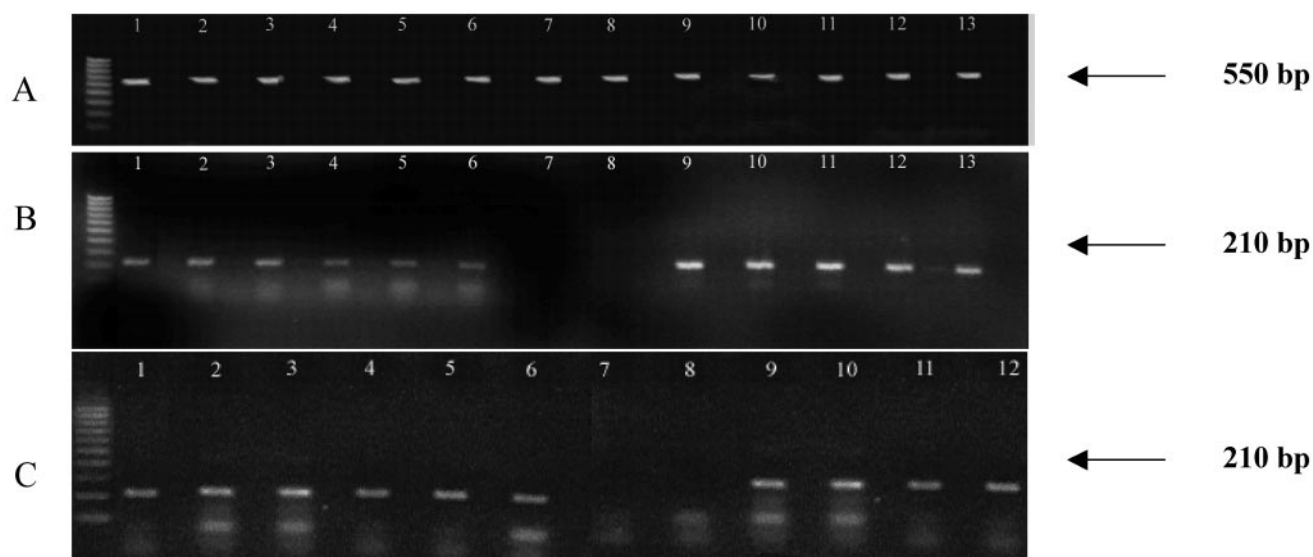


FIG. 7. Gel electrophoresis of the reverse transcriptase PCR products obtained by using total RNA from *Z. mobilis* ATCC 10988 and CU1Rif2 cells during growth in medium containing 2% and 10% glucose, respectively, and primers F2 and R3 (B and C) or appropriate primers for *Z. mobilis* 16S rRNA (A). (A) Amplification product of a region of *Z. mobilis* 16S rRNA obtained by using total RNA of ATCC 10988 (lanes 1 to 6) or CU1Rif2 cells (lanes 7 to 13). (B) Amplification product of a region of the *Z. mobilis* operon obtained by using primers F2 and R3 and total RNA from CU1Rif2 cells. (C) Amplification product of a region of the *Z. mobilis* operon obtained by using primers F2 and R3 and total RNA from ATCC 10988 cells. Cells were grown on 2% (0.11 M) glucose (panel A, lanes 1 to 3 and 7 to 9; panel B, lanes 1 to 6; panel C, lanes 1 to 6) or on 10% (0.55 M) glucose (panel A, lanes 4 to 6 and 10 to 13; panel B, lanes 7 to 13; panel C, lanes 7 to 12) for 30 min (panel A, lanes 1, 4, 7, and 10; panel B, lane 1; panel C, lanes 1 and 7), 2 h (panel B, lane 2; panel C, lanes 2 and 8), 4 h (panel B, lane 3; panel C, lanes 3 and 9), 5 h (panel A, lanes 2, 5, 8, and 11), 6 h (panel B, lane 4; panel C, lanes 4 and 10), 8 h (panel B, lane 5; panel C, lanes 5 and 11), 10 h (panel A, lanes 3, 6, 9, and 12; panel B, lane 6; panel C, lanes 6 and 12), 22 h (panel B, lane 7), 24 h (panel B, lane 8), 26 h (panel B, lane 9), 28 h (panel B, lane 10), 30 h (panel B, lane 11), 32 h (panel B, lane 12), or 35 h (panel A, lane 13; panel B, lane 13).

ORF4 alone, which is the only ORF responsible for the glucose-tolerant phenotype and interacts with  $P_{glc}$ , also contributes to the restoration of glucose uptake and to the glucokinase activity in the mutant strain. To do this, the recombinant plasmid pLAFR5: $P_{pdc}$ -ORF4 was conjugally transferred in CU1Rif2, and the glucose uptake and glucokinase activity were measured as described above. The results showed that in the presence of ORF4 glucose uptake and glucokinase activity in CU1Rif2 were restored to the wild-type levels.

**Localization of the mutation in CU1Rif2.** The ability of ORF4 alone to complement the CU1 defective phenotype implies that the CU1 mutation may be located in the ORF4 sequence. To investigate this possibility, we attempted to isolate the equivalent *glc* operon from CU1Rif2 cells and to localize the putative mutation. This was accomplished by a PCR using primers orf1F and orf4R (Fig. 1 and Table 2) and CU1Rif2 genomic DNA as the template. The amplified product obtained was approximately 900 bp larger than the expected size (data not shown). Ten repetitions of the same PCR gave identical results. Further amplification of each individual ORF, the flanking areas, and the promoter region using appropriate sets of primers (Table 2 and Fig. 1) revealed that ORF4 of CU1Rif2 contained an approximately 900-bp insert. The same insert also appeared in the original mutant, strain CU1 (data not shown). This insert was subcloned in the plasmid vector pUC18 and sequenced. The sequence revealed that the exact size of the insert was 848 bp and that the insert was located at nucleotide 2467 (Fig. 1). This DNA fragment exhibited 93% and 88% homology at the nucleotide level with two

regions of plasmid 1 of *Z. mobilis* strain ZM4 (44) (accession number AY057845), as well as 88% homology with two regions of *Z. mobilis* strain CP4 putative helicase II (*uvrD*) and a putative *Zymomonas glutaredoxin 2* (*glu2*) homologue (2) (accession number AY083904). The insert hybridized strongly with chromosomal DNA from both wild-type and mutant *Z. mobilis* strains but not with their plasmid DNA (data not shown). We concluded that the 848-bp insertion found in the ORF4 region in both our mutant strains (CU1 and CU1Rif2) had a chromosomal origin and was probably generated by some type of genetic rearrangement in the wild-type strain. This insertion could possibly explain the inability of CU1 and its derivative to grow on a medium containing 10% glucose since it disrupts the sequence of ORF4, which, as demonstrated above, is responsible for restoration of the glucose-defective phenotype.

**Theoretical analysis of the proteins encoded by the four ORFs.** To develop a hypothesis for the possible function of the products of the *glc* operon structural genes, we constructed a thorough alignment of the predicted products of the four ORFs with known proteins from data banks, which revealed the following. ORF1 and ORF2 exhibited strong homology with IspD (4-diphosphocytidyl-2C-methyl-D-erythritol synthase) (16, 37, 38) and IspF (2C-methyl-D-erythritol 2,4-cyclo-diphosphate synthase) (22, 51), respectively, which are enzymes involved in the nonmevalonate (DOXP) pathway for the biosynthesis of isoprenoids (Fig. 8a and b), a pathway found to be present in certain bacteria (including *Z. mobilis*), as well as in the plastids of plants and protozoans (17, 39, 40). The



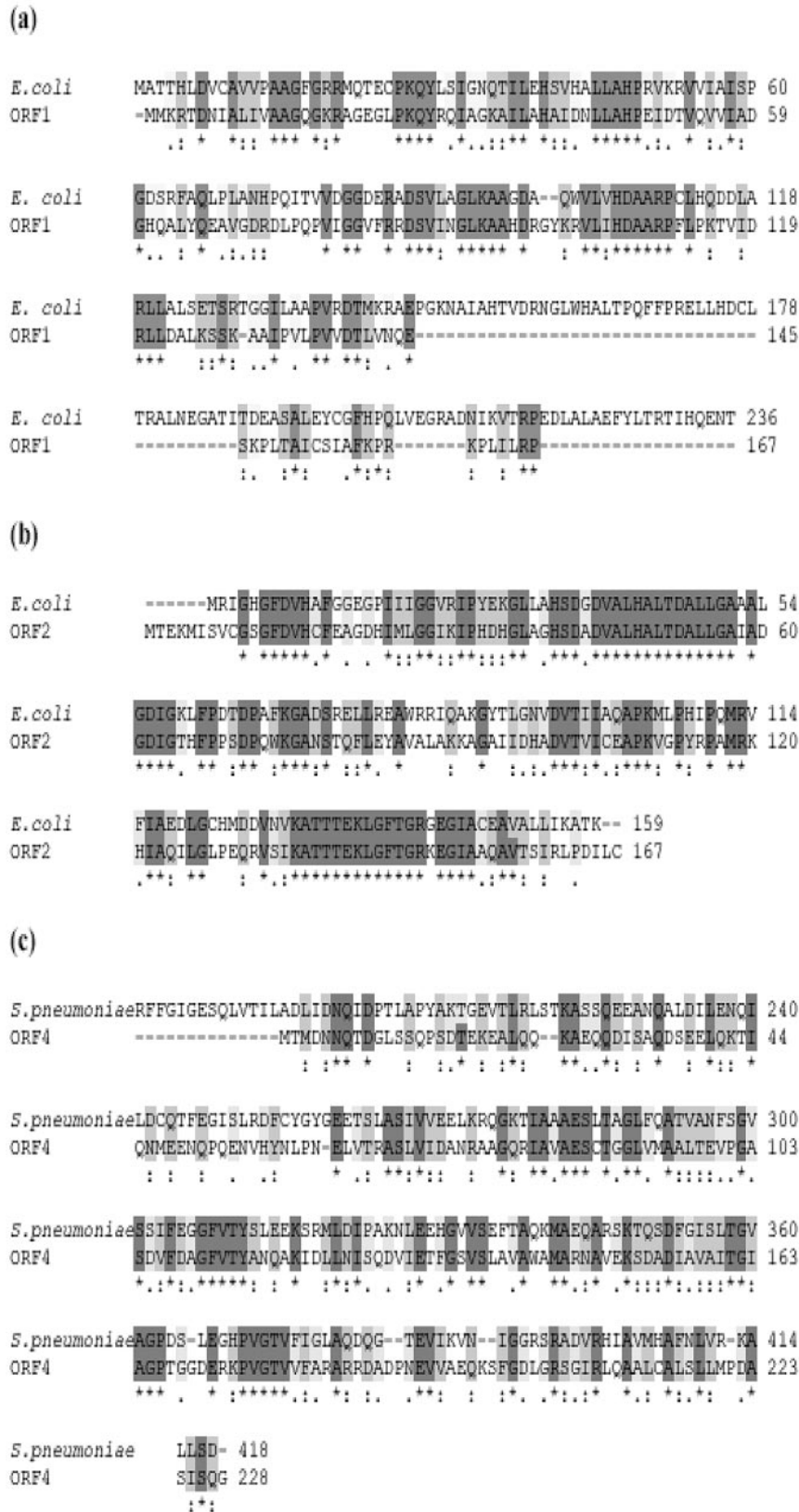


FIG. 8. Alignment of the predicted products of ORF1, ORF2, and ORF4 of the *glc* operon with representative homologues from other bacteria. No homologues were found for ORF3 in the databases searched. (a) ORF1 and IspD protein of *E. coli*; (b) ORF2 and IspF protein of *E. coli*; (c) ORF4 and CinA protein of *Streptococcus pneumoniae*. Dark shading and asterisks indicate identical amino acids; medium shading and colons indicate similar amino acids; and light shading and periods indicate amino acids that are less similar.

product of ORF3 appears to be a novel protein, as it did not show any detectable homology with any known sequence in all available databases. Finally, the product of ORF4 was found to be homologous with the CinA protein (competence-damaged protein [32]) (Fig. 8c), which is part of the competence-induced operon (*cin* operon). CinA is a membrane protein thought to be specifically required at some stage of the process of transformation. What relationship the function of ORF4 might have with CinA is not known at this time. The fact that none of the predicted products of the four ORFs of the *glc* operon is directly involved in glucose metabolism makes these observations really puzzling. However, the autoosmoregulated expression of this operon may justify the existence of at least ORF1 and ORF2, because these ORFs encode key enzymes of the isoprenoid biosynthesis pathway, and increased expression of these ORFs may be required for membrane adaptations, to compensate for the increased osmotic pressure. In the natural environment of *Z. mobilis*, osmotic pressure is usually caused by glucose or other sugars. Nevertheless, this is not the only case in which genes of a biosynthetic pathway are found as part of a cluster of apparently nonrelevant genes. In *Z. mobilis* a third isoprenoid biosynthesis gene is located in a different operon (19). At high glucose concentrations the functional product of ORF4 stimulates the expression of this and perhaps other operons involved in growth adaptations to a continuously changing osmotic environment.

In conclusion, it is very likely that there is a regulation mechanism in *Z. mobilis* which is derepressed in the presence of high glucose concentrations and is responsible for at least restoration of expression of the *glc* operon structural genes. This mechanism is apparently subject to osmotic regulation and does not function in mutant strain CU1 due to inactivation of ORF4. However, the impairment of growth of CU1 at high sugar concentrations, as reported previously (12), is specific for glucose, and a signal is generated by the increased glucose concentrations in the presence of the product of ORF4 and in a fashion that remains to be elucidated. It is pertinent to point out that the CU1 mutation only delays the growth in the presence of high glucose concentrations in liquid media. Consistent with our previous finding that growth is restored by a diffusible proteinaceous factor accumulating in high-glucose medium, we concluded that the product of ORF4 may act as a transcriptional regulator that controls expression of the *glc* operon along with other operons, including the putative gene encoding the hypothetical diffusible growth factor. The delayed growth can be explained by the inability of the CU1 mutant to stimulate expression of the relevant genes fast enough and allow undelayed growth with elevated levels of glucose. To date, such a glucose tolerance mechanism has not been reported for any microorganism.

#### ACKNOWLEDGMENT

This work was supported financially by the Greek General Secretariat of Research and Technology (program PENED 1999; contract 99ED67).

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