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# A *Zymomonas mobilis* Mutant with Delayed Growth on High Glucose Concentrations

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**Exponentially growing cells of *Zymomonas mobilis* normally exhibit a lag period of up to 3 h when transferred from 0.11 M (2%) to 0.55 M (10%) glucose liquid medium. A mutant of *Z. mobilis* (CU1Rif2), fortuitously isolated, showed more than a 20-h lag period when grown under the same conditions, whereas on 0.55 M glucose solid medium, it failed to grow. The growth of CU1Rif2 on elevated concentrations of other fermentable (0.55 M sucrose or fructose) or nonfermentable (0.11 M glucose plus 0.44 M maltose or xylose) sugars appeared to be normal. Surprisingly, CU1Rif2 cells grew without any delay on 0.55 M glucose on which wild-type cells had been incubated for 3 h and removed at the beginning of their exponential phase. This apparent preconditioning was not observed with medium obtained from wild-type cells grown on 0.11 M glucose and supplemented to 0.55 M after removal of the wild-type cells. Undelayed growth of CU1Rif2 on 0.55 M glucose previously conditioned by the wild type was impaired by heating or protease treatment. It is suggested that in *Z. mobilis*, a diffusible proteinaceous heat-labile factor, transitionally not present in 0.55 M glucose CU1Rif2 cultures, triggers growth on 0.55 M glucose. Biochemical analysis of glucose uptake and glycolytic enzymes implied that glucose assimilation was not directly involved in the phenomenon. By use of a wild-type *Z. mobilis* genomic library, a 4.5-kb DNA fragment which complemented in low copy number the glucose-defective phenotype as well as glucokinase and glucose uptake of CU1Rif2 was isolated. This fragment carries a gene cluster consisting of four putative coding regions, encoding 167, 167, 145, and 220 amino acids with typical *Z. mobilis* codon usage, −35 and −10 promoter elements, and individual Shine-Dalgarno consensus sites. However, strong homologies were not detected in a BLAST2 (EMBL-Heidelberg) computer search with known protein sequences.**

*Zymomonas mobilis*, a strictly fermentative gram-negative ethanologenic bacterium, obtains its metabolic energy anaerobically via the Entner-Doudoroff pathway (9, 20, 31, 44, 50, 51). It is an ideal organism for studying unclarified aspects of glycolytic flux in conjunction with sugar tolerance mechanisms. Its carbohydrate range is limited to glucose, fructose, and sucrose, with the last being hydrolyzed to its component hexoses via two extracellular hydrolases (37, 56). Both glucose and fructose are taken up by a low-affinity-, high-velocity-facilitated diffusion system (11, 34, 53) encoded by *glf* (2). However, *Z. mobilis* definitely prefers the former, as indicated by the much higher affinity of the transport system for glucose as well as by the inhibition of fructose kinase by glucose (35). *Z. mobilis* as a typical saccharophilic organism may thrive on exceptionally high concentrations of sugars (45, 50). The ability of *Z. mobilis* to counteract detrimental osmotic effects when grown on sucrose or mixtures of glucose and fructose has been attributed to the formation of sorbitol (25, 27) as a result of the activity of glucose-fructose oxidoreductase (GFOR) (57). However, sorbitol or any other compatible solute is not formed by *Z. mobilis* when grown on glucose as a sole carbon source, at least not to amounts sufficient to account for osmotic protection (27). On the other hand, all strains of *Z. mobilis* tested so far could grow on 1.11 M (20%) glucose within 34 h, whereas some strains were able to grow on up to 2.22 M (40%) glucose after a long lag phase of 4 to 20 days (50). It appears that *Z. mobilis* cells can be adjusted to grow on glucose following a lag period, the length of which depends upon the glucose concentration. For instance, strain ATCC 10988 proliferates on 0.55 and 1.11 M glucose media after lag periods of 3 and 40 h, respectively (12). The ability of *Z. mobilis* to grow on high glucose concentrations was originally explained by a rapid equilibration of the external and internal glucose concentrations achieved by the glucose facilitator system (11, 48). However, later findings showed that the internal concentration of glucose in growing *Z. mobilis* cells remained low (19), whereas after analysis with $^{13}C$ nuclear magnetic resonance spectroscopy, no other major compatible solutes were found (27). The basis of this phenomenon has not been studied before for *Z. mobilis*. For the clarification of this puzzle, the availability of *Z. mobilis* mutants with impaired growth on high glucose concentrations is indispensable. In the present report, the ability of *Z. mobilis* to grow on elevated glucose concentrations is investigated by use of a derivative of strain ATCC 10988 with delayed growth on high glucose concentrations (1, 12).

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## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** *Z. mobilis* wild-type ATCC 10988 (50) and mutants CU1 (12) and CU1Rif2 (1) were grown semianaerobically at 30°C in complete liquid or solid medium as described before (1). To avoid caramelization, carbohydrate solutions were sterilized separately as concentrated stock solutions and then added to liquid medium at the desired concentrations. Exponentially growing cells were used as inocula to yield a starting liquid culture of approximately $10^7$ cells per ml. Growth was monitored turbidimetrically at a wavelength of 600 nm. An optical density at 600 nm ($OD_{600}$) of 0.9 corresponds to 0.35 mg of dry cell weight · $ml^{-1}$. Dry cell weight was determined as described by Loos et al. (27). For minimal medium cultures, a chemically defined solution was used as described by Galani et al. (17). When needed, complete or minimal medium with 0.55 M glucose was conditioned with ATCC 10988 prior to inoc-



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ulation with CU1Rif2 cells. In these cases, ATCC 10988 inoculum was removed by centrifugation (6,000 × *g*, 10 min), and the medium supernatant was filtered (0.2-μm-pore-diameter Millipore filter) to remove unsedimented cells. The filtrate was used untreated, heated, or incubated with hydrolytic enzymes for 1 h before being inoculated. *Escherichia coli* DH5α (18) was grown at 37°C in Luria broth (29). The low-copy-number cosmid pLAFR5 (21) ($Tc^r$; 20 μg/ml) was used for expression in *Z. mobilis*. Plasmid pZY507 (53) ($Cm^r$; 25 μg/ml) was used for expression in *E. coli* ZSC112LΔpts (53), and pUC18 (Boehringer Mannheim Biochemicals) ($Ap^r$; 100 μg/ml) was used for subcloning and sequencing. Transconjugants of *Z. mobilis* CU1Rif2 were selected with tetracycline (40 μg/ml) and rifampin (20 μg/ml).

**Estimation of glucose concentrations.** The amount of glucose consumed during inoculation was calculated by subtracting the amount of glucose remaining in the culture broth at the time of assay from the initial amount of glucose. The amount of glucose was estimated with a hexokinase Olympus System Reagent Kit (Olympus Diagnostics GmbH, Hamburg, Germany).

**Lipid analysis.** For phospholipid and fatty acid analysis, cells were harvested in the late exponential phase by centrifugation (6,000 × *g*, 10 min, 4°C), washed with distilled water, lyophilized, and extracted by the method of Bligh and Dyer (5). The amounts of phospholipids and fatty acids were determined as described previously (23). Hopanoids were analyzed by gas-liquid chromatography following three extractions (1 h each) of freeze-dried cells under reflux with chloroform-methanol (2:1 [vol/vol] and treatment with $H_5IO_6$-$NaBH_4$ as described by Rohmer et al. (38).

**Enzyme assays.** Cells from 200 ml of liquid culture were harvested at the mid-exponential phase by centrifugation (6,000 × *g*, 10 min), washed with enzyme assay buffer containing mercaptoethanol (14 mM), resuspended in 1 ml of the same buffer, and disrupted in a Mini Bead Beater (Biospec Products, Bartlesville, Okla.) essentially as previously reported (22). The homogenate was centrifuged (10,000 × *g*, 5 min), and the supernatant was used as the crude cell extract. Glucokinase (GLK) and glucose-6-phosphate dehydrogenase were assayed as described by Scopes et al. (42). GFOR was assayed comparatively for wild-type and mutant cells essentially as described by Zachariou and Scopes (57) by coupling the reaction with indigenous gluconolactonase activity. Pyruvate decarboxylase (PDC) was assayed by coupling to alcohol dehydrogenase (ADH) and measuring the oxidation of NADH at 340 nm as described by Neale et al. (32). ADH was assayed by determining the production of ethanol as described by Conway et al. (10). All enzyme reactions were initiated by adding the cell extract, and enzyme activities were expressed in micromoles per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al. (28).

**Glucose uptake assays. (i) *Z. mobilis*.** Cells were harvested at the mid-exponential phase, 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-$^{14}$C]glucose (291 mCi/mmol; Amersham, Buckinghamshire, England) at concentrations ranging from 0.25 to 50 mM. *Z. mobilis* cells (50 μl) and fivefold-concentrated radiolabelled glucose (12.5 μl) were preincubated separately at 20°C, mixed together to yield the appropriate glucose concentration, and vortexed immediately. Uptake was stopped by the addition of 10 ml of cold (−2.5°C) phosphate buffer (100 mM, pH 7.5) containing 500 mM unlabelled glucose. Cells were immediately filtered and washed with 10 ml of the same buffer. The uptake rate was expressed as nanomoles of glucose taken up per minute per milligram of total protein.

**(ii) *E. coli*.** Glucose uptake was assayed with 2-deoxy-D-[U-$^{14}$C]glucose (308 mCi/mmol; Amersham) at a final concentration of 5 mM at 10°C essentially as described by Weisser et al. (53) with the following modifications. Briefly, *E. coli* was grown in M9 minimal medium (40) supplemented with 0.5% gluconate, thiamine (1 μg/ml), and chloramphenicol (25 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added at an $OD_{600}$ of 0.2 to induce the *tac* promoter. Cells were harvested in the late logarithmic phase ($OD_{600}$, 0.7) and assayed for uptake. The uptake reaction was stopped at different times by the addition of 0.1 M phosphate buffer (pH 7.5) containing 500 mM glucose and rapid filtration.

**Bacterial conjugation.** Conjugal transfer of recombinant plasmids in *Z. mobilis* CU1Rif2 was performed as described previously (1) with double-donor filter matings and pRK2013 (14) ($Km^r$; 50 μg/ml) as a helper plasmid.

**DNA methods.** Preparation of plasmids from *E. coli*, restriction enzyme digestions, ligations, DNA electrophoresis, and Southern blot analysis were performed by standard protocols (40). Plasmid DNA was isolated from *Z. mobilis* as previously described (43). Transformations of *E. coli* were done by chemical treatment (24). DNA was isolated from agarose gels by use of GeneClean II (Bio 101, Inc., La Jolla, Calif.). DNA labelling and hybridization were performed by the digoxigenin nonradioactive labelling method (Boehringer). Genomic libraries of *Z. mobilis* CP4 (G. A. Sprenger, Forschungszentrum, Jülich, Germany) and ATCC 10988 (C. Drainas) were prepared by digestion of *Z. mobilis* genomic DNA with *Sau*3A (~25-kb fragments). The digested fragments were ligated into the *Bam*HI-*Sca*I sites of the cosmid vector pLAFR5 (21) and in vitro packaged by use of a Stratagene Gigapack II packaging extract according to the instructions of the manufacturer. Following transfection in *E. coli* DH5α, two libraries of approximately 500 cell clones each were produced. All transfected *E. coli* cells tested had recombinant cosmids with inserts ranging from 24 to 35 kb.

Subcloning of a DNA fragment(s) which complements the phenotype of CU1Rif2 was carried out with restriction fragment replacements on pLAFR5. Initially, the 25-kb fragment(s) was digested with *Bgl*II, and a 9.5-kb DNA fragment was isolated and recircularized by self-ligation (pLAFR595; Table 1); see Fig. 2 for subsequent restriction steps. For construction of pLAFR589, pLAFR509, pLAFR512, pLAFR518, pLAFR508, or pLAFR516, restriction fragments were excised from pLAFR595 and inserted into the *Bam*HI or *Hin*dIII sites of pLAFR5. Plasmid pLAFR551 or pLARF545 was constructed by insertion of the *Sal*I-*Sal*I fragment of pLAFR595 or the *Bam*HI-*Sal*I fragment of pLAFR551, respectively, into the corresponding sites of pUC18 (i.e., pUC1851 or pUC1845, respectively; Table 1), followed by subcloning of the segments into pLAFR5 by *Eco*RI-*Pst*I restriction fragment replacement. A similar procedure was used for the construction of pLAFR531 and pLAFR520, the relevant fragments of which had been excised from pLAFR595 by *Apa*I-*Sal*I restriction, filled in with T4 DNA polymerase, and inserted into the *Sma*I site of pUC18. Finally, pLAFR533 was constructed by partial *Hin*dIII digestion of pLAFR545 which had been linearized with *Bam*HI and subcloning of the 3.3-kb *Bam*HI-*Hin*dIII fragment into pLAFR5.

Additionally, plasmid pZY50733 was constructed as a pZY507 derivative by the same method as that used for pLAFR533 (see above). Plasmid pLAFR5*glf* was derived from pUC18*glf* by *Hin*dIII restriction and insertion of the 2.3-kb restriction fragment into the corresponding site of pLAFR5 (Table 1).

TABLE 1. Plasmids

| Plasmid | Antibiotic resistance marker | *Z. mobilis* genomic insert (kb) | Source or reference |
|---|---|---|---|
| pUC1851 | $Ap^r$ | 5.1 | This study |
| pUC1845 | $Ap^r$ | 4.5 | This study |
| pUC18*glf* | $Ap^r$ | 2.3 (*glf*) | 46 |
| pLAFR595 | $Tc^r$ | 9.5 | This study |
| pLAFR551 | $Tc^r$ | 5.1 | This study |
| pLAFR509 | $Tc^r$ | 0.9 | This study |
| pLAFR512 | $Tc^r$ | 1.2 | This study |
| pLAFR518 | $Tc^r$ | 1.8 | This study |
| pLAFR508 | $Tc^r$ | 0.8 | This study |
| pLAFR516 | $Tc^r$ | 1.6 | This study |
| pLAFR531 | $Tc^r$ | 3.1 | This study |
| pLAFR520 | $Tc^r$ | 2.0 | This study |
| pLAFR589 | $Tc^r$ | 8.9 | This study |
| pLAFR545 | $Tc^r$ | 4.5 | This study |
| pLAFR533 | $Tc^r$ | 3.3 | This study |
| pLAFR5*glf* | $Tc^r$ | 2.3 (*glf*) | This study |
| pZY507*glf* | $Cm^r$ | 1.4 (*glf*) | 53 |
| pZY50733 | $Cm^r$ | 3.3 | This study |

**DNA sequencing.** Sequencing of the 4.5-kb *Z. mobilis* DNA fragment of pUC1845 was done by the dideoxy termination method (41) on an automated DNA sequencer (Applied Biosystems ABI Prism model 211) at the UCLA DNA Sequencing Facility (Erik Avaniss-Aghajani).

**Computer analysis.** A computer search for homologies to known nucleotide or protein sequences was performed with the BLAST2 program at the EMBL-Heidelberg website (13a). Analysis of the *Z. mobilis* sequences was aided by use of IntelliGenetics PC/Gene software (Oxford Molecular).

**Nucleotide sequence accession number.** The complete nucleotide sequence of the 4.5-kb *Z. mobilis* DNA fragment has been submitted to the EMBL database under accession no. AJ009974.

## RESULTS AND DISCUSSION

**Growth of wild-type *Z. mobilis* and derivative CU1Rif2 on media with high sugar concentrations.** Mutant CU1 was fortuitously isolated by mild acridine orange treatments in the course of screening for plasmid-cured isolates of *Z. mobilis* ATCC 10988 (12). This strain, appearing to have lost one of the smaller cryptic plasmids, was checked for growth on various concentrations of glucose and found to exhibit a pronounced lag period when grown in complex or minimal liquid medium with 0.55 M glucose, whereas it failed to grow at all on either solid medium. Mutant CU1Rif2 is a rifampin-resistant derivative of CU1 isolated to facilitate conjugal transfers from *E. coli* to *Z. mobilis* (1) and has been used in our laboratory since isolation as a routine experimental strain. CU1 and CU1Rif2 have identical phenotypes for growth on various

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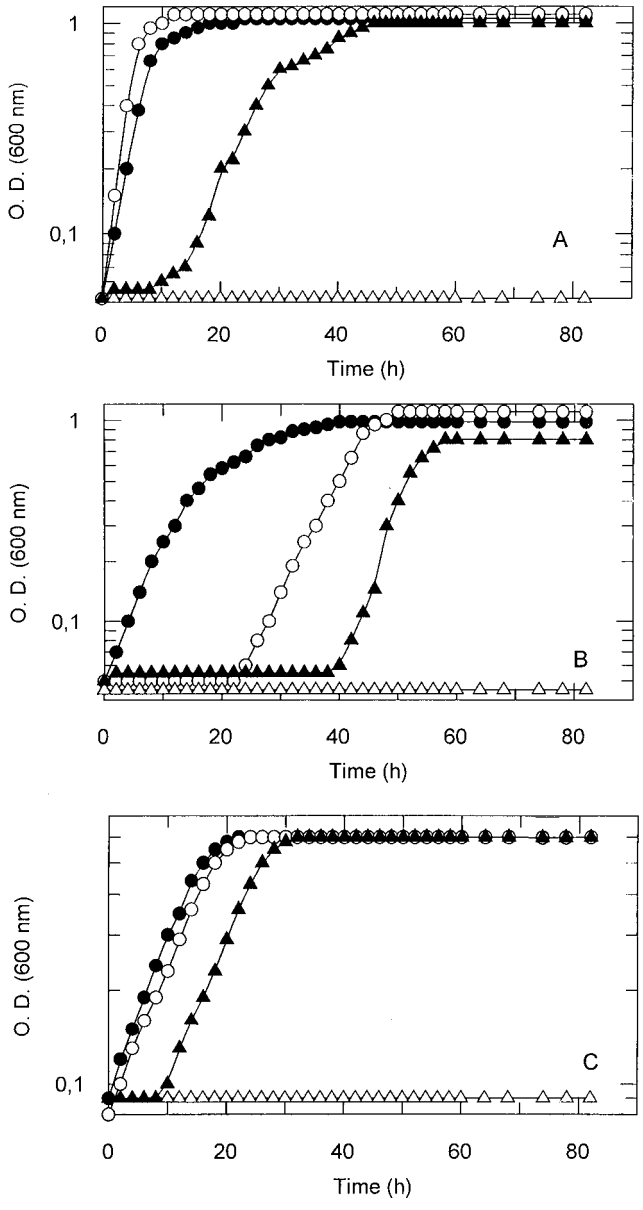


FIG. 1. Growth curves for *Z. mobilis* ATCC 10988 (A), CU1Rif2 (B), and CU1Rif2/pLAFR545 (C). After being precultured on 0.11 M glucose, cells were inoculated into liquid complete medium containing 0.11 M glucose (●) or 0.55 M glucose or 0.11 M glucose plus 0.44 M DOG (○). After being precultured in the presence of 0.55 M glucose or after 20 h of incubation with 0.55 M DOG, cells were inoculated into liquid complete medium containing 0.55 M glucose (●), 1.38 M glucose (△), or 1.38 M glucose plus 50 mM sorbitol (▲). Each experiment was repeated six times with a standard error of less than 5%.

sugar concentrations, plasmid content, lipid content, glucose uptake, and glycolytic enzyme activities. Therefore, only CU1Rif2 is discussed here.

We checked the growth of CU1Rif2 on high concentrations of various carbon sources. As shown in Fig. 1B, a pronounced extension of the lag phase (up to 20 h) was observed for the mutant when grown in liquid media with 0.55 M glucose, whereas it failed to grow at all on similar solid media. Its growth rate was unaffected by high concentrations of other fermentable sugars, such as fructose (up to 0.55 M), sucrose (up to 0.55 M), or glucose plus fructose (0.11 and 0.44 M, respectively). Similarly, the presence of high concentrations (up to 0.44 M) of nonfermentable sugars, such as maltose (not taken up) or xylose (taken up but not metabolized) (48, 54), in 0.11 M glucose media did not affect the growth of CU1Rif2 (data not shown). On the contrary, a similar extension of the lag phase (20 h) was observed in cultures containing 0.11 M glucose and a 0.44 M concentration of the glucose analog 2-deoxyglucose (DOG), which does not support growth (Fig. 1B). The addition of sorbitol at 50 mM did not reduce the lag period on 0.55 M glucose but expedited the exponential growth of CU1Rif2 in the presence of higher glucose concentrations (1.38 M), as in the wild-type strain (Fig. 1A and B). The growth of CU1Rif2 was the same in complete or minimal medium.

**Detection of a diffusible factor affecting growth on high glucose concentrations.** Once the growth of the mutant was established on 0.55 M glucose, or after 20 h of incubation in medium with 0.55 M DOG, growth occurred as for the wild type upon transfer to fresh solid or liquid medium with 0.55 M glucose (Fig. 1B). This result was observed even when the transferred mutant cells were washed with fresh liquid medium prior to inoculation, implying that they had at least phenotypically changed. However, subculturing of such cells through a growth cycle on 0.11 M glucose medium once again revealed the mutant phenotype of a long lag in the high-glucose liquid medium and no growth on the high-glucose solid medium. These results argue against reversion or suppression occurring during the lag phase. Instead, it appeared that a preconditioning of the medium was involved. Thus, when the wild-type strain was incubated to the beginning of exponential growth (3 h) in 0.55 M glucose complete or minimal medium and the cells were then removed (see Materials and Methods; the glucose concentration at this point was 0.54 M), mutant cells grew without delay. When the wild-type strain was preincubated in 0.11 M glucose medium which was supplemented to 0.55 M glucose after removal of the cells, a normal delay again occurred with mutant cells, as if the preconditioning required the high glucose concentration. The same phenomenon of preconditioning of the medium also occurred with the mutant culture itself; incubation of the mutant in 0.55 M glucose medium to the end of the 20-h lag phase (instead of the 2 h required for the wild type), followed by removal of the cells, allowed growth without delay of fresh mutant cells not exposed to high glucose. The putative growth lag factor was lost after treatment at 50°C for 30 min, at 75°C for 5 min, or with proteinase K (150 mU/ml) for 1 h but was stable after 1 h of treatment with phospholipase D (50 mU/ml), phospholipase A2 (500 mU/ml), DNase (150 U/ml), or RNase (75 U/ml). None of these treatments affected the growth of the wild type.

**Lipid composition of *Z. mobilis* CU1Rif2.** Due to the unusual lipid composition and the reported correlation with high sugar tolerance (6, 7), we examined the lipid composition of the *Z. mobilis* strains used in this work. No significant differences in the major phospholipid, fatty acid, and hopanoid contents of the wild type, CU1, and CU1Rif2 were observed.

**Glucose uptake and enzyme activities.** Glucose transport was measured in cells taken from 0.11 M glucose medium and subcultured for 3 h in medium with 0.11 or 0.55 M glucose. A decrease in transport activity was found in the mutant (see fig. 3B) but not in the wild type (see fig. 3A). In both cases, analysis of uptake kinetics (data not shown) did not reveal clear differences in $K_m$ values (for wild type versus mutant or for 0.11 versus 0.55 M glucose). The apparent $K_m$ values were between 5.55 and 15.7 mM, in agreement with earlier reports (11, 35, 48), although the $V_{max}$ values of 15 to 25.4 nmol · $min^{-1}$ · mg of $protein^{-1}$ were low. Enzyme activities measured for similarly treated cells (Table 2) showed no differences (for wild

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TABLE 2. Enzyme activities in *Z. mobilis* extracts grown on various glucose concentrations[a]

| Strain | Glucose (M) | GLK $K_m$ with glucose (mM) | Sp act (nmol of NADH · mg of protein$^{-1}$ · min$^{-1}$) of: | | | |
|---|---|---|---|---|---|---|
| | | | GLK | G6P | PDC | ADH |
| ATCC 10988 | 0.11 | 0.117 ± 0.011 | 550 ± 18 | 160 ± 28 | 2,135 ± 289 | 775 ± 35 |
| | 0.27 | 0.110 ± 0.008 | 770 ± 15 | 162 ± 10 | 2,234 ± 250 | 780 ± 40 |
| | 0.55 | 0.112 ± 0.011 | 1,050 ± 70 | 150 ± 14 | 2,425 ± 210 | 800 ± 113 |
| CU1Rif2 | 0.11 | 0.110 ± 0.006 | 420 ± 20 | 145 ± 7 | 2,335 ± 106 | 790 ± 98 |
| | 0.27 | 0.090 ± 0.005 | 370 ± 20 | 150 ± 12 | 2,310 ± 107 | 785 ± 95 |
| | 0.55 | 0.100 ± 0.010 | 400 ± 15 | 170 ± 18 | 2,155 ± 346 | 790 ± 98 |
| CU1Rif2/pLAFR545 | 0.11 | 0.107 ± 0.005 | 520 ± 15 | 170 ± 30 | 2,170 ± 75 | 830 ± 50 |
| | 0.27 | 0.116 ± 0.009 | 710 ± 14 | 165 ± 23 | 2,170 ± 65 | 835 ± 35 |
| | 0.55 | 0.114 ± 0.011 | 910 ± 14 | 170 ± 22 | 2,160 ± 45 | 830 ± 25 |

[a] *Z. mobilis* cells were grown in complete liquid medium containing 0.11 M glucose, harvested at the mid-logarithmic phase, and incubated in the same medium containing the indicated glucose concentrations for an additional 3 h. Values were estimated from four independent measurements (means ± standard deviations) (4). G6P, glucose-6-phosphate dehydrogenase.

type versus mutant or for glucose concentrations) for glucose-6-phosphate dehydrogenase, PDC, or ADH. GLK activity, on the other hand, was approximately doubled in the wild type exposed to the higher glucose concentration but not in the mutant; no differences were seen in $K_m$ values with glucose (Table 2). Although the differences in transport and GLK activity between the wild type and the mutant are likely to be related to the lag in mutant growth on high glucose, they offer no clear explanation for the phenomenon.

**Isolation of a DNA fragment which complements the phenotype of CU1Rif2.** A genomic library from *Z. mobilis* CP4 was transferred to strain CU1Rif2 by bacterial conjugation, and 600 transconjugant colonies were isolated. Each colony was tested for sensitivity to media containing 0.55 M glucose, and two clones resistant to 0.55 M glucose were selected. Restriction analysis of the DNA fragments (~25 kb each) isolated from both transconjugants indicated that they contained overlapping regions. One of them was further subcloned in pLAFR5 (Fig. 2). The resulting pLAFR5 recombinants were transformed in DH5α and transferred by conjugation to CU1Rif2, and transconjugants were tested again for growth on 0.55 M glucose medium (Fig. 1C). Initially, a 9.5-kb fragment complementing CU1Rif2 was isolated. Further subcloning of this fragment led to the isolation of functional subfragments of 8.9, 5.1, and 4.5 kb, whereas the *Hin*dIII and *Apa*I-*Sal*I fragments were not functional (Fig. 2). The 9.5-kb fragment hybridized strongly with chromosomal DNAs from both *Z. mobilis* CP4 and *Z. mobilis* ATCC 10988. Furthermore, a similar clone was isolated from the ATCC 10988 genomic library by hybridization under high-stringency conditions with the 4.5-kb fragment as a probe. Analysis of the ATCC 10988 clone revealed the same restriction pattern and complementing function as for the CP4 4.5-kb fragment.

The GLK activity and glucose uptake rate of complemented strain CU1Rif2/pLAFR545 (Table 2 and Fig. 3C) followed the same pattern as in the wild-type strain. The same result was obtained with all DNA fragments that complement CU1Rif2 phenotypically on solid 0.55 M glucose medium (data not

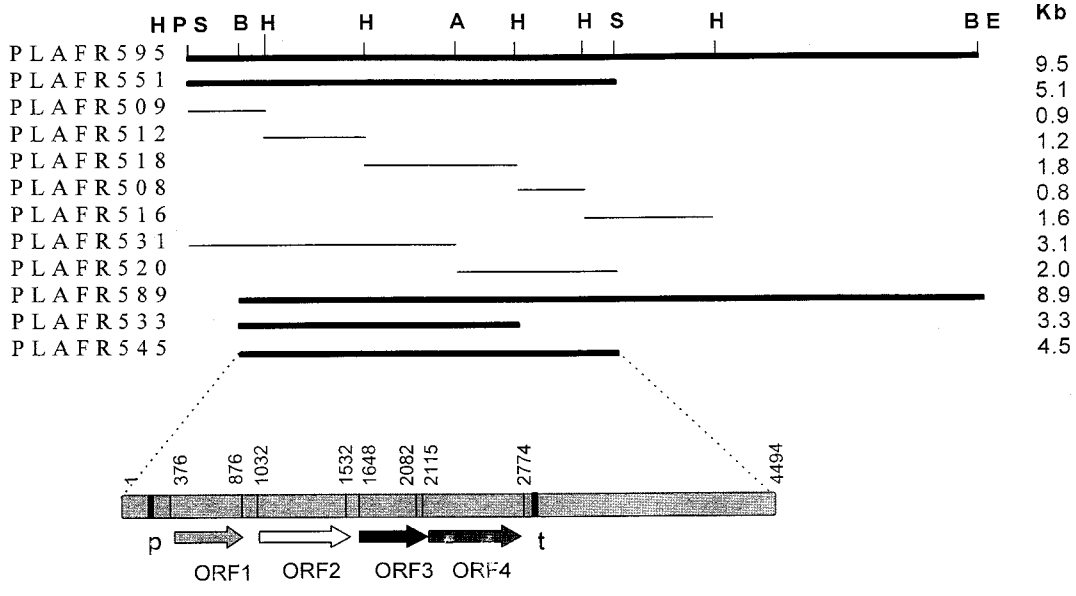


FIG. 2. Restriction analysis and subcloning of the 9.5-kb DNA fragment, which restores the glucose-defective phenotype of CU1Rif2, in pLAFR5 (see Table 1 for designations of plasmids). Restriction enzyme sites: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. The genetic map of the sequenced 4.5-kb fragment is given in detail. p, promoter; t, terminator. Thick lines indicate functional fragments; thin lines indicate nonfunctional fragments.

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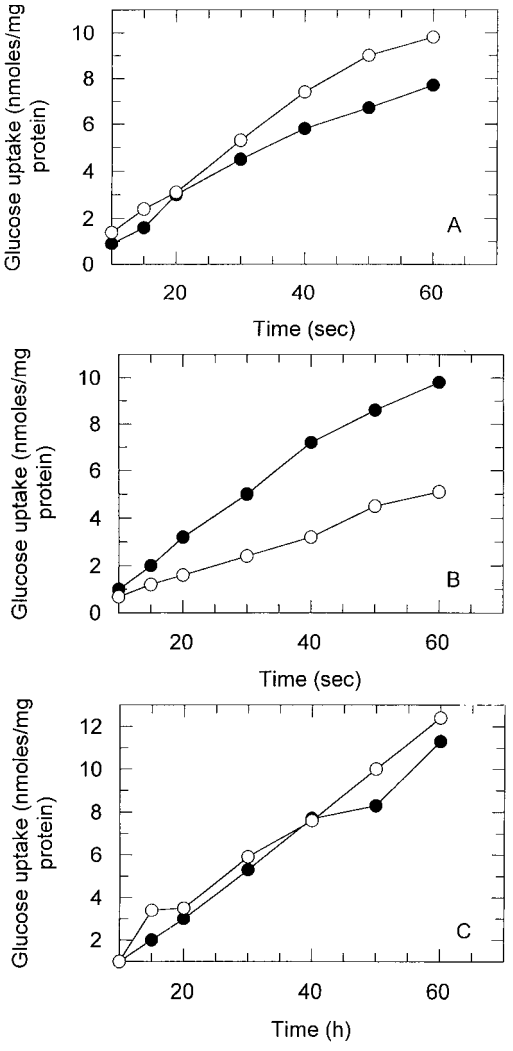


FIG. 3. Glucose uptake of *Z. mobilis* ATCC 10988 (A), CU1Rif2 (B), and CU1Rif2/pLAFR545 (C). Cells were grown in liquid medium containing 0.11 M glucose, harvested at the midexponential phase, and incubated for 3 h in the same medium containing 0.11 M (●) or 0.55 M (○) glucose. Labelled glucose was added at a concentration of 5 mM, and uptake was measured at 20°C. Each experiment was repeated six times with a standard error of less than 5%.

shown). Furthermore, CU1Rif2 cells grew without delay on 0.55 M glucose culture medium which had been preincubated with CU1Rif2/pLAFR545 cells.

Thus, the 4.5-kb fragment should contain a minimal sequence complementing CU1Rif2; this fragment was subcloned in pUC18 (pUC1845) for further DNA sequence analysis.

**Nucleotide sequence of the 4.5-kb *Z. mobilis* fragment in pUC1845.** Both strands were sequenced throughout the 4.5-kb region, and the sequence revealed the existence of four putative coding regions (open reading frames [ORFs]) (Fig. 2). ORF 1 consists of 501 bp (nucleotides 376 to 876), ORF 2 has 501 bp (nucleotides 1032 to 1532), ORF 3 has 435 bp (nucleotides 1648 to 2082), and ORF 4 contains 660 bp (nucleotides 2115 to 2774). Each of the four ORFs is preceded by potential ribosome-binding sites (Shine-Dalgarno consensus sequences). All four predicted ORFs begin with an ATG start codon, which is typical for *Z. mobilis* protein-coding sequences, and use a TAA stop codon (except for ORF 1, which uses TGA), which appears to be the most commonly used in *Z. mobilis*. Furthermore, each of the four ORFs displays synonymous codon usage statistics, a finding which is typical for protein-coding sequences of *Z. mobilis* (data not shown).

The overall organization of the cluster of the four ORFs suggests that they may be cotranscribed as an autonomous operon, as in the cases of *glf-zwf-edd-glk* for glucose metabolism (3) and *gluEMP* for glutamate transport (36). Putative −35 and −10 *Z. mobilis* promoter elements (47) are located at bases 261 to 273 and 298 to 306, respectively, i.e., 70 bp upstream of the start codon of ORF 1, whereas a putative transcription terminator sequence is found immediately downstream of the stop codon of ORF 4 (bases 2819 to 2851).

The 3.3-kb *Bam*HI-*Hin*dIII segment of pUC1845 containing all four putative coding regions along with the promoter and terminator sequences was transferred to pLAFR5 (pLAFR533; Table 1), expressed in CU1Rif2, and found to be sufficient for complementation of the glucose-sensitive phenotype. On the other hand, complementation was not achieved with pLAFR531 (Fig. 2), in which the cluster sequence is disrupted at the *Apa*I site of ORF 4 (nucleotide 2582). Removal of the 260-bp 5′-terminal sequence of the 4.5-kb fragment by partial *Hin*dIII digestion resulted in abrogation of the complementing phenotype, probably due to disruption of the −35 promoter sequence. The possibility that products of the cloned DNA fragment could be involved with the expression of secondary glucose uptake in *Z. mobilis* was ruled out because the functional 3.3-kb fragment subcloned in the pZY507 vector did not complement *E. coli* ZCL11LΔpts for glucose uptake as it did strain ZCL11LΔpts/*glf* (53; data not shown). Additionally, plasmid pLAFR5*glf* (Table 1) was transferred to *Z. mobilis* CU1Rif2 and found to be negative in functional tests, indicating that *glf* does not restore the phenotype of CU1Rif2.

A computer search for similar sequences deposited in the database at the EMBL-Heidelberg website (13a) yielded no information on the functions of the proteins. No sequence similarities between any of the four ORFs and cloned DNA fragments encoding glucose transporters (33) or other outer membrane proteins associated with glucose uptake (39, 55) were identified. However, the search revealed a set of proteins from other species highly homologous to the products of ORF 1, ORF 2, and ORF 4. ORF 1 and ORF 2 show the strongest homology to a *Rhodobacter capsulatus* ORF upstream of the *nifR3* nitrogen regulatory gene (15). The ORF 1 product shows 40% sequence identity over 137 residues (positions 10 to 146 of the *R. capsulatus* ORF), and the ORF 2 product shows 59% sequence identity over 160 residues (positions 226 to 376 of the *R. capsulatus* ORF). In addition, ORF 2 has 50 to 54% sequence identity with a set of sequences homologous to the C-terminal half (positions 226 to 376) of the *R. capsulatus* ORF, including a 158-amino-acid ORF immediately upstream of the *gltX* glutamyl-tRNA synthetase gene in *Bacillus subtilis* (16) and a 159-amino-acid ORF in the region upstream of a cluster of four genes involved in stationary-phase survival in *E. coli* (26). No significant homology to known sequences was detected for the ORF 3 product. Finally, the ORF 4 product displays 40 to 46% sequence identity over its last 150 to 170 residues with an ORF of 160 to 165 nucleotides identified upstream of the homologous *recA* DNA recombination gene in *E. coli* (13), *Enterobacter agglomerans*, and *Pseudomonas putida*. A similar degree of homology is found between ORF 2 and an ORF of 177 amino acids located upstream of the *cheA*, *cheW*, and *cheY* chemotactic factor genes in *Thermotoga maritima* (49); interestingly, *cheY* is often encountered upstream of a *recA* gene (8). The same region of ORF 2 has 33 to 35% sequence identity with the C-terminal half of an ORF (*cinA*) encoding a putative competence-damage protein in *Streptococ-*

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*cus pneumoniae* or *B. subtilis*; *cinA* is also located upstream of the *recA* gene in these gram-positive bacteria (30).

In conclusion, a new phenomenon has been observed: the impaired adaptation of a mutant to growth on a high glucose concentration appears to be related, surprisingly, to the delayed production of a presumably proteinaceous and diffusible factor produced in response to the high glucose concentration. The factor may be related to the as-yet-unexplained mechanism of adaptation of the wild-type strain to growth on high glucose. In keeping with the knowledge that sorbitol does not accumulate during growth on high glucose (in contrast to growth on sucrose), the mutant has been shown not to be defective in GFOR (data not shown), and changes in other functions, such as glucose transport and GLK activity, are no more than suggested. A cluster of genes of unknown function, with similarity only to unassigned sequences in other organisms, complements in low copy numbers the various phenotypes of the mutant. Hence, analysis of these genes may reveal the mutant lesion and mechanism of adaptation.

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