Glycerol utilization by *Rhizobium leguminosarum* requires an ABC transporter and affects competition for nodulation

Hao Ding,1 Cynthia B. Yip,1 Barney A. Geddes,2 Ivan J. Oresnik2 and Michael F. Hynes1

Correspondence
Michael F. Hynes
hynes@ucalgary.ca

1Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, AB T2N 1N4, Canada
2Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

Plasmid curing has shown that the ability to use glycerol as a carbon source is plasmid-encoded in *Rhizobium leguminosarum*. We isolated the locus responsible for glycerol utilization from plasmid pRleVF39c in *R. leguminosarum* bv. *viciae* VF39. This region was analyzed by DNA sequencing and mutagenesis. The locus encompasses a gene encoding GlpR (a DeoR regulator), genes encoding an ABC transporter, and genes *glpK* and *glpD*, encoding a kinase and dehydrogenase, respectively. All the genes except the regulatory gene *glpR* were organized into a single operon, and were required for growth on glycerol. The *glp* operon was strongly induced by both glycerol and glycerol 3-phosphate, as well as by pea seed exudate. GlpR repressed the operon in the absence of inducer. Mutation of genes encoding the ABC transporter abolished all transport of glycerol in transport assays using radiolabelled glycerol. This confirms that, unlike in other organisms such as *Escherichia coli* and *Pseudomonas aeruginosa*, which use facilitated diffusion, glycerol uptake occurs by an active process in *R. leguminosarum*. Since the *glp* locus is highly conserved in all sequenced *R. leguminosarum* and *Rhizobium etli* strains, as well as in *Sinorhizobium* spp. and *Agrobacterium* spp. and other alphaproteobacteria, this process for glycerol uptake is probably widespread. Mutants unable to use glycerol were deficient in competitiveness for nodulation of peas compared with the wild-type, suggesting that glycerol catabolism confers an advantage upon the bacterium in the rhizosphere or in the infection thread.

**INTRODUCTION**

Rhizobia are agriculturally important bacteria that are capable of forming nitrogen-fixing nodules on roots of leguminous plants, such as peas, beans, clovers and lentils. The genes required for nodulation and nitrogen fixation are often found on symbiotic plasmids (pSym) or on symbiosis islands. In addition to the pSym, members of the genus *Rhizobium* usually have additional plasmids carrying genes for traits such as bacteriocin production, lipopolysaccharide synthesis, exopolysaccharide synthesis, plasmid transfer, utilization of different carbon sources, and synthesis or utilization of specific vitamins (Baldani et al., 1992; Brom et al., 1992, 2000; Finan et al., 1986; Hynes & McGregor, 1990; Phillips et al., 1998; Streit et al., 1996; Yost et al., 2006). Many of these plasmid-encoded genes have been shown to confer advantages for bacterial survival in the rhizosphere and for competition for nodule occupancy.

Glycerol is one of the most universal carbon sources for both fast-growing and slow-growing rhizobia (Arias & Martínez-Drets, 1976). The biochemical pathways of glycerol metabolism in rhizobia have been well studied. In *Rhizobium*, glycerol is first converted to L-α-glycerolphosphate (glycerol 3-phosphate or G3P) by glycerol kinase (EC 2.7.1.30) and then oxidized by glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) to dihydroxyacetone phosphate (DHAP), which is able to enter glycolysis. However, the mechanism of glycerol uptake by *Rhizobium* has not been reported in the literature.

In *Escherichia coli*, glycerol uptake is mediated by glycerol facilitator (GlpF)-catalysed facilitated diffusion (Sweet et al., 1990). Once inside the cell, glycerol is immediately phosphorylated by glycerol kinase and trapped inside the cell as G3P. The same type of glycerol transport system has also been found in a variety of other Gram-negative and Gram-positive bacteria, such as *Pseudomonas aeruginosa*.
In this paper, we describe a plasmid-borne operon on pRleVF39c that is responsible for active glycerol uptake and metabolism. The expression of the glycerol operon is inducible by glycerol or G3P, and is negatively regulated by GlpR, a self-regulated DeoR-type transcription regulator. We demonstrate for the first time that in *Rhizobium leguminosarum* bv. *viciae*, glycerol uptake is actively mediated by a putative ATP-binding cassette transporter rather than a glycerol facilitator as in *E. coli* or *Pseudomonas*. Bioinformatic analysis revealed that this type of glycerol transporter is conserved among alphaproteobacteria. We also show that the presence of the glycerol operon enhances the nodulation competition ability of the rhizobia on its host plant.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in the study are listed in Table 1. *R. leguminosarum* strains were grown at 30 °C on TY as a complex medium (Beringer, 1974), or on Robertsen’s minimal medium (RMM) as a defined medium (Robertsen et al., 1981). Mannitol, glycerol or G3P (or a combination of two carbon sources) was used as a carbon source in RMM, as needed for the experiments. Carbon sources were filter-sterilized and added to RMM to a final concentration of 0.2 % (w/v and v/v for mannitol and glycerol, respectively). *E. coli* strains were grown at 37 °C on LB medium (Sambrook et al., 1989). When required, *R. leguminosarum* strains were grown at the following antibiotic concentrations: 600 μg streptomycin (Sm) ml⁻¹, 200 μg tetracycline (Tc) ml⁻¹, 10 μg neomycin (Nm) ml⁻¹ and 30 μg gentamicin (Gm) ml⁻¹. *E. coli* strains were grown at the following antibiotic concentrations: 10 μg Tc ml⁻¹, 50 μg Km ml⁻¹ and 15 μg Gm ml⁻¹.

**DNA manipulations and sequencing.** Standard techniques were used for DNA manipulations (Sambrook et al., 1989). Plasmids were isolated using an EZ-10 Spin Column Plasmid DNA MiniPreps kit (Bio Basic). Total DNA was isolated using mi-Bacterial Genomic DNA Isolation kit (Metabion). Primers were synthesized by Sigma-Genosys (Sigma-Aldrich). PCR reactions were set up using either a Taq PCR Master Mix kit (Qiagen) or Phusion High-Fidelity DNA Polymerase (New England Biolabs) based on experimental requirements, and used according to manufacturers’ instructions. PCR reactions were carried out using a MultiGene II thermal cycler (Labnet International). Restriction endonucleases were purchased from Invitrogen or Fermentas and were used according to the manufacturers’ specifications. DNA fragments were isolated from agarose gels using a QIAquick Gel Extraction kit (Qiagen). Southern hybridizations were performed using the DIG labelling system supplied by Roche Applied Science. The VF39SM whole-genome shotgun sequencing was performed by the National Research Council of Canada, Plant Biotechnology Institute (Saskatoon, SK) on a 454 GS FLX sequencer with Titanium chemistry. Sanger sequencing was performed by Quintara Biosciences.

**Mutagenesis of glycerol utilization genes.** In vitro transposition reactions were carried out with cosmid pCos138 (which is unable to complement the inability of LRS393401 to use glycerol as the sole carbon source) using an EZ-Tn5 <Kan-2> Insertion kit (Epipcent Biotechnologies), followed by transforming *E. coli* S17.1 competent cells. EZ-Tn5 insertion sites were determined by sequencing using EZ-Tn5 internal primers. pCos138 derivatives with EZ-Tn5 inserted within glpS, glpT, glpV and glpKup (intergenic region between glpV and glpK) were individually selected and mobilized into VF39SM. Plasmid pHH1, which is incompatible with pCos138, was then introduced to each VF39SM carrying pCos138::EZ-Tn5, forcing the gene replacement via homologous recombination, as described in Ruvkun & Ausbel (1981). Correct gene replacement was confirmed by Southern hybridization analysis or PCR.

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The construction of mutants VF39SMglpR, VF39SMglpD, VF39SMglpK and VF39SMglpKch was accomplished by the use of pQ200SK or pQ200mp18 (Quandt & Hynes, 1993). For glpR, a 1.7 kb BanHI/Sall fragment containing the entire glpR gene was subcloned from pCos138 to pQ200SK, followed by the insertion of a Km-resistance cassette from pBSL99 into the SstI site within the glpR gene. For glpD, a 2.2 kb Apal/SrfI fragment containing the glpD gene was subcloned from pCos138 into pQ200SK, followed by the insertion of the Km cassette from pBSL99 (Alexeyev et al., 1995) into the Xmal site within the glpD gene. For glpK, a 2.4 kb Psfl fragment was subcloned from pCos138 into pQ200mp18, followed by the insertion of the Km-resistance cassette into the Xhol site within the glpK gene. The chromosomal glpK (glpKch) gene was PCR-amplified and then cloned into pQ200SK using restriction sites (Xmal/SstI) that were incorporated within the PCR primers. A Tc-resistance cassette (Alexeyev et al., 1995; Prentki & Krisch, 1984) was inserted into the Xhol site within the glpKch gene. The mutated genes were introduced into the genome of VF39SM by homologous recombination, as described by Quandt & Hynes (1993). For generating the double mutant VF39SMglt/p/glKch, each mutated gene copy was sequentially introduced into VF39SM. Southern hybridization or PCR was done for each gene to confirm the gene replacement.

**RNA methods.** Total RNA was extracted from VF39SM, grown in RMM with glycerol as sole carbon source for 48 h, using the Ambion RiboPure-Bacteria kit (Applied Biosystems). cDNA synthesis was accomplished by reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions using specific primers instead of the kit-supplied primer mixture (see Supplementary Table S1 available with the online version of this paper for primer sequences).

**Construction of gene fusions and β-glucuronidase (gusA) assay.** To construct gene fusions, PCR was performed using specific primers targeting the promoter regions of glpR (PglpR) and glpD (PglpD) (Table S1). Restriction sites for subsequent cloning were designed and added to the 5′ end of each primer. The resulting PCR fragment was directionally cloned into pFUS1, and confirmed by DNA sequencing. A 1.9 kb BanHI/SphI fragment containing the glpR promoter, the entire glpR gene and the glpD promoter was also cloned into pFUS1, to construct pFUS1::Pglp. A graphic representation of the location of each promoter cloned upstream of *gusA* is shown in Fig. 1 as solid arrows, with the arrow pointing towards the direction of transcription.

All transcriptional fusion constructs were mobilized into *Rhizobium* by conjugation. The wild-type and mutant strains carrying the fusion constructs were grown in minimal medium supplemented with different carbon sources. The β-glucuronidase assay was performed as described by Yost et al. (2004).

Pea seed exudate was prepared as described elsewhere (Mulligan & Long, 1985), with slight modifications. Briefly, 50 pea seeds were surface-sterilized and soaked in 200 ml sterile distilled water with gentle shaking at 4 °C. Pea seed exudates were collected after 12 h and filter-sterilized. A final 1:10 dilution was used in RMM as sole carbon source.

**Glycerol transport assay.** Transport assays were carried out as previously described (Geddes et al., 2010; Poysti & Oresnik, 2007).
Sugar uptake was measured using $[^1-14]C$ glycerol (5500 MBq mmol$^{-2}$, American Radiolabeled Chemicals). Briefly, cells were grown in defined media containing either 15 mM mannitol, or 15 mM glycerol and 15 mM mannitol. Cells were washed twice and resuspended to a final OD$$_{600}$ of 0.1–0.3 in defined media containing 15 mM mannitol. To initiate the assay, $[^14]C$ glycerol was added to the samples to a final concentration of 2 mM. Aliquots of 0.5 ml were withdrawn immediately and at 20 s intervals up to 80 s. They were rapidly filtered through a Millipore 0.45 µm HV filter on a Millipore sampling manifold. The amount of radioactivity retained by the cells on the filters was measured with a liquid scintillation counter (Beckman LS6500). Uptake was normalized to total cell protein using a Lowry protein assay (Stoscheck, 1990). Rates were generally linear for 40 s before showing typical saturation kinetics.

Glycerol kinase assay. Cultures were first grown overnight in TY and then used to inoculate a fresh broth culture of defined medium containing 0.4 % mannitol. Cells were incubated for 72 h.

### Table 1. Bacterial strains and plasmids used in this study and their relevant characteristics

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5x</td>
<td>endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1 (argF-lacZYA), U169, $\phi$80dlacZ $\Delta$M15</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S1.7</td>
<td>Sp', RP4 tra region, mobilizer strain</td>
<td>Simon <em>et al.</em> (1983)</td>
</tr>
<tr>
<td><strong>R. leguminosarum strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VF39SM</td>
<td>Biovar vicieae, Sm', containing six plasmids from pRleVF39a to f</td>
<td>Priefer (1989)</td>
</tr>
<tr>
<td>LRS393401</td>
<td>VF39 derivative, with both pRleVF39c and pRleVF39d cured</td>
<td>Schlüter <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>VF39SM$^{glpR}$</td>
<td>Km cassette from pBSL99 inserted within $glpR$ gene</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpD}$</td>
<td>Km cassette from pBSL99 inserted within $glpD$ gene</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpK}$</td>
<td>Km cassette from pBSL99 inserted within $glpK$ gene</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpS}$</td>
<td>EZTn5 insertion in $glpV$ and $glpK$ intergenic region</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpT}$</td>
<td>EZTn5 insertion in $glpT$</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpK}/glpKch}$</td>
<td>Km cassette from pBSL99 inserted within $glpK$, Tc cassette from pBSL193 inserted within $glpKch$</td>
<td>This work</td>
</tr>
<tr>
<td>VF39SM$^{glpKch}$</td>
<td>Tc cassette from pBSL193 inserted into $glpKch$, the chromosomal $glpK$ gene</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pCos138</td>
<td>pRK7813 cosmid from wild-type VF39SM cosmid bank, complements LRS393401 for glycerol utilization</td>
<td></td>
</tr>
<tr>
<td>pFus1</td>
<td>pMP220 derivative with promoterless gusA, Tc'</td>
<td>Reeve <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td>Suicide vector, Gm'</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pBlueScript II SK</td>
<td>Cloning vector, Ap'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSL199</td>
<td>Contains Km-resistance cassette</td>
<td>Alexeyev <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>pH11J</td>
<td>IncP plasmid, Gm'</td>
<td>Beringer <em>et al.</em> (1978)</td>
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<td>pFus1::$P^{glpR}$</td>
<td>Putative promoter region of $glpR$ directionally cloned in pFUS1</td>
<td>This work</td>
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<tr>
<td>pFus1::$P^{glpD}$</td>
<td>Putative promoter region of $glpD$ directionally cloned in pFUS1</td>
<td>This work</td>
</tr>
<tr>
<td>pFus1::$P^{glp}$</td>
<td>Region spanning from $glpR$ promoter to $glpD$ promoter including intact $glpR$, directionally cloned in pFUS1</td>
<td>This work</td>
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</table>

Illustration:

**Fig. 1.** The glycerol catabolic region in *R. leguminosarum* bv. *vicieae* VF39SM, including the $glp$ operon (white arrows) and the upstream regulatory gene $glpR$. The triangles above the genes represent insertions of either a Km cassette (black) or EZTn5$^{kan2}$ (white). The growth of each corresponding mutant in RMM with glycerol as sole source of carbon is indicated as ‘+’, ‘−’, and ‘Δ’, representing wild-type level growth, no growth, and reduced growth (see text for details). The solid arrows represent different promoter regions cloned in pFUS1 for gene expression studies.

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cultures were additionally supplemented with 0.2 % glycerol after 48 h. Cell-free extracts were prepared by using 50 ml culture and centrifuging at 4000 r.p.m. for 10 min. Cells were then washed in 1 M NaCl and subsequently in 1 × PBS, pH 7.4. The washed pellets were suspended in 500 μl 1 × PBS (pH 7.4), supplemented with 1 mM β-mercaptoethanol, and disrupted by ultrasonic treatment (Ultrasonic Cell Disruptor XL2000, Microson) at 4 °C (kept on ice, 10 × 10 s treatments with intensity gradation 12). The mixture was centrifuged at 10 000 g for 2 min and the supernatant was collected and kept on ice.

Glycerol kinase activity was determined by measuring the reduction of NAD⁺ in the presence of added glycerol phosphate dehydrogenase (Arias & Martinez-Drets, 1976; Freedberg & Lin, 1973). The assay mixture contained (final volume 3.0 ml): ATP, 2.5 μmol; glycerol, 30 μmol; NAD⁺, 1.0 μmol, hydrazine monohydrate (pH 9.8), 3120 μmol; glycine-MgCl₂ buffer (pH 9.8), 600 μmol and 6 μmol, respectively, x-glycerol-3-phosphate dehydrogenase from rabbit muscle (Sigma), 12 μg protein; and cell extract, 200 μl. The reaction began with the addition of the cell extract to all the other reagents, and readings were taken every 10 s for 5 min at 340 nm in a UV spectrophotometer. Enzyme activity was normalized by determining total cell protein using a Lowry protein assay (Stoscheck, 1990).

**RESULTS**

**Nodulation competition test.** Nodulation competition tests were performed as described by Yost et al. (2006). Briefly, pea seeds (Pisum sativum cv. Trapper) were surface-sterilized and germinated on water agar plates in the dark for 3 days. The germinated seedlings were then transferred to modified magenta jars (two seedlings per jar) mimicking Leonard jars containing sterile vermiculite as a solid support, and a 200 ml reservoir of nitrogen-free Hoagland's plant medium (Hoagland & Arnon, 1938). Rhizobia were inoculated onto the seedlings at concentrations between 10⁸ and 10⁹ bacteria per magenta jar. A mixture of two strains was prepared in an approximate ratio of 1:1 based on optical density. The actual numbers of each strain in the inoculum were determined by plate counts. Plants were harvested after 4 weeks, and nodules were collected for analysis. Nodules were crushed and their contents spotted on media with and without Nm in order to differentiate between the two competing strains. The recovery ratio was calculated from plate counts on the two different media. Chi-square tests were performed for each competition with P set at 0.001.

**Glycerol utilization genes in R. leguminosarum VF39SM**

In *R. leguminosarum* bv. *viciae* VF39SM, the third-smallest plasmid, pReVF39c (~370 kb), was previously demonstrated to be required for utilization of glycerol as the sole carbon source (Yost et al., 2006). In the closely related strain *R. leguminosarum* bv. *viciae* 3841, whose genome has been completely sequenced (Young et al., 2006), a putative glycerol utilization locus is present on pRL9J1 (~350 kb), the analogous plasmid to pReVF39c. In the VF39SM genome sequencing project, which has generated a draft sequence with approximately 30-fold coverage, we identified a contig containing a region with 99 % nucleotide sequence identity to the putative glycerol utilization locus on pRL9J1. The sequence containing the putative glycerol utilization locus has been deposited in Genbank (accession no. JN390944). As shown in Fig. 1, the putative glycerol locus contains genes encoding putative glycerol catabolic functions, including *glpD*, encoding glycerol-3-phosphate dehydrogenase, and *glpK*, encoding glycerol kinase. In addition, there are six genes (*glpS, T, P, Q, U* and *V*) between *glpD* and *glpK* that encode putative ABC transporter components. All of these genes are in the same transcriptional orientation. A putative DeoR-type transcription regulatory gene is located immediately upstream of *glpD*. Southern hybridization using both *glpD* and *glpK* internal probes located the glycerol utilization locus to plasmid pReVF39c (data not shown), consistent with our previous observation that plasmid pReVF39c-cured VF39SM derivatives are not able to grow on defined medium using glycerol as sole source of carbon (Yost et al., 2006).

Since this type of glycerol utilization locus seems different from what has been reported for glycerol catabolism in *E. coli*, *P. aeruginosa*, *S. clavuligerus* and *B. subtilis*, we wished to establish how widespread was this arrangement of glycerol catabolism-related genes. Bioinformatic searches revealed that this variety of glycerol locus was well conserved among members of the family *Rhizobiales*. It is found on plasmids pRetCFN42b, pRL132504 and pRLG204 in *Rhzobium etli* CFN42, and *R. leguminosarum* bv. * trifolii* strains WSM1325 and WSM2304, respectively. In *Agrobacterium tumefaciens* C58, the locus is on the linear chromosome, whereas in *Mesorhizobium loti* MAF303099 and *Chelatovarans* sp. BNC1 it is located on the main chromosome. The conservation also extended to some other members of the alphaproteobacteria, such as *Azospirillum* sp. B510 (plasmid pAB510e), *Hoeflea pottophtropha* DFL-43 (chromosome), *Rhodopseudomonas palustris* BioA53 (chromosome), and *Roseobacter* sp. AzwK-36 (chromosome) (Fig. S1). Interestingly, members of the genera *Sinorhizobium* and *Bradyrhizobium* have similar glycerol utilization loci, including *glpD* and genes encoding the putative ABC transporter, but lack the *glpK* gene in the vicinity.

Based on the fact that all genes from *glpD* to *glpK* are in the same orientation, and the close proximity of start and stop codons of each consecutive gene, we predicted that these genes were within the same operon. To test this hypothesis, transcript analysis was performed. Total RNA was isolated from a VF39SM culture that had been grown on RMM with glycerol for 48 h. Reverse transcription was performed using *glpK* reverse primer (Table S1). The reaction mixture was then subjected to PCR, using primers targeting *glpK* and the upstream genes *glpV, glpQ, glpT, glpD* and *glpR*. Amplification of upstream genes was detected for *glpK, glpV, glpQ, glpT and glpD* but not *glpR*, confirming the operon structure from *glpD* to *glpK*. To exclude the possibility that cDNA synthesis from the *glpK* reverse primer might not extend to *glpR*, thus leading to no template for *glpR* amplification, we also used the *glpD* reverse primer to carry out reverse transcription followed by PCR using *glpR* internal primers. It was confirmed that *glpR* was outside the *glp* operon. The transcript analysis implies that any mutation within the *glp* operon would
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have a polar effect on the downstream genes within the glp operon, but that mutations in glpR would not affect glycerol utilization.

To characterize the function of this putative glycerol utilization locus on VF39SM, we isolated two cosmids by hybridizing a glpK internal probe to a VF39SM genomic cosmid library (Yost et al., 1998). One of the cosmids, pCos138, was able to complement strain LRS393401 (VF39SM cured of both pRleVF39c and pRleVF39d) (Schlueter et al., 1997) for its inability to use glycerol as sole carbon source. To delineate the region responsible for glycerol utilization, in vitro transposon mutagenesis was carried out on pCos138. The resulting mutants were individually isolated, mobilized into LRS393401, and tested for their ability to allow LRS393401 to grow in RMM with glycerol as carbon source. It was found that when EZTn5 was inserted within the region spanning from glpS to glpV, the resulting cosmid was unable to restore LRS393401 glycerol catabolism. Interestingly, when the EZTn5 was inserted in the intergenic region between glpV and glpK, the resulting cosmid allowed very limited growth of LRS393401in RMM with glycerol. When EZTn5 was inserted upstream of glpD or downstream of glpK, the resulting mutated cosmids were able to restore the ability of LRS393401 to grow on RMM with glycerol as sole source of carbon (Fig. 1). Each of these mutated cosmids was then introduced into VF39SM, and recombination of the mutated locus into pRleVF39c was selected for using the method of Ruvkun & Ausubel (1981). The resulting homogenotized mutants were tested for their glycerol utilization abilities. It was found that none of the strains VF39SMglpS, VF39SMglpT and VF39SMglpV was able to use glycerol as sole source of carbon. VF39SMglpKup (with EZTn5 inserted within the intergenic region between glpV and glpK) was able to grow in RMM with glycerol but at a significantly slower rate, with an OD600 about 15 % of that of VF39SM after 300 h. This is consistent with the observation that pCos138 with EZTn5 inserted in the glpV–glpK intergenic region was capable of supporting limited growth of LRS393401 in RMM with glycerol. When EZTn5 was inserted in orf13 or orf14, the resulting mutants were able to utilize glycerol.

Since none of the EZTn5 mutants that we screened had lesions within glpR, glpD or glpK, pIJ2005K-based mutagenesis (Quandt & Hynes, 1993) was carried out using versions of these genes interrupted by antibiotic-resistance cassettes. The resulting mutant VF39SMglpD was unable to utilize glycerol, whereas VF39SMglpK was found to be the same as VF39SMglpKup, which was able to grow on glycerol as sole carbon source but at a much slower rate. This could be explained by the presence of a chromosomal copy of glpK in VF39SM, an orthologue of RL3568 in R. leguminosarum 3841. Both VF39SMglpKup and VF39SMglpK would have unaffected glpDSTPQUV genes; therefore, an extra copy of glpK might be able to restore some of the kinase activity. To verify this possibility, we constructed a VF39SMglpK/glpKch double mutant, and determined that it was completely unable to grow on glycerol, confirming that the chromosomal glpK product had at least some glycerol kinase activity. The growth of a single mutant in glpKch on glycerol was virtually indistinguishable from that of the wild-type VF39SM, indicating that the glpK gene in the glpD operon on pRleVF39c was the major kinase contributing to the ability to grow on glycerol. VF39SMglpR was found to be able to utilize glycerol as sole source of carbon, consistent with our transcript analysis data that confirmed that glpR is outside the glpD operon.

Glycerol kinase activities

To confirm the activity of a chromosomal gene encoding a putative glycerol kinase (glpKch, orthologous to RL3586 in strain 3841), we carried out glycerol kinase assays on cell extracts prepared from VF39SM, VF39glpK, VF39glpKch and the double mutant VF39glpK/glpKch. In multiple assays, glycerol kinase activities were detected for wild-type VF39SM and the glpK mutant, though the results were variable depending on media and growth conditions. In RMM with mannitol, VF39SM gave a specific activity of 0.0241 ± 0.0050 μmol min⁻¹ mg⁻¹, whereas under inducing conditions (addition of glycerol) the activity was 0.0311 ± 0.0054. The glpK mutant gave uninduced and induced activities of 0.0146 ± 0.0064 and 0.0163 ± 0.0035 μmol min⁻¹ mg⁻¹, respectively. There was no background or inducible activity in the double mutant, supporting the observation that both glpK–encoding genes were functional. However, surprisingly, the glpKch mutant had extremely low levels of glycerol kinase activity (below 10 % of wild-type VF39SM) under all conditions tested.

Expression of the glp operon can be induced by glycerol, G3P or pea seed exudates

To study the regulation of glp operon expression, we constructed a transcriptional fusion by cloning the glp operon (PglpD) into pFUS1 to drive the expression of a promoterless gusA gene, encoding β-glucuronidase (Reeve et al., 1999). β-Glucuronidase assays showed that in VF39SM, the expression of the glp operon in RMM with glycerol was about 5.5-fold that in RMM with mannitol (significant, P<0.0001, Student’s t test) (Fig. 2a), suggesting that the glp operon is inducible by glycerol. Since mannitol is the most commonly used carbon source in Rhizobium cultivation (Stowers, 1985), it was of interest to examine whether glycerol utilization was subject to catabolic repression by mannitol. To test this, VF39SM was grown in RMM with a mixture of both mannitol and glycerol. The expression of the glp operon was found to be about three times higher than in RMM with mannitol alone (significant, P<0.0001, Student’s t test), indicating that the expression of the glp operon is not subject to strong catabolic repression by mannitol, although the expression values did remain somewhat lower than with glycerol alone.

In E. coli, glycerol transport and utilization is inducible by both glycerol and G3P (Beijer et al., 1993; Sanno et al.,
To investigate whether G3P also induces glycerol transport and utilization in *Rhizobium*, we measured the expression of the *glp* operon in VF39SM when growing in RMM with mannitol (0.1%, w/v) and DL-α-glycerolphosphate (0.4%, w/v). It was found that the expression of the *glp* operon was about 3.4-fold higher in RMM with mannitol (0.1%, w/v) and DL-α-glycerolphosphate (0.2%, w/v) than in RMM with mannitol alone (Fig. 2a) (significant, *P* < 0.001, Student’s *t* test). These results suggest that G3P is able to induce transcription of the *glp* operon.

When pea (*P. sativum* cv. Trapper) seed exudate was used as sole carbon source in RMM, the expression of the *glp* operon was found to be about 5.5-fold higher than in RMM with mannitol (Fig. 2a; significant, *P* < 0.001, Student’s *t* test). This is not surprising, since glycerol has been reported to be present in legume seed exudates (Nelson, 2004; Zheng & Kawabata, 2000). However, we could not rule out the possibility that substances other than glycerol or G3P in seed exudates could induce the operon.

**GlpR is a self-regulated transcriptional repressor of the *glp* operon**

In *R. leguminosarum* bv. *viciae* 3841, the gene (pRL90073) immediately upstream of the *glp* operon was annotated as a DeoR-type transcriptional regulator. DeoR-type transcriptional regulators are often found to be the negative regulators for uptake and metabolism of sugars, and usually control the transcription of the neighbouring genes (Gaigalat *et al.*, 2007). To investigate the potential regulatory function of GlpR on the *glp* operon, we made an insertional mutation in the *glpR* gene and tested its effect on *glp* operon expression. As shown in Fig. 2(a), in the VF39SM *glpR* background, when GlpR was disrupted, the *glp* operon was constitutively expressed in RMM at high levels (about six times the expression of the *glp* operon in wild-type VF39SM in RMM with mannitol), regardless of the carbon source used, i.e. mannitol or glycerol. This suggested that GlpR was acting as a transcriptional repressor of the *glp* operon, and that the induction of *glp* by glycerol was likely to be a result of derepression of GlpR.

Regarding the expression of *glpR* itself, we constructed a transcriptional fusion of the putative *glpR* promoter region (PglpR) to the promoterless *gusA* gene in pFUS1 (Fig. 1). The expression of *glpR* was found to be inducible by glycerol (Fig. 2b). In the absence of GlpR (i.e. in VF39SMpglpR) and glycerol, the expression of *glpR* in RMM with mannitol increased to almost the same level as the expression level in the presence of glycerol. Therefore, the *glpR* gene seems to be negatively self-regulated. Similarly to the *glp* operon, the induction of *glpR* by glycerol is likely a result of derepression of GlpR.

Since GlpR was found to act as a transcriptional repressor and we measured the expression of *glp* in the multi-copy pFUS1 plasmid, a titration effect would be expected during...
induction/derepression by glycerol. To address this, we constructed pFUS1::Pglp (Fig. 1) by cloning the whole region spanning from the glpR promoter region to the middle of the glpD gene. Therefore, pFUS1::Pglp provided the same glpR gene copy number as the glp operon promoter. In this construct, gusA gene expression is still under the control of the glpD promoter. The expression of pFUS1::PglpD and pFUS1::Pglp was then measured in RMM with 0.1 % mannitol with decreasing concentrations of glycerol (Fig. 2c). At glycerol concentrations of 0.1 and 0.02 %, v/v, similarly high levels of pFUS1::PglpD and pFUS1::Pglp expression were observed. This suggests that 0.02 % glycerol was sufficient to support derepression in the cell, and hence there was no titration effect. However, when the glycerol concentration was decreased to 0.001 % the titration effect became evident. When the repressor gene glpR and glp promoter were at the same copy number, 0.001 % glycerol was not sufficient to derepress the GlpR in the cell, resulting in a very low expression level. In contrast, when there was a single copy of the glpR gene and multiple copies of the glp promoter (in pFUS1::PglpD), the same amount of glycerol was able to derepress all the GlpR in the cell, resulting in a fivefold increase in expression (significant, P=0.002, Student’s t test).

**Transport of glycerol is inducible and is carried out by the ABC transporter**

BLASTX analysis (Altschul et al., 1990) suggested that glpSTPQV encode an ABC-type transporter. Since these genes were embedded within the operon together with glpD and glpK, we suspected that these genes might function in glycerol catabolism and therefore carried out transport assays using [1-14C]glycerol. Wild-type VF39SM was found to be able to take up glycerol from the culture medium, even in the presence of mannitol (Fig. 3a). This was consistent with the expression analysis results showing that glycerol utilization is not subject to catabolic repression by mannitol. Uptake of glycerol was inducible by glycerol.

To determine whether the proteins encoded by glpSTPQUV were responsible for the transport of glycerol, VF39SMglpS, selected as a representative transporter mutant, was shown to be unable to transport glycerol (Fig. 3b), whereas VF39SMglpKup, which carried a mutation downstream of the transporter genes, had transport rates similar to those of VF39SM (data not shown). When pCos138 was introduced into VF39SMglpS, it restored the glycerol transport ability of VF39SMglpS to a level similar to that of the wild-type (Fig. 3b).

**Mutants unable to utilize glycerol are less competitive in nodulation occupancy**

To assess whether the plasmid-borne glycerol catabolic locus affects competition for nodulation occupancy, VF39SMglpD, selected as the representative glycerol utilization mutant, was first determined to be able to form healthy nitrogen-fixing nodules on pea roots, based on the observation that plants inoculated with VF39SMglpD were green and healthy, and indistinguishable from the plants and nodules inoculated by wild-type VF39SM. The number of nodules on plants inoculated with mutants was also not

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**Fig. 3.** Glycerol transport experiments. Strains were grown to mid-exponential phase in defined medium containing either mannitol (open symbols) or mannitol and glycerol (closed symbols). The initial uptake rate was determined using [1-14C]glycerol. Symbols: squares, VF39SM (wild-type); triangles, VF39SMglpS (transporter mutant); diamonds, VF39SMglpS with pCos138 (complemented strain). (a) Comparison of glycerol uptake between VF39SM grown on mannitol and VF39SMglpS with pCos138. (b) Comparison of glycerol uptake of the induced wild-type (VF39SM), transporter mutant (VF39SMglpS) and complemented transporter mutant (VF39SMglpS with pCos138). The data represent the means of three independent experiments; error bars (SEM) smaller than the symbols are not displayed.
significantly different from that of the wild-type (data not shown).

In competition studies, the percentages of wild-type VF39SM of the initial inocula and of all the nodules recovered were determined (Fig. 4). An increase of the VF39SM percentage would indicate decreased nodulation competitiveness of the competitor strain VF39SM glpD. In both trials with different initial inocula, the final percentage of VF39SM recovered from nodules increased (significant, Chi-square test with \(P<0.001\)), indicating that VF39SM glpD was less competitive than VF39SM in nodule occupancy.

**DISCUSSION**

Genes involved in glycerol utilization in *R. leguminosarum* bv. *viciae* VF39SM are located on its third-smallest plasmid, pRleVF39c (Yost et al., 2006). In this paper, we isolated the cosmid pCos138, which contains DNA from pRleVF39c, and is able to restore the ability of LRS393401, lacking pRleVF39c, to utilize glycerol as the sole source of carbon. Sequence analysis revealed that the putative glycerol utilization locus on pRleVF39c was composed of genes encoding a complete pathway for glycerol utilization, including an ABC transporter for glycerol uptake (glpSTPQUV), glycerol kinase (GlpK) and G3P dehydrogenase (GlpD). Mutagenesis studies revealed that all of these genes are required for glycerol utilization in *R. leguminosarum*, except for glpK, whose function could be partially replaced by a chromosomal copy of glpK. The chromosomal glpK gene (glpKch) did not support wild-type growth levels on glycerol, but its activity was confirmed in glycerol kinase assays, where the presence of significant levels of kinase activity in the glpK mutant, plus the absence of any detectable activity in the double mutant, allowed us to conclude that glpKch encodes a functional glycerol kinase. The low levels of activity in the glpKch mutant seem to contradict the growth experiments, which attribute the role of the major glycerol kinase to GlpK rather than GlpKch. The reason for this contradiction remains unclear.

The gusA fusion studies of pFUS1::PglpD showed that the expression of the operon is inducible by both glycerol and G3P, the product immediately downstream from glycerol in the glycerol catabolic pathway (Fig. 3a). The physiological inducer of the operon is most likely G3P, as it is in *E. coli*, since we found that G3P is still able to induce expression of a glpD fusion in a glpK/glpKch double mutant, whereas there was no induction of the glpD fusion by glycerol in this genetic background (data not shown).

It was also interesting to see that the glp operon was inducible when pea seed exudates were used as sole source of carbon, which implied that the glp locus plays a role during the interaction with the plant host, as we have seen in rhamnose and erythritol catabolism (Oresnik et al., 1998; Yost et al., 2006). In nodule occupancy competition assays comparing VF39SM and VF39SM glpD on peas, we showed that the strain deficient in glycerol utilization was less competitive (Fig. 4). However, whether the glp operon is responsible for bacterial competitiveness in nodule occupancy with other host plants remains unknown.

By measuring the expression of pFUS1::PglpD in a VF39SM glpR background (Fig. 2b), we confirmed that GlpR is a transcriptional repressor of the glp operon, consistent with sequence analysis showing that glpR encodes a DeoR-type transcription regulator. A simple mechanism for the glpR-regulated glp operon could then be proposed. In the absence of inducer (glycerol or G3P), the expression of the glp operon is repressed by GlpR, presumably by binding to the operator sequences, thus blocking the transcription initiation of the glp operon. When the inducer becomes available, it interacts with GlpR, allowing the derepression of the glp operon.

Based on current literature, the uptake of glycerol by a variety of bacteria has been reported to be mediated by GlpF-facilitated diffusion. The list of bacteria using this mechanism includes *E. coli*, *P. aeruginosa*, *S. clavuligerus* and *B. subtilis* (Baños et al., 2009; Beijer et al., 1993; Schweizer & Po, 1996; Sweet et al., 1990). In *R. leguminosarum* bv. *viciae* VF39SM, we were not able to identify any glpF orthologues. The most similar gene encoded a putative aquaporin, and the predicted amino acid sequence of this gene was quite clearly much more related to the *E. coli* aquaporin than to GlpF. Transport assays showed that, in VF39SM, glycerol uptake was mediated by an ABC transporter. This is, as far as we are aware, the first reported ABC transporter responsible for

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**Fig. 4.** Nodulation competition assays between VF39SM (wild-type) and VF39SM glpD (mutant unable to utilize glycerol as sole carbon source). VF39SM glpD was co-inoculated with wild-type VF39SM onto pea plant seedlings. Numbers are expressed as the percentage of the wild-type VF39SM. Open bars, percentage of wild-type VF39SM in the inocula as determined by plate counts; filled bars, percentage of nodules occupied by VF39SM as determined by bacterial isolation from mature nodules. Experiments were performed at least three times for each competition; error bars, SEM.
glycerol uptake. Bioinformatic analysis revealed that glycerol utilization loci orthologous to the one studied here are well conserved among members of the alphaproteobacteria and are often found on plasmids. This suggests that ABC transporter-mediated uptake of glycerol takes place in these bacteria as well. This system may have evolved to take advantage of very low concentrations of glycerol present in soil and rhizosphere environments.

In Sinorhizobium and Bradyrhizobium species, the glpK gene is missing at the 3’ end of the glpD operon. In these species, a single copy of glpK is found elsewhere in the genome, which is homologous to glpKch in VF39SM. This is consistent with our finding that glpKch in VF39SM was still able to produce a functional glycerol kinase; presumably, this version of the enzyme takes on the major kinase function involved in glycerol utilization in Sinorhizobium and Bradyrhizobium.

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