Effect of phosphoglycerate mutase and fructose 1,6-bisphosphatase deficiency on symbiotic *Burkholderia phymatum*

Wen-Ming Chen,[1] Jurgen Prell,[2] Euan K. James,[3] Der-Shyan Sheu[4] and Shih-Yi Sheu[4]

Correspondence
Shih-Yi Sheu
sys816@mail.nkmu.edu.tw

[1]Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung City 811, Taiwan, ROC

[2]Soil Ecology, Department of Botany, RWTH Aachen, 52056 Aachen, Germany

[3]The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

[4]Department of Marine Biotechnology, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung City 811, Taiwan, ROC

Burkholderia phymatum STM815 is a β-rhizobial strain that can effectively nodulate several species of the large legume genus *Mimosa*. Two Tn5-induced mutants of this strain, KM16-22 and KM51, failed to form root nodules on *Mimosa pudica*, but still caused root hair deformation, which is one of the early steps of rhizobial infection. Both mutants grew well in a complex medium. However, KM16-22 could not grow on minimal medium unless a sugar and a metabolic intermediate such as pyruvate were provided, and KM51 also could not grow on minimal medium unless a sugar was added. The Tn5-interrupted genes of the mutants showed strong homologies to *pgm*, which encodes 2,3-biphosphoglycerate-dependent phosphoglycerate mutase (dPGM), and *fbp*, which encodes fructose 1,6-bisphosphatase (FBPase). Both enzymes are known to be involved in obligate steps in carbohydrate metabolism. Enzyme assays confirmed that KM16-22 and KM51 had indeed lost dPGM and FBPase activity, respectively, whilst the activities of these enzymes were expressed normally in both free-living bacteria and symbiotic bacteroids of the parental strain STM815. Both mutants recovered their enzyme activity after the introduction of wild-type *pgm* or *fbp* genes, were subsequently able to use carbohydrate as a carbon source, and were able to form root nodules on *M. pudica* and to fix nitrogen as efficiently as the parental strain. We conclude that the enzymes dPGM and FBPase are essential for the formation of a symbiosis with the host plant.

INTRODUCTION

Nitrogen fixation by the legume–rhizobium symbiosis produces a significant amount of the available nitrogen in the biosphere, making it agronomically and ecologically important. Although it was generally accepted that legumes were nodulated exclusively by relatives of *Rhizobium* in the α-proteobacteria, the so-called 'α-rhizobia', over the last 10 years there has been an increasing number of reports of legumes being nodulated by members of the β-proteobacteria (the so-called 'β-rhizobia') (Gyaneshwar *et al.*, 2011). This is particularly true for species in the large genus *Mimosa* in the legume subfamily *Mimosoideae*, most of which are nodulated by β-proteobacteria (Chen *et al.*, 2005; dos Reis *et al.*, 2010).

In common with other α- and β-rhizobia, symbiotic genes (*nifH*, *nodA*, *nodC*) have been identified in *Burkholderia phymatum* STM815 (Gyaneshwar *et al.*, 2011), and these are involved in the formation and functioning of nitrogen-fixing nodules on legumes. Nodulation is a multistep process (van Rhijn & Vanderleyden, 1995; Chen *et al.*, 2003; Gibson *et al.*, 2008; Oldroyd & Downie, 2008), in which rhizobia (α- or β-) in the rhizosphere infect legume roots via root hairs, and are ultimately released into cells of the developing nodule primordium via endocytosis. Inside a nodule cell, the bacteria are enclosed in so called symbiosomes, in which they differentiate into bacteroids (Chen *et al.*, 2003). The C_4-dicarboxylate tricarboxylic acid (TCA) cycle intermediates succinate, fumarate and malate have generally been considered to be the principal carbon

Abbreviations: 2,3-BPGA, 2,3-biphosphoglycerate; dPGM, 2,3-biphosphoglycerate-dependent phosphoglycerate mutase; FBPase, fructose 1,6-bisphosphatase, iPGM, cofactor-independent phosphoglycerate mutase; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; PGM, phosphoglycerate mutase; TCA cycle, tricarboxylic acid cycle.

and energy sources supplied by the plant to bacteroids (Stowers, 1985). It is within the symbiosomes that the bacteria fix nitrogen and convert the fixed nitrogen (ammonia) into a form that can be released easily to the host plant (Brewin, 1991).

The pathways for carbon metabolism outlined in Fig. 1 are based primarily on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for *B. phymatum*. Phosphoglycerate mutase (PGM) is known to be an important enzyme for both glycolysis and gluconeogenesis (Fothergill-Gilmore & Watson, 1989). It catalyses the reversible isomerization of 2-phosphoglycerate (2-PGA) and 3-phosphoglycerate (3-PGA) in glycolysis and gluco-neogenesis, and it can be subdivided into two different types of evolutionarily unrelated enzymes (although both EC 5.4.2.1). The better-documented enzyme is the cofactor-dependent phosphoglycerate mutase (dPGM), due to its requirement for 2,3-biphosphoglycerate (2,3-BPGA). The second enzyme, called cofactor-independent phosphoglycerate mutase (iPGM), is a monomeric protein of ~60 kDa. There is no amino acid sequence similarity between these two types of PGMs, and their structures are also quite different, as are their catalytic mechanisms

(Jedrzejas, 2000). Upon comparative sequence and structure analysis, the former enzyme has been classified as a member of the phospho-histidine acid phosphatase superfamily (Hasemann *et al.*, 1996), and the latter as a member of the metal-dependent alkaline phosphatase superfamily (Galperin & Jedrzejas, 2001). Vertebrates, yeasts and various eubacterial species have only dPGM, whilst archaea, plants and some eubacteria possess only iPGM (Fothergill-Gilmore & Watson, 1989; van der Oost *et al.*, 2002). In addition, a small number of eubacteria appear to possess both enzymes (Fraser *et al.*, 1999).

Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) is an essential regulatory enzyme in gluconeogenesis. It converts fructose 1,6-bisphosphate to fructose 6-phosphate, an important precursor in various biosynthetic pathways. At present, five different classes of FBPases have been proposed based on their amino acid sequences (FBPases I–V) (Donahue *et al.*, 2000; Fraenkel & Horecker, 1965; Jules *et al.*, 2009; Sato *et al.*, 2004). Eukaryotes contain only the FBPase I-type enzyme, but all five types exist in various prokaryotes. Many organisms have more than one FBPase, and the type I FBPase is the most widely distributed among living organisms and is the primary FBPase in most
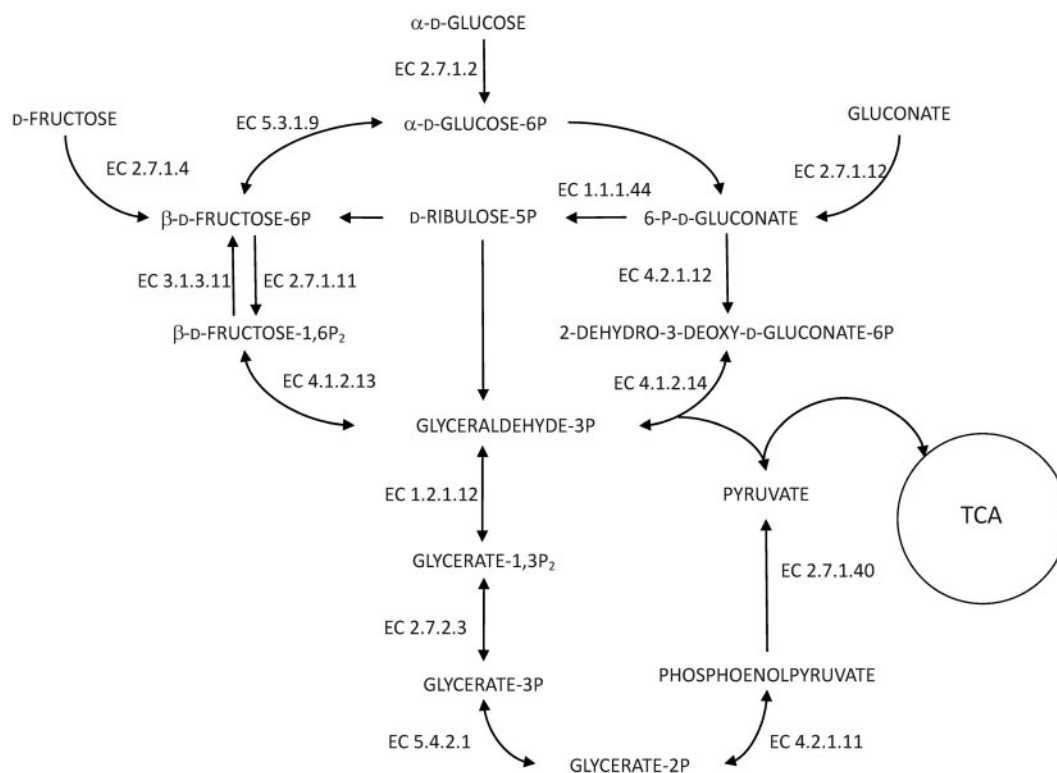

Fig. 1. Glycolytic and gluconeogenic pathway in *B. phymatum*. EC 1.1.1.44, 6-phosphogluconate dehydrogenase; EC 1.2.1.12, glyceraldehyde-3-phosphate dehydrogenase; EC 2.7.1.2, glucokinase; EC 2.7.1.4, fructokinase; EC 2.7.1.11, 6-phosphofructokinase; EC 2.7.1.12, gluconokinase; EC 2.7.1.40, pyruvate kinase; EC 2.7.2.3, phosphoglycerate kinase; EC 3.1.3.11, FBPase; EC 4.1.2.13, fructose 1,6-bisphosphate aldolase; EC 4.1.2.14, 2-dehydro-3-deoxyphosphogluconate aldolase; EC 4.2.1.11, enolase; EC 4.2.1.12, phosphogluconate dehydratase; EC 5.3.1.9, glucose-6-phosphate isomerase; EC 5.4.2.1, phosphoglycerate mutase.

bacteria (e.g. *Escherichia coli*), a few archaea, and all eukaryotes.

In this study, we report that *B. phymatum* mutants KM16-22 and KM51, respectively lacking PGM and FBPase activity because of a transposon insertion, are deficient in nodule formation and nitrogen fixation. These mutants are defective in carbohydrate metabolism, although they grow as efficiently as the wild-type strain on complex media, thus implying that PGM and FBPase are essential for effective symbiosis of *B. phymatum*.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids are listed in Table 1. *B. phymatum* STM815, KM16-22 and KM51 were grown in yeast extract mannitol (YEM) medium (Vincent, 1970) at 28 °C, and *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C. Antibiotics were used at the following final concentrations: chloramphenicol, 34 µg ml^{-1}; tetracycline, 12.5 µg ml^{-1}; kanamycin, 50 µg ml^{-1} and ampicillin, 50 µg ml^{-1}.

Transposon Tn5 mutagenesis. Introduction of a transposon Tn5 into *B. phymatum* STM815 was achieved by triparental mating (Figurski & Helinski, 1979; Matthysse *et al.*, 1996). Equal amounts of the donor *E. coli* S17-1 λ pir with pUTmini-Tn5gfp Matthysse *et al.* (1996), the helper strain *E. coli* HB101 with pRK2013 (Boyer & Roulland-Dussoix, 1969), and the recipient *B. phymatum* STM815 were mixed and spotted onto a nitrocellulose filter. After incubation on YEM plates, the chloramphenicol- and tetracycline-resistant colonies were selected.

Plant tests. *Mimosa pudica* cultivation and nodulation tests were carried out using the tube methods of Gibson (1963) and Somasegaran & Hoben (1994). After germination, the seedlings were inoculated with approximately 10^5 cells of either wild-type *B. phymatum* STM815 or the Tn5-induced mutants. The observation of infection threads was examined by the method of Vasse & Truchet (1984). Nitrogen fixation assays (acetylene-reduction assays) were carried out on plants at 28 days after inoculation according to the method of James & Crawford (1998).

Identification and cloning of the Tn5-interrupted gene. Total DNA from mutant strains was prepared and digested with *Eco*RI. Southern blotting was carried out by standard methodology (Sambrook & Fritsch, 1989) to confirm that the mutant strains were carrying a single copy of Tn5. Genomic DNA was prepared and digested with *Apa*I, and then ligated into pBluescript II SK(+). After transformation of *E. coli* DH5α, tetracycline-resistant colonies were selected. The Tn5-containing plasmids were purified and then subjected to restriction enzyme analysis and DNA sequencing.

Growth studies. Bacterial strains were tested for their ability to utilize various organic compounds. Washed bacterial suspensions (100 µl containing 2×10^9 cells) were placed into 10 ml minimal medium containing 0.05 % $K_2HPO_4.3H_2O$, 0.08 % $MgSO_4.7H_2O$, 0.02 % NaCl, 0.025 % $CaCl_2$, 0.1 % $(NH_4)_2SO_4$ and 0.01 % $FeCl_3.6H_2O$, pH 7.0, supplemented with the test compound(s) at 0.2 % (w/v) (when supplemented with mixture, each compound at 0.1 %). The cultures were incubated at 28 °C for up to 60 h. The OD_{600} values were recorded at intervals. In the 60 h culture period, if the maximum OD_{600} value of bacterial growth reached a value above 0.5 it was defined as positive '+' growth. In contrast, if the OD_{600} value in the same medium was below 0.1 it was defined as negative '−' growth.

PGM activity assay. The PGM activity in cells or in bacteroids was measured. Cells were lysed by sonication, and the lysate was cleared by centrifugation (30 000 *g*, 4 °C, 10 min). Bacteroid extracts were prepared according to the method of Chen *et al.* (2003). PGM activity

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Reference or source
***B. phymatum* strains**		
STM815	Wild-type, Cm^r, *nod*$^+$ on *M. pudica*	Elliott *et al.* (2007); Vandamme *et al.* (2002)
KM16-22	STM815::Tn5, Tc^r	This study
KM16-22/*pgm*	KM16-22 containing pBBR/*pgm*, Tc^r Km^r	This study
KM51	STM815::Tn5, Tc^r	This study
KM51/*fbp*	KM51 containing pBBR/*fbp*, Tc^r Km^r	This study
***E. coli* strains**		
S17-λpir	Containing pUTmini-Tn5gfp	de Lorenzo *et al.* (1990)
HB101	Containing pRK2013	Boyer & Roulland-Dussoix (1969)
DH5α	Transformation strain	Gibco-BRL
BL21(λDE3)	Expression strain	Novagen
Plasmids		
pUTmini-Tn5gfp	Tn5-based delivery plasmid with *gfp* gene, Ap^r Tc^r	Matthysse *et al.* (1996)
pRK2013	Helper plasmid used in triparental conjugation, Km^r	Figurski & Helinski (1979)
pBluescript II SK(+)	Cloning and sequencing vector, Ap^r	Stratagene
pBBR1MCS-2	Broad-host-range cloning vector, Km^r	Kovach *et al.* (1995)
pBBR/*pgm*	pBBR1MCS-2 containing the *pgm* ORF	This study
pBBR/*fbp*	pBBR1MCS-2 containing the *fbp* ORF	This study
pET-23b(+)	Expression vector, Ap^r	Novagen
pET/*pgm*	pET-23b(+) containing the *pgm* ORF	This study

*Abbreviations for antibiotics are as follows: Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin; Ap, ampicillin.

was measured after an overnight dialysis of the cleared cell or bacteroid lysate at 4 °C against 400 vols 10 mM Tris/HCl, pH 8.0, in order to remove potentially interfering metabolites. The activity was then measured by the coupled enzyme assay procedure described in Carreras *et al.* (1980), with some modifications. The reaction was followed upon the addition of 1.5 mM 3-PGA into a 1 ml quartz cuvette containing 50 mM HEPES, pH 7.6, 0.55 U rabbit muscle enolase (Sigma), 1 mM $MgCl_2$ and 50 mM KCl. The assay temperature was 25 °C. The coupling enzyme, enolase, converts the product of the PGM reaction, 2-PGA, to phosphoenopyruvate. The latter was measured by exploiting its UV absorbance at 240 nm (molar absorption coefficient 1310 M^{-1} cm^{-1}; Guerra *et al.*, 2004) using a spectrophotometer. One activity unit (U) is defined as the conversion of one micromole substrate per minute under standard conditions. In order to characterize the type of PGM, the enzyme activity of recombinant PGM protein (see below) was measured in the presence of potentially activating (0.6 mM 2,3-BPGA) or inhibiting compounds (50 μM $NaVO_3$, Sigma).

FBPase assay. FBPase activity was measured in a coupled spectrophotometric assay containing 2 mM $MgCl_2$, 100 mM KCl, 20 mM Tricine, pH 7.7, 0.25 mM $NADP^+$, yeast glucose-6-phosphate dehydrogenase (0.4 U ml^{-1}) and yeast phosphoglucoisomerase (0.7 U ml^{-1}). The assay was started with 0.75 mM fructose 1,6-bisphosphate as described for *E. coli* GlpX (Fraenkel & Horecker, 1965). Fructose 6-phosphate formed by the reaction of FBPase was converted to glucose 6-phosphate and subsequently to 6-phosphogluconate by coupling to phosphoglucoisomerase and glucose 6-dehydrogenase, and the concomitant formation of NADPH ($\varepsilon_{340\ nm}=6.22\ mM^{-1}\ cm^{-1}$) was followed at 340 nm.

Gene complementation. The *pgm* gene was amplified by PCR using Vent (exo−) proof-reading polymerase (New England Biolabs) from *B. phymatum* STM815, using the oligonucleotide primers 5′-ACGT**GG-GCCC**GGAAGATCCGCCGGAAAA-3′ (*pgm-Apa*, forward primer) and 5′-CATG**TCTAGA**TGAAATGAAGGCTGAGCC-3′ (*pgm-Xba*, reverse primer) (bold type indicates restriction sites). After restriction enzyme digestion, *pgm* was cloned into pBBR1MCS-2 (Kovach *et al.*, 1995). The resulting plasmid pBBR/*pgm* was used to transform *B. phymatum* KM16-22 according to the method of Vincze & Bowra (2006). The plasmid pBBR/*fbp* was constructed using the same procedure but with the oligonucleotide primers 5′-ACGT**GGGCCC**CCTGCGTTTTTCCA-GCGA-3′ (*fbp-Apa*, forward primer) and 5′-CATG**TCTAGA**TCAACT-TTTCTTTTCGAGAT-3′ (*fbp-Xba*, reverse primer).

Construction of the bacterial expression system. The *pgm* gene was amplified by PCR from *B. phymatum* STM815 using Vent (exo−) proof-reading polymerase and the oligonucleotide primers 5′-TGACTG**CATATG**TACAAACTCGTTCTC-3′ (*pgm-Nde*, forward primer) and 5′-ACGT**GCGGCCGC**TAAGCCTGCCTTGCCTT-3′ (*pgm-Not*, reverse primer). The amplified gene was digested with NdeI and NotI, and ligated into the corresponding sites of the expression vector pET23b(+), allowing expression of PGM with a six-histidine tag at the C terminus. The resulting plasmid pET/*pgm* was introduced into *E. coli* BL21(λDE3). To purify recombinant PGM, cleared cell lysate from a transformed *E. coli* BL21 culture was loaded onto a His Excellose Spin column (YB Real Proteomics, ECOS), and subsequently washed and eluted.

RESULTS

Isolation of Tn5-induced mutants that cannot form root nodules on *M. pudica*

B. phymatum Tn5 insertion mutants were screened for a symbiotic phenotype by inoculating them onto *M. pudica*.

The parental strain STM815 could induce nitrogen-fixing nodules by 28 days after inoculation (Fig. 2a, left). Two of the Tn5-induced mutants, designated KM16-22 and KM51, had no ability to form root nodules (e.g. KM16-22, which is shown in Fig. 2a, right), even up to 56 days after inoculation. The roots from plants inoculated either with STM815 or with the Tn5-induced mutants were examined by light microscopy at 14 days after inoculation. Roots inoculated with STM815 were clearly becoming nodulated by this time (Fig. 2b), whereas the roots of *M. pudica* inoculated with strains KM16-22 and KM51 and those without bacterial inoculation were not (Fig. 2c–e).

However, in contrast to the uninoculated roots, those inoculated with KM16-22 or KM51 had abundant distorted and curled root hairs (Fig. 2c, d), which suggests that although they had lost their ability to form nodules, both mutants still retained their ability to cause root hair deformation, which is one of the earliest steps in the rhizobial infection of legumes. To examine whether infection threads were formed in these root hairs or not, the roots of *M. pudica* inoculated with either STM815 or the Tn5-induced mutants were stained with methylene blue; infection threads were only detected in plants inoculated with STM815 (data not shown).

Identification of the Tn5-interrupted genes

Cloning and sequencing revealed that mutant KM16-22 carried a single Tn5 insertion in a gene identified as Bphy_0266 (*B. phymatum* STM815 chromosome 1, CP001043) and encoding a 2,3-BPGA-dependent PGM (88–94 % amino acid identity to *Burkholderia* spp., 78–82 % identity to *Cupriavidus/Ralstonia* spp., 66 % identity to *E. coli*, 48–46 % identity to *Rhizobium etli*, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Mesorhizobium loti* and *Bradyrhizobium japonicum*). This putative *pgm* gene is 747 bp long, and encodes a protein of 248 aa. Furthermore, all of the active site residues proposed for *E. coli* in dPGM (Bond *et al.*, 2001) have been conserved in the *B. phymatum* dPGM enzyme.

In mutant KM51, the sequence flanking the Tn5 identified the disrupted gene as Bphy_0685 (*B. phymatum* STM815 chromosome 1, CP001043), which encodes a putative FBPase (85–95 % amino acid identity to *Burkholderia* spp., 72–73 % identity to *Cupriavidus/Ralstonia* spp., 48 % identity to *S. meliloti* and 45 % identity to *B. japonicum*). This putative *fbp* gene is 1017 bp long and encodes a protein of 338 aa.

Tn5-induced mutants are defective in carbohydrate metabolism

PGM and FBPase are known as important enzymes for both glycolysis and gluconeogenesis. In order to elucidate their metabolic ability, the *B. phymatum* mutants KM16-22, KM51 and their parental strain STM815 were cultured on various media (Table 2). Mutant KM16-22 could not
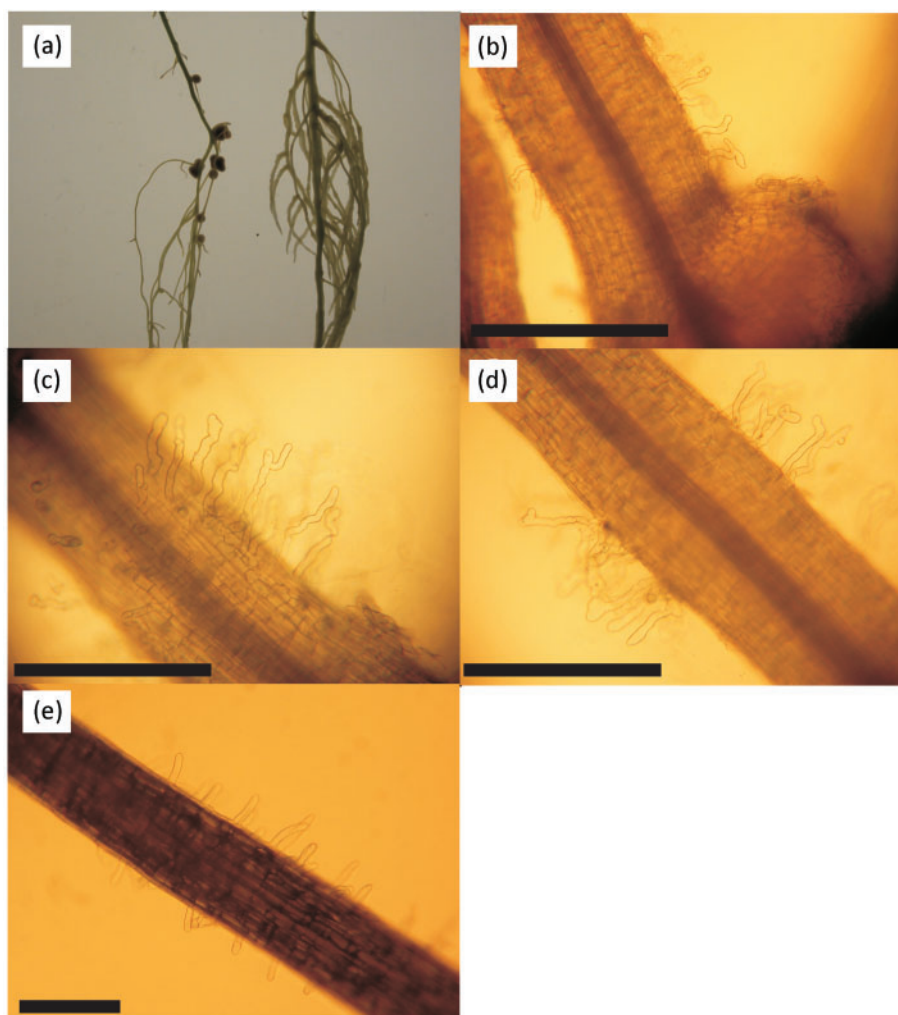
Fig. 2. Nodulation test of the parental strain and Tn5-induced mutants of *B. phymatum*. (a) Nodules induced by *B. phymatum* STM815 (left) and the mutant strain KM16-22 (right) at 28 days after inoculation on the roots of *M. pudica*; (b) wild-type STM815 can form root nodules on *M. pudica*; (c) mutant KM16-22 and (d) mutant KM51 have no ability to form root nodules, but they still cause root hair deformation; (e) *M. pudica* root without bacterial inoculation. Bars, 1 mm.

grow on a minimal medium containing glucose, fructose, mannose, gluconate or mannitol as sole carbon source. It could also not grow on minimal medium with gluconeogenic precursors such as pyruvate, succinate, fumarate, malate or glutamate. However, KM16-22 could grow as efficiently as the parental strain when minimal media containing mixtures of glucose or mannitol in combination with pyruvate, malate or glutamate were used. Mutant KM51 could not grow on a minimal medium unless glucose, fructose, mannose, gluconate or mannitol was provided.

KM16-22 has lost PGM activity and KM51 has lost FBPase activity

The database search suggested that the Tn5-interrupted gene in KM16-22 may encode PGM and that in KM51 may encode FBPase; these possibilities are consistent with the carbohydrate utilization studies. To help confirm these identifications, we performed a PGM activity assay with the parental strain STM815 and KM16-22, and an FBPase assay with STM815 and KM51. The PGM-specific activities detected in the cell lysate and bacteroid lysate of STM815 were 110 ± 6 and 113 ± 8 µmol min^{-1} (mg protein)$^{-1}$, respectively, for the conversion of 3-PGA to 2-PGA. KM16-22 was found to contain greatly reduced PGM activity, amounting to only 8–14 % of the activity determined in the cell lysate of the parental strain (Table 3). With regard to FBPase, the specific activities detected in the cell lysate and bacteroid lysate of STM815 were 256 ± 7 and 195 ± 5 µmol min^{-1} (mg protein)$^{-1}$, respectively, and that detected in KM51 was 62 ± 3 µmol min^{-1} (mg protein)$^{-1}$. These results indicate a significant decrease in activity in the mutant strain KM51, amounting to 24.2 % of the activity determined in the parental strain (Table 3).

Table 2. Growth of *B. phymatum* STM815, KM16-22 and KM51 on complex and minimal media containing various carbon sources

ND, Not determined.

Medium	Growth of strain		
	STM815	KM16-22	KM51
Yeast extract mannitol	+	+	+
Luria–Bertani	+	+	+
Minimal medium with compound:			
Glucose, fructose, mannose, gluconate or mannitol	+	− (+)*	+
Minimal medium with compound:			
Pyruvate, succinate, fumarate, malate or glutamate	+	− (+)*	− (+)†
Minimal medium with compound mixture:			
Glucose and pyruvate	+	+	ND
Glucose and malate	+	+	ND
Glucose and glutamate	+	+	ND
Mannitol and pyruvate	+	+	ND
Mannitol and malate	+	+	ND
Mannitol and glutamate	+	+	ND
Pyruvate and malate	+	−	−
Pyruvate and glutamate	+	−	−
Glucose and gluconate	+	−	−
Glucose and mannitol	+	−	−

*Results in parentheses were obtained with strain KM16-22/*pgm* harbouring the wild-type *pgm* gene.
†Results in parentheses were obtained with strain KM51/*fbp* harbouring the wild-type *fbp* gene.

In summary, enzyme assays confirmed that although activities of PGM and FBPase are normal in both free-living and symbiotic bacteroidal forms of STM815, the mutant strains KM16-22 and KM51 have lost most of their PGM and FBPase activities.

PGM activity and FBPase activity are essential for effective symbiosis of *B. phymatum* with *M. pudica*

The phenotypes imparted by a mutated gene can usually be complemented by introduction of the corresponding wild-type gene into the cell. Therefore, pBBR/*pgm* was subsequently constructed and introduced into mutant strain KM16-22, which was then designated KM16-22/*pgm*. When the cell lysate extracted from strain KM16-22/*pgm* was assessed for PGM activity, it had activity exceeding that of the parental strain STM815 (Table 3), and had recovered its ability to utilize hexoses and related compounds as carbon sources (Table 2). To further analyse its symbiotic capacity, strain KM16-22/*pgm* was inoculated onto *M. pudica*. The results from the nodulation test and nitrogen-fixation assays

Table 3. Relative enzyme activity and nodulation response of the parental strain, mutant strain and the mutant strain harbouring the uninterrupted enzyme gene

Bacterial strain	Relative enzyme activity (%)*	Nodulation response†
STM815	100	+
KM16-22	12.1‡	−
KM16-22/*pgm*	190.7‡	+
KM51	24.2§	−
KM51/*fbp*	98.2§	+

*Each value is the mean of six independent experiments.
†+, Nitrogen-fixing nodules; −, no nodules.
‡Enzyme activities were assayed by PGM activity assay. The specific enzyme activity of the parental strain STM815 was adjusted to 100 %, and the relative enzyme activities of the mutants were calculated.
§Enzyme activities were assayed by FBPase activity assay. The specific enzyme activity of the parental strain STM815 was adjusted to 100 %, and the relative enzyme activities of the mutants were calculated.

demonstrated that strain KM16-22/*pgm* had the same phenotype as its parental strain, STM815 (Table 3).

A similar method was used to clone the *fbp* gene of *B. phymatum* STM815, and thus to obtain strain KM51/*fbp*. The cell lysate extracted from strain KM51/*fbp* was assessed for FBPase activity. Strain KM51/*fbp* had almost the same level [251 ± 5 µmol min^{-1} (mg protein)$^{-1}$] of FBPase activity as STM815 (Table 3). Growth studies showed that strain KM51/*fbp* could grow as well as the parental strain when cultured on minimal medium. After its inoculation onto *M. pudica*, the symbiotic capacity of strain KM51/*fbp* in terms of nodulation and nitrogen fixation was also restored (Table 3).

These studies confirmed that the phenotypes of KM16-22 and KM51 resulted from the insertion of Tn5 into the *pgm* and *fbp* genes, respectively. They also demonstrated that PGM and FBPase activities are essential for effective symbiosis of *B. phymatum*.

The Tn5-interrupted gene in KM16-22 encodes a 2,3-BPGA-dependent PGM

To confirm that the putative *pgm* gene belongs to the cofactor-dependent class, this gene was fused to a six-histidine tag at the C terminus of the protein using plasmid pET23b(+). The resulting plasmid, pET/*pgm*, was introduced into *E. coli* BL21. Lysates of the transformed *E. coli* BL21 cells showed a strong band with the expected molecular mass of about 30 kDa that was not seen in controls (data not shown). The recombinant PGM proteins were purified to homogeneity with a nickel affinity column and subjected to assays for detailed enzymic characterization. The purified recombinant PGM had a specific activity of 30.8 ± 0.9 U (mg protein)$^{-1}$. In the presence of 2,3-BPGA

this activity was significantly increased to 50.8 ± 1.5 U (mg protein)$^{-1}$, which constitutes a 165 % enhancement of activity by the addition of the cofactor. Vanadate is known to be a potent inhibitor of a number of 2,3-BPGA-dependent PGMs, but does not inhibit 2,3-BPGA-independent PGMs (Carreras et al., 1980). The PGM activity was strongly inhibited by >90 % when NaVO$_3$ (50 μM) was added to the enzyme assay. These results are similar to those reported for 2,3-BPGA-dependent PGM (Fraser et al., 1999), and thus provide evidence that the putative PGM gene encodes a 2,3-BPGA-dependent PGM.

DISCUSSION

PGM is known to be an important enzyme for both glycolysis and gluconeogenesis (Fothergill-Gilmore & Watson, 1989). As the insertion of Tn5 into the pgm gene of B. phymatum STM815 resulted in loss of PGM activity in the resulting mutant strain KM16-22, it suggested that the lower part of the glycolytic pathway and the capacity for gluconeogenesis from various carbon compounds were both abolished in this mutant. The growth of mutant KM16-22 was severely compromised on minimal medium containing glucose, fructose, mannose, gluconate or mannitol, and a number of gluconeogenic precursors such as pyruvate, succinate, fumarate, malate or glutamate, compared with the parental strain STM815. On the other hand, the mutant KM16-22 grew as well as the parental strain on minimal medium when it was supplemented with combinations of a sugar and a metabolic intermediate (Table 2). In this respect, this B. phymatum mutant behaves similarly to E. coli enolase (eno), 3-phosphoglycerate kinase (pgk) and glyceraldehyde-3-phosphate dehydrogenase (gap) mutants (Irani & Maitra, 1977), as well as pgm mutants of Bacillus subtilis (Leyva-Vazquez & Setlow, 1994) and Pseudomonas syringae pv. tomato (Morris et al., 1995). All of the aforementioned mutants obligately require two carbon sources for growth, one source above and one below the metabolic block. In contrast, the growth characteristics of this B. phymatum pgm mutant are different from those of pgk or gap mutants of Pseudomonas aeruginosa (Banerjee et al., 1987) and Rhizobium meliloti (now renamed Sinorhizobium meliloti) (Finan et al., 1988), as neither is able to grow well on a number of intermediates in hexose catabolism. Possible explanations for the different carbon requirements have been discussed elsewhere (Banerjee et al., 1987), but it is likely that the glycolytic pathway of glucose or gluconate available in P. aeruginosa does not absolutely require either pgk or gap, although they are essential for gluconeogenesis. In summary, PGM has been proven in E. coli, Bacillus subtilis, P. syringae pv. tomato and B. phymatum STM815 (this study) to be an obligate enzyme for both glycolysis and gluconeogenesis.

dPGM enzymes have previously been identified in some bacteria, such as Haemophilus influenzae (Fleischmann et al., 1995), Streptomyces coelicolor (White et al., 1992) and Zymomonas mobilis (Yomano et al., 1993), whereas only

the iPGM enzymes are found in archaea (Graham et al., 2002; Potters et al., 2003). Surprisingly, both dPGM and iPGM are also found in some bacteria, such as E. coli and Bacillus subtilis, although only one form is predominantly active (Fraser et al., 1999; Watabe & Freese, 1979). In order to determine whether the enzyme that was detected in free-living B. phymatum STM815 and its symbiotic bacteroids was a dPGM or an iPGM, the gene encoding PGM was expressed as a recombinant enzyme in E. coli. The catalytic properties of the enzyme were determined, and as it was stimulated by 2,3-BPGA and was susceptible to vanadate; it thus corresponded to a dPGM. Moreover, the amino acid sequence has substantial identity to those of other dPGMs from various organisms. The phylogenetic analysis clustered this protein together with those of other betaproteobacterial dPGMs, and it was relatively distant from E. coli dPGM and very distant from alphaproteobacterial dPGMs. In addition, several Burkholderia spp., including Burkholderia cepacia, Burkholderia mallei, Burkholderia pseudomallei and B. phymatum, have their complete genome sequences available in GenBank, and from these complete genome sequence data, only dPGM has so far been annotated. In the specific case of B. phymatum STM815, only one copy of dPGM has been annotated on chromosome 1 (CP001043). Taken together, these results suggest that Burkholderia spp., including B. phymatum STM815, may possess genes for dPGM only.

In addition to their obvious deficiencies in carbohydrate metabolism, pgm mutant bacteria have also shown some incomplete physiological properties. For example, without a functional pgm gene, P. syringae pv. tomato cannot grow on its host plant and cause disease (Morris et al., 1995), and if its pgm gene is abolished, Bacillus subtilis cannot commit itself to sporulation (Leyva-Vazquez & Setlow, 1994). In addition, Reutz et al. (1982) reported that PGM is absolutely essential for autotrophic carbon metabolism in Alcaligenes eutrophus, which is a facultatively chemo-lithoautotrophic hydrogen bacterium. In our study of the β-rhizobial strain B. phymatum STM815, we found that PGM is essential for the formation of a nodulating symbiosis when it is inoculated onto its plant host, M. pudica. Interestingly, however, other mutant rhizobial strains affected in gluconeogenesis, including phospho-enolpyruvate carboxykinase (pck) mutants of R. legumino-sarum, S. meliloti and Rhizobium NGR234, may induce the formation of root nodules, even though in some of these rhizobial–legume combinations the resulting nodules contain few or uninvaded host cells (Finan et al., 1991; Mckay et al., 1985; Osterås et al., 1991). As suggested elsewhere (Finan et al., 1991), these different symbiotic behaviours may reflect differences in the composition or amount of the metabolites made available by each legume to their symbiotic bacteria. Tatè et al. (2004) have reported that fructose 1,6-bisphosphate aldolase (fba) and pck mutants of R. etli induce only a few abortive nodules. It is proposed that gluconeogenesis is strictly required for the growth and symbiosis of R. etli with its plant host,

Phaseolus vulgaris. In our study, we also found a fructose 1,6-biphosphatase (*fbp*) mutant, KM51, which was affected in gluconeogenesis, and it had lost the ability to form nodules and to fix nitrogen. In addition, several studies have elucidated that FBPase is required for virulence in *Mycobacterium tuberculosis* and *Leishmania major*, and for growth under different carbon sources in *Salmonella enterica* and *Lactococcus lactis* (Dougherty *et al.*, 2006; Looijesteijn *et al.*, 1999; Naderer *et al.*, 2006), and FBPase plays an important role in the production of lysine and glutamate in *Corynebacterium glutamicum* (Becker *et al.*, 2005).

As far as we are aware, these are the first *pgm* and *fbp* mutants of β-rhizobia to be reported. Why KM16-22 and KM51 cannot nodulate their plant host is unknown. One possibility is that the loss of PGM or FBPase activity leads to the accumulation of an interfering metabolite that blocks the process of nodulation. Alternatively, and most likely, it is possible that gluconeogenesis for growth is indispensable in STM815 during the early steps of nodulation. Mutant strain KM16-22 could grow efficiently only when both hexose and TCA cycle intermediates were supplied. On the other hand, mutant KM51 could grow on hexoses, but was not able to grow on pyruvate and the TCA cycle intermediates. Strains KM16-22 and KM51 thus appear to be lacking a hexose carbon source supplied from the host plant for nodulation. C_4-dicarboxylates have generally been considered to be the principal carbon and energy sources supplied by the plant to nitrogen-fixing bacteroids (Stowers, 1985), and they may also be limiting during the early stages of infection. Future experiments will be aimed at constructing specific gene-knockout mutations in the same metabolic pathway in *B. phymatum*, followed by a detailed physiological analysis. Simultaneously, we are focusing on comparing the interaction of *B. phymatum* STM815 with *M. pudica* with those of its *pgm* and *fbp* mutants, using high-resolution light and electron microscopy.

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