Biosynthesis of branched-chain amino acids is essential for effective symbioses between betarhizobia and *Mimosa pudica*

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*Burkholderia phymatum* STM815 and *Cupriavidus taiwanensis* LMG19424 are betaproteobacterial strains that can effectively nodulate several species of the large legume genus *Mimosa*. A Tn5 mutant, derived from *B. phymatum* STM815 (KM60), and another derived from *C. taiwanensis* LMG19424 (KM184-55) induced Fix- nodules on *Mimosa pudica*. The Tn5-interrupted genes of the mutants showed strong homologies to *ilvE*, which encodes a branched-chain amino acid aminotransferase, and *leuC*, which encodes the large subunit of isopropylmalate isomerase. Both enzymes are known to be involved in the biosynthetic pathways for branched-chain amino acids (BCAAs) (leucine, valine and isoleucine). The *B. phymatum* *ilvE* mutant, KM60, was not auxotrophic for BCAAs and could grow well on minimal medium with pyruvate as a carbon source and ammonia as a nitrogen source. However, it grew less efficiently than the wild-type (WT) strain when ammonia was substituted with valine or isoleucine as a nitrogen source. The BCAA aminotransferase activity of KM60 was significantly reduced relative to the WT strain, especially with isoleucine and valine as amino group donors. The *C. taiwanensis* *leuC* mutant, KM184-55, could not grow on a minimal medium with pyruvate as a carbon source and ammonia as a nitrogen source, but its growth was restored when leucine was added to the medium. The isopropylmalate isomerase activity of KM184-55 was completely lost compared with the WT strain. Both mutants recovered their respective enzyme activities after complementation with the WT *ilvE* or *leuC* genes and were subsequently able to grow as well as their parental strains on minimal medium. They were also able to form nitrogen-fixing nodules on *M. pudica*. We conclude that the biosynthesis of BCAAs is essential for the free-living growth of betarhizobia, as well as for their ability to form effective symbioses with their 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 class Alphaproteobacteria, the so-called 'alpharhizobia', over the last 10 years there have been an increasing number of reports of legumes being nodulated by members of the Betaproteobacteria (the so-called 'betarhizobia') (Gyaneshwar et al., 2011). *Cupriavidus taiwanensis* LMG19424 and *Burkholderia phymatum* STM815 were among the first identified nodulating betaproteobacterial strains (Gyaneshwar et al., 2011) and both are highly effective nitrogen-fixing symbionts of *Mimosa* species (Chen et al., 2003, 2005; Elliott et al., 2007; dos Reis et al., 2010).

Nodulation is a multistep process (van Rijn & Vanderleyden, 1995; Chen et al., 2003; Gibson et al., 2008; Oldroyd & Downie, 2008), in which rhizobia (alpha or beta) 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 symbiosomes, in which they...
differentiate into bacteroids (Chen et al., 2003). The C4-dicarboxylate intermediates of the tricarboxylic acid cycle, succinate, fumarate and malate, have generally been accepted as the principal carbon and energy sources supplied by the plant to bacteroids (Stowers, 1985). It is within the symbiosomes that the bacteria fix nitrogen in the form of ammonia, which is then released to the host plant (Brewin, 1991). In addition, it has been proposed that nitrogen fixation requires the cycling of amino acids between the bacteroid and plant compartments (Lodwig et al., 2003; Prell & Poole, 2006). However, more recently, Prell et al. (2009) have emphasized that plants control branched-chain amino acid (BCAA) supply to bacteroids, regulating their development and persistence, at least in the symbioses of Rhizobium leguminosarum with Pisum sativum and Phaseolus vulgaris (Prell et al., 2010).

Several mutants of members of the genus Rhizobium (and related alphahizobia genera) that are affected in their biosynthesis of isoleucine, valine and leucine have repeatedly been demonstrated to be defective in either nodulating their host plants or fixing nitrogen when nodules are formed (Aguilar & Grasso, 1991; de las Nieves Peltzer et al., 2008; Ferraioli et al., 2002; Hassani et al., 2002; Kerppola & Kahn, 1988; López et al., 2001; Pobigaylo et al., 2008; Sanjuan-Pinilla et al., 2002; Truchet et al., 1980). This is often generally the case for auxotrophic mutants, because the compounds that cannot be synthesized by the rhizobium might also not be provided by the plant in the rhizosphere or during the nodulation process. The symbiotic defect can often be corrected by adding the missing compound to the plant nutrient medium. However, in betahizobia the link between BCAA biosynthesis and the nodulation process has not yet been demonstrated.

The pathways for biosynthesis of BCAAs, outlined in Fig. 1, are based primarily on the KEGG pathway analysis for B. phymatum and C. taiwanensis. The metabolic pathway for the synthesis of valine and isoleucine consists of four successive steps, which are catalysed by acetolactate synthase (EC 2.2.1.6, ilvHI gene product), ketol-acid reductoisomerase (EC 1.1.1.86, ilvC gene product), dihydroxy acid dehydratase (EC 4.2.1.9, ilvD gene product) and BCAA aminotransferase (EC 2.6.1.42, ilvE gene product). Leucine is synthesized by the isopropylmalate (IPM) pathway. Three enzymes convert α-ketoisovalerate, an intermediate in valine biosynthesis, to α-ketoisocaproate, the immediate precursor of leucine. The first enzyme in the pathway is α-IPM synthase (EC 2.3.3.13), which is encoded by leuA. The next enzymes unique to leucine synthesis are IPM isomerase (EC 4.2.1.33), encoded by leuC (large subunit) and leuD (small subunit), and β-IPM dehydrogenase (EC 1.1.1.85), encoded by leuB. For the formation of leucine, BCAA aminotransferase reversibly transfers one α-amino group from glutamate to α-ketoisocaproate, finally forming leucine.

In this study, we present the successful use of transposon mutagenesis in betahizobia and show that both KM60, a

Fig. 1. Predicted BCAA biosynthesis in B. phymatum and C. taiwanensis. Structural gene designations and the encoded enzymes are: ilvHI, acetolactate synthase; ilvC, ketol-acid reductoisomerase; ilvD, dihydroxy acid dehydratase; ilvE, BCAA aminotransferase; leuA, α-isopropylmalate synthase; leuC, isopropylmalate isomerase large subunit; leuD, isopropylmalate isomerase small subunit; leuB, β-isopropylmalate dehydrogenase. 

B. phymatum mutant with reduced BCAA aminotransferase activity, and KM184-55, a C. taiwanensis mutant lacking IPM isomerase activity, are deficient in nitrogen fixation. Our data imply that the biosynthesis of BCAAs is important for either nodule invasion, bacteroid formation or bacteroid maturation, and mutations affecting BCAA biosynthesis lead to non-nitrogen-fixing nodules. We provide genetic evidence to demonstrate that BCAA aminotransferase activity and IPM isomerase activity are essential for the formation and function of effective symbioses by B. phymatum and C. taiwanensis with their host plant, M. pudica.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used are listed in Table 1. B. phymatum strains STM815 (wild-type, WT) and KM60, and C. taiwanensis strains LMG19424 (WT) and KM184-55 were grown in yeast extract mannitol (YM) medium (Vincent, 1970) at 28 °C and Escherichia coli strains were grown in Luria–Bertani medium at 37 °C. Antibiotics were used for these bacterial strains at the following final concentrations: 34 μg chloramphenicol ml⁻¹, 12.5 μg tetracycline ml⁻¹, 50 μg kanamycin ml⁻¹ and 50 μg ampicillin ml⁻¹.

**Tn5 mutagenesis.** Introduction of a Tn5 transposon into B. phymatum STM815 and C. taiwanensis LMG19424 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 (either B. phymatum STM815 or C. taiwanensis LMG19424) were mixed and spotted onto a nitrocellulose filter. After incubation on YEM plates, chloramphenicol- and tetracycline-resistant colonies were selected.

**Plant tests.** M. 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...


Table 1. Bacterial strains and plasmids used in this study

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>B. phymatum</strong></td>
<td></td>
<td></td>
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<tr>
<td>STM815</td>
<td>Wild-type, Cm&lt;sup&gt;+&lt;/sup&gt;, Nod&lt;sup&gt;+&lt;/sup&gt; on M. pudica</td>
<td>Elliott et al. (2007); Vandamme et al. (2002)</td>
</tr>
<tr>
<td>KM60</td>
<td>STM815::Tn5, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>KM60/ilvE</td>
<td>KM60 containing pBBR/ilvE, Tc&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>C. taiwanensis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMG19424</td>
<td>Wild-type, Cm&lt;sup&gt;+&lt;/sup&gt;, Nod&lt;sup&gt;+&lt;/sup&gt; on M. pudica</td>
<td>Chen et al. (2001)</td>
</tr>
<tr>
<td>KM184-55</td>
<td>LMG19424::Tn5, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>KM184-55/leuC</td>
<td>KM184-55 containing pBBR/leuC, Tc&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td><strong>E. coli</strong></td>
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<tr>
<td>S17-1 pir</td>
<td>Containing pUTmini-Tn5gfp</td>
<td>de Lorenzo et al. (1990)</td>
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<td>HB101</td>
<td>Containing pRK2013</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
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<td>DH5α</td>
<td>Transformation strain</td>
<td>Gibco-BRL</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>gfp</td>
<td>Tn5-based delivery plasmid with gfp gene, Ap&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Matthisse et al. (1996)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid used in triparental conjugation, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pBluescript II SK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Cloning and sequencing vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBBR1MCS-2</td>
<td>Broad-host-range cloning vector, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kovach et al. (1995)</td>
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<tr>
<td>pBBR/ilvE</td>
<td>pBBR1MCS-2 containing the ilvE ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR/leuC</td>
<td>pBBR1MCS-2 containing the leuC ORF</td>
<td>This study</td>
</tr>
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</table>

Identification and cloning of the Tn5-interrupted genes. Total DNA from mutant strains was prepared and digested with EcoRI. Southern blotting was carried out by standard methodology (Sambrook & Fritsch, 1989) to confirm that the mutant strains were carrying a single copy of Tn5. The genomic DNA was prepared and digested with Apal and it was ligated to pBluescript II SK<sup>+</sup>. 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. Washed bacterial suspensions (100 μl containing 2 × 10<sup>9</sup> cells) were used to start 10 ml minimal medium cultures containing 0.05% K₂HPO₄·3H₂O, 0.08% MgSO₄·7H₂O, 0.02% NaCl, 0.025% CaCl₂, 0.1% (NH₄)₂SO₄ and 0.01% FeCl₃·6H₂O (pH 7.0) supplemented with pyruvate at 0.2% (w/v). The cultures were incubated at 28 ℃ for 24 or 60 h. The OD<sub>600</sub> values were recorded at intervals and represented as the mean of four independent experiments. To test the ability of mutant strains to grow on various nitrogen sources, growth studies were performed in a minimal medium containing isoleucine, valine or leucine at 0.02% (w/v) as substitutes for (NH₄)₂SO₄.

Enzyme assay. The BCAA aminotransferase or the IPM isomerase activity in cells was measured. Cells were grown to exponential phase (2 × 10<sup>9</sup> cells ml<sup>-1</sup>) and then harvested by centrifugation, after which they were resuspended in 2 ml lysis buffer (25 mM Tris/HCl, pH 7.5; 1 mM EDTA). Cells were lysed by sonication and the lysate was cleared by centrifugation (30,000 g, 4 ℃, 10 min). The BCAA aminotransferase activity was assayed by an aminotransferase reaction as described by Thage et al. (2004). Reaction mixtures with a final volume of 250 μl contained 56 mmol potassium phosphate buffer l<sup>-1</sup> (pH 7.4), 0.05 mmol pyridoxal-5'-phosphate l<sup>-1</sup>, 6 mmol 2-ketogluutaric acid l<sup>-1</sup>, 1 mmol leucine, isoleucine or valine l<sup>-1</sup> and 50 μl (8.2 μg protein μl<sup>-1</sup>) of the bacterial lysate. The enzymic reaction was performed for 15 min at 37 ℃. The reaction was stopped by heating at 80 ℃ for 15 min. The amount of glutamic acid formed during the reaction was measured using a colorimetric l-glutamic acid kit from Roche (R-Biopharm; formerly Boehringer Mannheim) according to the manufacturer’s instructions. The glutamic acid content was measured after 30 min at room temperature using a spectrophotometer.

IPM isomerase was assayed with z-IPM as the substrate and by following the appearance of the intermediate dimethyl citraconate at 235 nm using a spectrophotometer. The procedure of Gross et al. (1963) was modified such that the reaction mixture contained 5 μmol neutralized z-IPM, 20 μmol potassium phosphate (pH 7.0) and 50 μl cell-free extract in a total volume of 1 ml. The enzymic reaction was performed for 5 min at room temperature.

Gene complementation. The ilvE gene was amplified by PCR using Vent<sub>R</sub> exonuclease proofreading DNA polymerase (New England Biolabs) from B. phymatum STM815, using these oligonucleotide primers: 5'-ACGTGGCCCTCGCCGATGCAGCGCC3'- (ilvE-Apa, forward primer, Apal site in bold) and 5'-CATGTCTAGATCGGATTCGCGAAG-3' (ilvE-Xba, reverse primer, XbaI site in bold). After restriction enzyme digestion, the ilvE gene was cloned into pBBR1MCS-2 (Kovach et al., 1995). The resulting plasmid pBBR/ilvE was used to transform B. phymatum KM60 according to the method described by Vincze & Bowra (2006). The plasmid pBBR/leuC was constructed following the same procedure but using the...
gene amplified from *C. taiwanensis* LMG19424 with the primers 5'-ACGTTGGCCCTTGAATAACGCCCCTC-3' (*leuC*-Apa, forward primer, ApaI site in bold) and 5'-CATGTTCTAGATCGCCCGA-GCTTGCGGA-3' (*leuC*-Xba, reverse primer, XbaI site in bold).

**RESULTS**

**Tn5-induced mutants form Fix⁻ root nodules on *M. pudica***

Two WT strains, *B. phymatum* STM815 and *C. taiwanensis* LMG19424, together with about 6000 presumptive Tn5 insertion mutants were screened for their symbiotic phenotypes by inoculation onto *M. pudica*. The parental strains STM815 and LMG19424 could induce Fix⁺ nodules on the roots of *M. pudica* 28 days post-inoculation (Fig. 2a, c). Twenty-eight and 56 days post-inoculation, two of the Tn5-induced mutants, designated KM60 and KM184-55, derived from *B. phymatum* STM815 and *C. taiwanensis* LMG19424, respectively, had only produced white nodules (Fig. 2b, d). The mean dry weights of *M. pudica* inoculated with strain KM60 or KM184-55 were significantly lower than those of the plants inoculated with their parental strains (data not shown). When comparing the roots of *M. pudica* inoculated with strain KM60 or KM184-55 and those inoculated with their parental strains, it could be seen that both mutant strains induced fewer nodules than their parental strains and neither showed any nitrogenase (acetylene reduction) activity (Table 2). Mature nodules from *M. pudica* inoculated with the parental strains were as described previously (Chen et al., 2003; Elliott et al., 2007) i.e. they were typically indeterminate, with a distinct meristem, invasion zone and nitrogen fixation zone, and the infected cells were packed with nitrogen-fixing bacteroids. However, in contrast with those induced by the parental strains, nodules induced by the two mutants were devoid of invasion and nitrogen-fixation zones and they contained no bacteroids (data not shown).

**Table 2.** Symbiotic features of Tn5-induced mutants and of the WT

Values are means ± SD for three trials. Results are numbers of nodules per plant 28 days after inoculation of three plants. Activity is detected as reduction of acetylene to ethylene and expressed as nmol C₂H₄ h⁻¹ per plant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>No. of nodules</th>
<th>Nitrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM815</td>
<td>Wild-type</td>
<td>56 ± 11</td>
<td>571 ± 27.4</td>
</tr>
<tr>
<td>KM60</td>
<td><em>ilvE</em>::Tn5</td>
<td>29 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>KM60/<em>ilvE</em></td>
<td>pBBR/*ilvE</td>
<td>55 ± 15</td>
<td>582 ± 23.5</td>
</tr>
<tr>
<td>LMG19424</td>
<td>Wild-type</td>
<td>46 ± 7</td>
<td>72 ± 5.4</td>
</tr>
<tr>
<td>KM184-55</td>
<td><em>leuC</em>::Tn5</td>
<td>23 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>KM184-55/<em>leuC</em></td>
<td>pBBR/*leuC</td>
<td>56 ± 12</td>
<td>70 ± 7.2</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Identification of the Tn5-interrupted genes

Cloning and sequencing revealed that mutant KM60 carried a single Tn5 insertion in a gene identified as Bphy_0456 (B. phymatum STM815 chromosome 1, CP001043) (Fig. 3a) and encoding a BCAA aminotransferase (92–95% amino acid identity to Burkholderia spp., 77–78% identity to Cupriavidus/Ralstonia spp., 45% identity to E. coli and 44% identity to Mesorhizobium loti). This putative ilvE gene is 924 bp long and encodes a protein of 307 amino acids. Mutant KM60 contained a transposon insertion at base 308 of the 924 bp ilvE gene. The two ORFs located upstream of the putative ilvE gene were Bphy_0458 and Bphy_0457 and they encode the BCAA transporter and BCAA efflux pump, respectively, whereas the ORF located downstream was Bphy_0454 which encodes the lipopolysaccharide heptosyltransferase II. In addition, in B. phymatum STM815 only one ilvE gene was identified and no parologue could be found.

In mutant KM184-55, the sequence flanking Tn5 identified the disrupted gene as RALTA_A2120 (C. taiwanensis LMG19424 chromosome 1, NC_010528) (Fig. 3b), which encodes a putative IPM isomerase large subunit (90–96% amino acid identity to Cupriavidus/Ralstonia spp. and 83–84% identity to Burkholderia spp.). This putative leuC1 gene is 1410 bp and encodes a protein of 469 amino acids. Mutant KM184-55 contained a transposon insertion at base 1219 of the 1410 bp leuC1 gene. The ORF located upstream of the putative leuC1 gene was RALTA_A2121 and it encodes a high-affinity BCAA transport protein, whereas the two ORFs located downstream were RALTA_A2119 and RALTA_A2118 which encode the IPM dehydratase small subunit and β-IPM dehydrogenase, respectively. However, there are two paralogues in C. taiwanensis LMG19424, one strong parologue (70% amino acid identity) named leuC2 (RALTA_A0672, chromosome 1, NC_010528) and another parologue (30% identity) named leuC3 (RALTA_B0053, chromosome 2, NC_010530).

Tn5-induced mutants are defective in the metabolism of BCAAs

Both BCAA aminotransferase and the IPM isomerase large subunit are involved in the synthetic pathway of BCAAs (Fig. 1). The former catalyses the reversible transamination of α-amino groups and is essential for anabolism and catabolism of the three amino acids, leucine, isoleucine and valine. IPM isomerase catalyses the conversion of α-IPM to β-IPM, the second step in leucine biosynthesis. In order to elucidate their metabolic ability, the mutants KM60 and
KM184-55 and their parental strains STM815 and LMG19424 were cultured on minimal medium containing pyruvate and ammonia as carbon and nitrogen sources, respectively. Surprisingly, this experiment showed that KM60 was not an auxotroph for BCAAs, as it could grow as well as the parental strain, STM815 (Fig. 4). In contrast, KM184-55 is a true auxotroph, as it could not grow at all under these conditions (Fig. 5).

When isoleucine, valine or leucine were substituted as nitrogen sources, KM60 grew less well than the parental strain, STM815, in the presence of isoleucine and valine, but grew as well as STM815 in the presence of leucine (Fig. 4). Furthermore, the growth of KM184-55 could be restored when using leucine, but not isoleucine or valine, as a nitrogen source (Fig. 5). These results indicated that KM60 was deficient in its utilization of isoleucine and valine, and that KM184-55 was a leucine-auxotrophic mutant. Both mutants KM60 and KM184-55 were defective in their metabolism of BCAAs.

**KM60 has reduced BCAA aminotransferase activity and KM184-55 lost IPM isomerase activity**

A database search suggested that the Tn5-interrupted gene in the *B. phymatum* mutant strain KM60 may code for BCAA aminotransferase. This possibility is consistent with the aforementioned BCAA utilization studies. In order to confirm this, we performed an enzyme assay on KM60 in comparison with the parental strain STM815. BCAA aminotransferase-specific activities were detected in the cell lysate of strain STM815 when using isoleucine, valine or leucine as an amino group donor, but the mutant KM60 had relatively lower activities (21.4–78.6%) (Table 3). These residual activities explain why KM60 is not an auxotroph and why leucine, when used as the sole nitrogen source, allows better growth compared with valine and isoleucine.

In the case of the *C. taiwanensis* mutant KM184-55, the Tn5 insertion interrupted the gene coding for the large subunit of IPM isomerase which is involved in the pathway of leucine synthesis. The leucine growth requirement of KM184-55 was consistent with this, and so we performed an IPM isomerase assay with KM184-55 and the parental strain LMG19424 to confirm this. Compared with LMG19424, the level of IPM isomerase activity dropped in KM184-55 to almost undetectable levels (1.7% of that of LMG19424). This explains why KM184-55 is a true leucine auxotroph.

In summary, enzyme assays confirmed that KM60 had only partially lost BCAA aminotransferase activity and thus was not a true auxotroph, but it was defective in its utilization of isoleucine and valine, whereas KM184-55 had no IPM isomerase activity and thus was a true leucine auxotroph.

**BCAA aminotransferase activity and IPM isomerase activity are essential for effective symbioses between betarhizobia and *M. pudica***

The phenotypes imparted by a mutated gene can usually be complemented by introduction of the corresponding WT gene into the cell. To test this, we generated two recombinant plasmids by cloning the *ilvE* gene of the *B. phymatum* parental strain STM815 and by cloning the *leuC* gene of the *C. taiwanensis* parental strain LMG19424 into the broad-host-range vector pBBR1MCS-2. The two recombinant plasmids (pBBR/ilvE and pBBR/leuC) were then introduced into the respective mutants KM60 and KM184-55 and the resulting strains were named KM60/IlvE and KM184-55/leuC. Both were then used for further examination.

The cell lysates extracted from strains KM60/ilvE and KM184-55/leuC were assayed for their respective enzyme activities. The results from the aminotransferase reaction
Table 3. BCAA aminotransferase enzyme assay

<table>
<thead>
<tr>
<th>Amino acid substrate as amino group donor</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td></td>
<td>STM815</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>100</td>
</tr>
<tr>
<td>Valine</td>
<td>89.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>98.5</td>
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</table>

Enzyme activities were assayed by the aminotransferase reaction. The specific activity using isoleucine as an amino group donor of the parental strain STM815 was adjusted to 100% and the activities of the mutants were calculated relative to this. Each value is the mean of six independent experiments. ND, not determined.

assay showed that KM60/ilvE had recovered its activity to a level slightly exceeding that of the parental strain STM815 when using isoleucine or valine as amino group donors (Table 3). In the IPM isomerase assay, KM184-55/leuC had almost the same activity as the parental strain, LMG19424 (112 versus 100%). In the ensuing growth studies, strain KM60/ilvE showed that it had recovered its ability to utilize isoleucine or valine as nitrogen sources and KM184-55/ilvE regained its prototrophy when cultured on a minimal medium containing pyruvate and ammonia (data not shown). To further analyse their symbiotic capacity, KM60/ilvE and KM184-55/leuC were inoculated onto M. pudica. The results from the nodulation tests and nitrogen fixation assays demonstrated that KM60/ilvE and KM184-55/leuC had the same symbiotic phenotype as their respective parental strains, STM815 and LMG19424 (Table 2).

These studies confirmed that the phenotypes of the mutant strains KM60 and KM184-55 resulted from the insertion of Tn5 into the genes ilvE and leuC, respectively. They also demonstrated that biosynthesis of BCAAs is essential for free-living growth of betarhizobia, as well as for their ability to form an effective symbiosis with their host legume.

DISCUSSION

In this study, both betarhizobial mutants generated by transposon mutagenesis produced Fix− nodules on M. pudica. Both mutant strains were mutated in genes involved in BCAA biosynthesis. These data are consistent with the results obtained from previous studies on alpharhizobial mutants with altered BCAA biosynthesis, which were also defective in nodulating their host legumes (Aguilar & Grasso, 1991; de las Nieves Peltzer et al., 2008; Ferraioli et al., 2002; Hassan et al., 2002; Kerppola & Kahn, 1988; López et al., 2001; Pobigaylo et al., 2008; Sanjuán-Pinilla et al., 2002; Truchet et al., 1980).

The B. phymatum STM815 mutant, KM60, was mutated in its ilvE gene, the gene encoding BCAA aminotransferase, which is the last enzyme of the BCAA biosynthetic pathway. Interestingly, however, we did not obtain an auxotrophic mutant, which suggests that the parental strain STM815 may have at least one functionally redundant ilvE copy. Two BCAA aminotransferases (IlvE1 and IlvE2) catalysing the last step of the BCAA biosynthesis pathway have been demonstrated as being functional in Sinorhizobium meliloti (de las Nieves Peltzer et al., 2008) and in R. leguminosarum bv. viciae (J. Prell, unpublished data). In B. phymatum STM815 only one ilvE gene was identified and no parologue could be found. So while KM60 was not an auxotroph for BCAAs and it grew less well than the WT strain when using minimal media with pyruvate and valine or isoleucine (Fig. 4), it still showed significant residual BCAA aminotransferase activities (Table 3). Based on this, there must be an unknown enzyme that partially compensates for the lost BCAA aminotransferase activity. Therefore, it is surprising that KM60 lost its capacity to effectively nodulate the roots of M. pudica (Fig. 2b). The fact that these phenotypes were caused by a mutation in the ilvE gene which could be complemented by the introduction of the corresponding WT gene indicates that BCAA aminotransferase activity is essential for an effective symbiosis between B. phymatum STM815 and its host plant. However, it is possible that the unidentified enzyme compensating for IlvE in cultures is not expressed during nodulation.

The C. taiwanensis LMG19424 mutant, KM184-55, is a true leucine auxotroph due to a mutation in the leuC1 gene encoding the enzyme catalysing the second step in leucine biosynthesis. The mutated leuC1 gene has two paralogues, leuC2 and leuC3, as described above. In this study, a single mutation in the leuC1 gene led to leucine auxotrophy, indicating that the leuC2 and leuC3 genes are not required for the pathway under the conditions tested.

Truchet et al. (1980) reported a leucine-auxotrophic mutant in S. meliloti L5-30 that formed ineffective nodules, inside which mutants were sequestered within infection threads. It was also found that the capacity of this mutant to invade host cells and fix nitrogen was restored when leucine was added to the plant medium. Another S. meliloti leucine auxotroph (strain 104A14) that formed very small, white nodules was characterized by Kerppola & Kahn (1988); addition of leucine to the medium around plants infected with this mutant did not restore the WT phenotype. Two other leucine-auxotrophic mutants of S. meliloti, strains GR4 and 1021, both defective for host
legume nodulation, were identified by Sanjuan-Pinilla et al. (2002). Both strains were mutated in leuA1, the gene encoding the first enzyme of the leucine biosynthesis pathway, and the addition of leucine to the plant growth medium allowed the mutants to nodulate alfalfa roots, although with slower kinetics than the parental strain. Two leucine auxotrophs of S. meliloti Rm2015 were isolated and characterized by Hassani et al. (2002). Based on cross-feeding and intermediate accumulation studies, they were designated leuC(leuD and leuB) mutants. These two leucine auxotrophs could induce nodules, but did not fix nitrogen, and the addition of leucine to the plant medium did not restore the symbiotic effectiveness of the auxotrophs. Ferraioli et al. (2002) also reported a leucine-auxotrophic mutant of Rhizobium etli CE3 which was mutated in the leuC gene. The leucine auxotroph induced up to fivefold fewer nodules than the parental strain and had very low nitrogenase activity.

More recently, de las Nieves Peltzer et al. (2008) tested a set of mutants that were auxotrophic for leucine and were affected in almost all enzymic steps of the biosynthesis pathway. These leucine auxotrophs, including leuA1, leuC, leuD and leuB mutants, exhibited severe nodulation defects. For example, nodules induced by these mutants appeared on roots from approximately 7 to 20 days after those induced by the parental strain, the number of nodules was low and they did not fix nitrogen. Interestingly, however, in the presence of exogenous leucine, nodules induced by these mutants were invaded in the normal way and fixed nitrogen (de las Nieves Peltzer et al., 2008). A leuD mutant of R. leguminosarum bv. viciae Rv3841 did not nodulate pea plants, but if 1 mM leucine was added to the nodulation medium (Prell et al., 2009), peas infected with the leuD mutant were indistinguishable from plants infected with the WT strain. Interestingly, however, in Bradyrhizobium japonicum a leucine-auxotrophic mutation did not lead to a nodulation defect on soybean (Kummer & Kuyckendall, 1989), thus indicating that negative effects of auxotrophy on symbioses are not universal even among the alpharhizobia.

The leucine auxotroph of C. taiwanensis LMG19424, KM184-55, which is the first described leucine auxotroph among the betarhizobia, had a Nod+ Fix− phenotype on its host legume, M. pudica (Fig. 2d). These observations suggest that the host cells do not provide leucine to the invading bacteria at any point during the infection or nodule invasion process. The fact that different leucine-auxotrophic mutants have similar phenotypes indicates that the reason for the symbiotic defect is the disruption of the metabolic pathway in which the products of all four genes are involved. As with studies on rhizobia and sinorhizobia (de las Nieves Peltzer et al., 2008; Sanjuan-Pinilla et al., 2002; Truchet et al., 1980; Prell et al., 2009), we also observed that the nodulation deficiency of some leucine mutants was essentially restored by the addition of leucine to the plant growth medium. However, in our study, the supplementation of leucine (at 0.2–1 mM) to the plant medium did not restore symbiotic effectiveness to the leucine auxotrophs (data not shown). This demonstrates that either leucine from the nodulation medium did not reach the invading bacteria or leucine was limiting in the rhizosphere of their host plants. Extending this study to other auxotrophic mutants will give a more complete picture of the BCAAs available to betarhizobia during the development of symbioses with compatible hosts.

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REFERENCES


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