Cloning, sequencing, and mutational analysis of the Bradyrhizobium japonicum fumC-like gene: evidence for the existence of two different fumarases

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The Bradyrhizobium japonicum fumarase gene (fumC-like) was cloned and sequenced, and a fumC deletion mutant was constructed. This mutant had a Nod+ Fix+ phenotype in symbiosis with the host plant, soybean, and growth in minimal medium with fumarate as sole carbon source was also not affected. The cloned B. japonicum fumC gene fully complemented an Escherichia coli Fum- mutant, strain JH400, for growth in minimal medium with fumarate. The predicted amino acid sequence of the FumC protein showed strong similarity to the E. coli FumC protein, Bacillus subtilis CitG protein, Saccharomyces cerevisiae Fum1 protein, and the mammalian fumarases. The B. japonicum FumC protein accounted for about 40% of the total fumarase activity in aerobically grown cells. The remaining 60% was ascribed to a temperature-labile fumarase. These data suggest that B. japonicum possesses two different fumarase isoenzymes, one of which is encoded by fumC. Besides E. coli, which has three fumarases, B. japonicum is thus the second bacterium for which there is genetic evidence for the existence of more than one fumarase.

Introduction

Biological nitrogen fixation demands a substantial amount of energy and reductant. In the nitrogen-fixing Rhizobium-legume symbiosis this requirement is met exclusively by the photosynthate supply from the host plant. The elucidation of the central rhizobial pathways by which the endosymbiotic bacteroids metabolize the plant-derived carbon sources is a subject of considerable interest in order to understand the physiology of root nodule symbiosis.

There are several lines of evidence that a functional citric acid cycle plays an essential role in bacteroid carbon metabolism. For example, Rhizobium meliloti mutants defective in 2-oxoglutarate dehydrogenase (Duncan & Fraenkel, 1979) or in succinate dehydrogenase (Gardiol et al., 1982) induced nitrogen non-fixing (Fix-) nodules on alfalfa. The results from studies on the uptake of radiolabelled carbon substrates performed with isolated bacteroids were consistent with the existence of a functional tricarboxylic acid (TCA) cycle (Stovall & Cole, 1978). Moreover, the specific activities of several TCA cycle enzymes in different rhizobial bacteroids were shown to increase in parallel with the increase of nitrogenase activity (Robertson & Taylor, 1973; Kurz & LaRue, 1977; Karr et al., 1984; McKay et al., 1989). Finally, the C₄-dicarboxylates were recognized as the most effective substrates for respiration and for promoting nitrogen fixation (Bergersen & Turner, 1967), which indirectly supported the assumption that the TCA cycle played a major role in bacteroid metabolism. This was confirmed genetically by the observation that C₄-dicarboxylate transport (det) mutants of Bradyrhizobium japonicum (Humbeck & Werner, 1988), R. meliloti (Bolton et al., 1986; Engelke et al., 1989), Rhizobium leguminosarum biovar vicieae (Finan et al., 1983), and R. leguminosarum biovar trifolii (Ronson et al., 1981) formed ineffective nodules on their host plants.

Fumarase (fumarate hydratase; EC 4.2.1.2) catalyses the interconversion of fumarate and L-malate and functions as an integral step of the TCA cycle in aerobic metabolism and, in some organisms, in the reductive branch of the cycle during fermentation and anaerobic fumarate respiration. Recent work has revealed the existence of two classes of fumarases (Woods et al., 1988b; Yumoto & Tokushige, 1988). Class I fumarases

Abbreviations: ORF, open reading frame; TCA, tricarboxylic acid.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M38241.
are thermostable homodimers of \( M \), 120000 (2 \times 60000) carrying an Fe–S cluster, and include the highly homologous products of the *Escherichia coli* *fumA* (Miles & Guest, 1984) and *fumB* genes (Bell et al., 1989) and probably also an immunologically related fumarase of *Euglena gracilis* (Shibata et al., 1985; Woods et al., 1988). Class II fumarases are thermostable homotetramers of \( M \), 200000 (4 \times 50000). This class includes the structurally related products of the *E. coli fumC* (Woods et al., 1986), *Bacillus subtilis citG* (Moir et al., 1984; Miles & Guest, 1985) and *Saccharomyces cerevisiae* FUM1 genes (Wu & Tzagoloff, 1987), and of the human liver (Kinsella & Doonan, 1988), rat liver (Suzuki et al., 1989) and porcine heart (Sacchettini et al., 1988) fumarase genes. In fact, all animal fumarases characterized to date exhibit the biochemical properties characteristic of class II fumarases. *E. coli* appears to be the only proven case for the presence of both class I and class II fumarases in one and the same organism.

Here we report the cloning, sequencing and mutational analysis of the fumarase gene (*fumC*) from the soybean root nodule bacterium *B. japonicum*. Contrary to our expectations, the *B. japonicum fumC* mutant strain was not affected in symbiotic nitrogen fixation. This led to the discovery of a second fumarase activity that could functionally substitute for the missing FumC protein.

### Methods

**Bacterial strains, vectors and recombinant plasmids.** These are listed in Table 1. *B. japonicum* strain 110spc4 is a spectinomycin-resistant derivative of strain 311b10 (US Department of Agriculture, Beltsville, MD, USA) and is called “wild type” throughout this paper. Strain FC5 is a chromosomal *fumC* deletion mutant of the wild-type in which two *fumC*-internal *SphI* fragments have been replaced by a Km\(^{\text{R}}\) fragment. *E. coli* strain JH400 is deleted for *fumA*, *fumB* and *fumC*. Plasmid pGS210 carries the *E. coli fumC* gene in pUC12 in the opposite orientation to the *lac* gene in the vector. The plasmids used to transform strain JH400 were first isolated from strain JM109 (\( r^{-}\), \( m^{+}\)).

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH400</td>
<td>Δ(lac-proAB) F′ (traD36 proAB+ lacI− ΔM15)</td>
<td>Henson et al. (1987)</td>
</tr>
<tr>
<td>JM101</td>
<td>hsdR17 recA1</td>
<td>Messing (1983)</td>
</tr>
<tr>
<td>JM109</td>
<td>hsdR17 recA1</td>
<td>Messing (1983)</td>
</tr>
<tr>
<td>S17-1</td>
<td>hsdR RP4-2kan::Tn7 tet::Mu (chromosomally located)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>B. japonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110spc4</td>
<td>Spc(^{\text{R}}) (referred to as wild-type)</td>
<td>Regensburger &amp; Hennecke (1983)</td>
</tr>
<tr>
<td>110rf15</td>
<td>Rif(^{\text{R}})</td>
<td>Regensburger &amp; Hennecke (1984)</td>
</tr>
<tr>
<td>259</td>
<td>Fix− Tn5 mutant of 110rf15; Km(^{\text{R}}) Rif(^{\text{R}})</td>
<td>Regensburger et al. (1986)</td>
</tr>
<tr>
<td>FC5*</td>
<td><em>fumC</em> deletion mutant (1.2 kb <em>SphI</em> fragment) of the wild-type; Km(^{\text{R}}) Spc(^{\text{R}})</td>
<td>This work</td>
</tr>
<tr>
<td>Vectors and recombinant plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13mp18</td>
<td>Sequencing vectors</td>
<td>Norrander et al. (1983)</td>
</tr>
<tr>
<td>M13mp19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBLS(KS)+</td>
<td>Ap(^{\text{R}})</td>
<td>Stratagene, La Jolla, CA, USA</td>
</tr>
<tr>
<td>pGS210</td>
<td><em>E. coli fumC</em> in pUC12</td>
<td>Woods &amp; Guest (1987)</td>
</tr>
<tr>
<td>pJF118EH</td>
<td>lacI− derivative of the expression vector pKK223-3; Ap(^{\text{R}})</td>
<td>P. Grob, ETH Zürich (1979)</td>
</tr>
<tr>
<td>pRK290pol2</td>
<td>pKK290 TcR Mob+ with the pBLS polylinker in the EcoRI site</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km(^{\text{R}}) Tra+ for bi-parental mating</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pSU202</td>
<td>Ap(^{\text{R}}) Km(^{\text{R}}) Om(^{\text{R}}) from pR4</td>
<td>Pharmacia, Uppsala Sweden</td>
</tr>
<tr>
<td>pUC4-KIXX</td>
<td>Ap(^{\text{R}}) Km(^{\text{R}}) source of the Km(^{\text{R}}) 1-2 kb <em>Smal</em> fragment</td>
<td>Regensburger (1986)</td>
</tr>
<tr>
<td>pL20-11H</td>
<td>Cosmid clone carrying the wild-type region corresponding to the Tn5 mutation of mutant 259</td>
<td></td>
</tr>
<tr>
<td>pJR2034†</td>
<td>3-5 kb BamHI−BglII subclone of pL20-11H in pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pJR2000†</td>
<td>3-7 kb PstI fragment of pRJ2034 in the PstI site of pBLS (KS)+</td>
<td>This work</td>
</tr>
<tr>
<td>pJR2001†</td>
<td>1-2 kb <em>SphI</em> deletion of pJR2200 replaced by 1-2 kb Km(^{\text{R}}) <em>Smal</em> fragment from pUC4-KIXX</td>
<td>This work</td>
</tr>
<tr>
<td>pJR2002†</td>
<td>3-7 kb <em>EcoRI−BamHI</em> fragment of pRJ2201 in the EcoRI/PstI sites of pSU202</td>
<td>This work</td>
</tr>
<tr>
<td>pJR2004†</td>
<td>2-7 kb <em>Xhol</em> fragment of pRJ2200 in the <em>SalI</em> site of pJF118EH with <em>tac</em> opposite to <em>fumC</em></td>
<td>This work</td>
</tr>
<tr>
<td>pJR205†</td>
<td>2-7 kb BamHI−<em>BamHI</em> fragment of pRJ2200 in the <em>BamHI</em>/<em>HindIII</em> sites of pJF118EH with <em>fumC</em> under the control of <em>tac</em></td>
<td>This work</td>
</tr>
<tr>
<td>pJR217†</td>
<td>2-9 kb PstI−<em>SalI</em> fragment of pRJ2034 in the PstI/<em>Xhol</em> sites of pRK290pol2</td>
<td>This work</td>
</tr>
</tbody>
</table>

* The genomic structure of this mutant is shown in Fig. 1.
† The inserts of these recombinant plasmids are shown in Fig. 1.
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Fig. 1. Restriction map of the B. japonicum wild-type DNA region containing fumC. The map also shows the Tn5 insertion site in mutant 259 (vertical arrow). The bold line below fumC indicates the sequenced region shown in Fig. 2, which was established by sequencing the M13 subclones shown above the map. The open arrows mark the SphI fragments deleted and replaced by a kanamycin resistance (KmR) gene in mutant FC5. In the plasmid constructs below the map, the shaded and filled arrowheads denote the positions and directions of the lac and tac promoters, respectively. The shaded box represents the DNA fragment carrying the kanamycin resistance gene.

Abbreviations: B, BamHI; Bg, BglII; (Bg), BglII site lost after cloning; E, EcoRI; N, NsiI; P, PstI; S, SalI; Sc, SacI; Sm, SmaI; Sp, SphI; X, XhoI.

Construction of the fumC mutant. B. japonicum mutant 259 was found after screening several thousand mutants generated by random Tn5 mutagenesis for a Nod+ and Fix− phenotype. The Tn5-mutagenized region of mutant 259 was cloned from total DNA, and used as a hybridization probe to find the corresponding wild-type region in a cosmid library (Regensburger, 1986). A 5.5 kb BamHI-BglII fragment of the cosmid clone pL20-1H containing the Tn5 insertion site was cloned in the BamHI site of pUC19, resulting in plasmid pRJ2034 (Fig. 1). For the purpose of constructing the SphI deletion in fumC, the PstI fragment of pRJ2034 containing fumC was first subcloned in the PstI site of pBLS(KS)+. The resulting clone, pRJ2200 (Fig. 1), carried fumC in the opposite orientation to the lac gene of the vector. Plasmid pRJ2200 was then digested with SphI, treated with T4 DNA polymerase to generate blunt ends, and ligated to the SmaI fragment of pUC4-KIXX containing the KmR gene. The orientation of the KmR gene in the resulting plasmid, pRJ2201, is opposite to that of fumC (Fig. 1). The EcoRI-BamHI fragment of pRJ2201 containing the mutated region was finally subcloned in pSUP202 digested with EcoRI and PstI. To achieve this, the BamHI site of the insert and the PstI site of the vector were treated with Klenow and T4 DNA polymerases, respectively, to generate blunt ends. The final construct, pRJ2202 (Fig. 1), carried fumC in the opposite orientation to the lac gene of the vector. Plasmid pRJ2202 was then transferred into B. japonicum by conjugation. Exconjugants in which a double crossover recombination event had occurred were selected for KmR CmR, and screened for loss of TcR. The correct genomic structure of the deletion mutation in strain FC5 was confirmed by appropriate Southern blot hybridizations (not shown).

Construction of complementing plasmids. The plasmid pRJ2204 was constructed by cloning the 2.7 kb XhoI fragment of pRJ2034 (Fig. 1) in the SalI site of the expression vector pJFl18EH, which carried the tac promoter as well as the lacP repressor gene. The orientation of the fumC gene in this construct was opposite to that of the tac promoter. In pRJ2205 (Fig. 1) the 3.7 kb BamHI–HindIII fragment of pRJ2200 was cloned in the BamHI–HindIII sites of pJFl18EH so that fumC was under the control of the tac promoter. Plasmid pRJ2217 was constructed by cloning the fumC-containing PstI–SalI fragment of pRJ2034 in the vector pRK290p201 digested with PstI and XhoI. The orientation of fumC in this clone was the same as the TcR gene of the vector.

Media and growth conditions. B. japonicum was grown aerobically in PSY medium (Regensburger & Hennecke, 1983) and anaerobically in YEM with 10 mM-KNO₃ (Daniel & Appleby, 1972). Growth with different carbon sources was tested in aerobic liquid cultures in modified H-M medium (Cole & Elkan, 1973). The modification consisted in adding K⁺ ions to the medium by replacing 0.12 g NaH₂PO₄·H₂O with 0.6 g NaH₂PO₄·2K₂HPO₄. Carbon sources (succinate, fumarate, L-malate, L-glutamate, and L-aspartate) were added to a final concentration of 20 mM. The E. coli strains were grown in complex LB medium or in M9 minimal medium (Miller, 1972). The complementation assay of the E. coli Fum− strain JH400 was done in aerobic liquid M9 salts medium (Miller, 1972) supplemented with 40 μg methionine ml⁻¹, and using 40 mM-fumarate as carbon source. Ampicillin (200 μg ml⁻¹) was added for plasmid selection.
Bacterial crosses. The pSUP202-derived plasmids were transferred into *B. japonicum* by conjugation using *E. coli* S17-1 as donor (Simon et al., 1983). The pRK290ps2-based plasmid or the vector itself were transferred from *E. coli* HR101 to *B. japonicum* by tri-parental matings using the helper plasmid pRK2013, which provided the transfer functions in trans (Ditta et al., 1980).

**Symbiotic nitrogen fixation assay.** The symbiotic nitrogen fixation (Fix) activity of the *B. japonicum* wild-type and mutant strains was tested in soybean plant infection tests as described previously (Hahn & Hennecke, 1984). Acetylene reduction (Turner & Gibson, 1980) was measured in 20-d-old plants.

**DNA work.** Cloning, restriction endonuclease digests, plasmid isolations, transformations, Southern blots, and hybridizations were performed using standard protocols (Maniatis et al., 1982). Radioactive labelling of DNA probes was done by random priming (Feinberg & Vogelstein, 1984). The *fumC* DNA was sequenced on both strands using overlapping M13 clones prepared by Bal31 nuclease deletions and by subcloning restriction fragments. The sequencing strategy is depicted at the top of Fig. 1. The sequencing reactions (Sanger et al., 1977) were performed using M13-specific fluorescent dye primers and the method and equipment for automated DNA sequencing (Applied Biosystems Sequencer model 370).

**Analysis of the sequences determined.** The analysis of the DNA sequences, as well as data bank searches, protein sequence comparisons and alignments were performed with the GCG sequence analysis software package release 6.2 (University of Wisconsin, Madison, WI, USA).

**Enzymology.** Crude cell extracts of *B. japonicum* were prepared from cultures grown to stationary phase. The cells were collected and washed three times with sodium phosphate buffer (100 mM, pH 7.3). The cells were then disrupted by passing them twice through a French pressure cell (124 MPa). The lysate was centrifuged at 30000 × g for 30 min and the supernatant was used for the enzyme assays. Fumarase activity was measured at 25 °C by monitoring the production of fumarate from L-malate as the increase in absorbance at 250 nm (Hill & Bradshaw, 1969). Activities were expressed as μmol fumarate min⁻¹ (mg protein)⁻¹ using a value of 1479 M⁻¹ cm⁻¹ for the absorption coefficient of fumarate at pH 7.3 and 250 nm (O'Hare & Doonan, 1985). Mesoaconase activity was measured at 25 °C using the same conditions as described for the fumarase assay but using DL-citramalate (100 mM) as substrate. Protein concentrations were determined as described by Bradford (1976).

**Results**

**Identification and sequencing of the *B. japonicum* fumC gene**

The *fumC* gene was found fortuitously in the course of studying a *B. japonicum* Tn5 mutant (strain 259) that was reported to have a pleiotropic defect in nodule development and symbiotic nitrogen fixation (Regensburger et al., 1986). The genomic region containing the Tn5 mutation of strain 259 was cloned as described in Methods. DNA sequence analysis of a region located about 1 kb adjacent to the mapped Tn5 insertion site revealed the existence of an open reading frame (ORF) that was identified as *fumC* (see text below and Fig. 1). Whilst the independent genetic characterization of mutant 259 was still in progress (Ebeling, 1990) we became interested in a more detailed characterization of *fumC*. In particular it was thought that the construction of a *fumC* mutant could provide a means to further substantiate the importance of C₄-dicarboxylate metabolism and the TCA cycle for symbiotic nitrogen fixation in *B. japonicum*.

A restriction map of the cloned region with the location of the *fumC* gene and the sequencing strategy are shown in Fig. 1. A 1647 bp DNA sequence is shown in Fig. 2. This sequence contained the 1461 bp *fumC* ORF that started at nucleotide 22. The ATG at this position was arbitrarily chosen as the *fumC* start codon because it was preceded by a well-conserved *E. coli*-like Shine–Dalgarno sequence, 5'-GAGGAG-3' (Shine & Dalgarno, 1975). However, it should be noted that there are other potential start codons at the beginning of *fumC* (Fig. 2).

The predicted amino acid sequence of the *fumC*-encoded protein is also shown in Fig. 2; it consisted of 487 amino acids and had a calculated M, of 52650. Protein sequence comparisons revealed that the FumC polypeptide had a high degree of similarity (72-76%) to fumarases of class II (Fig. 3). The percentage of identical amino acids between the *B. japonicum* FumC protein and all others aligned in Fig. 3 ranged from 53% to 64%. There was also a weaker, but significant homology to aspartase sequences from *E. coli* W (39% identity, 60% similarity; Takagi et al., 1985) and *Pseudomonas fluorescens* (44% identity, 64% similarity; Takagi et al., 1986b) (not shown).

**Complementation of an *E. coli* Fum⁻ mutant with the *B. japonicum* fumC gene**

The *B. japonicum* fumC gene was tested for its ability to complement the Fum⁻ phenotype of *E. coli* JH400. This mutant strain carried deletions in all three fumarase genes (Table 1). The strain was transformed with different plasmids, carrying either the *B. japonicum* fumC gene (pRJ2200, pRJ2204 and pRJ2205, see Fig. 1), the *E. coli* fumC gene (pGS210), or just the vector (pJF118EH). The transformants were tested for growth in minimal medium with fumarate as carbon source. Those transformants that carried a pJF118EH-based plasmid (i.e. pRJ2204 and pRJ2205) or the vector itself were also tested in the presence of 1 mM-IPTG to allow induction of transcription from the tac promoter located on the vector. Only pRJ2205 in the presence of IPTG and pGS210 (the positive control) were capable of complementing the aerobic Fum⁻ phenotype of JH400. This result indicated that the *B. japonicum* fumC gene, upon induction by a functional *E. coli* promoter, could be expressed in *E. coli* to produce an active fumarase capable of restoring the Fum⁻ phenotype of strain JH400.
Bradyrhizobium japonicum fumarase gene (fumC)

Fig. 2. DNA sequence containing the ORF of the \textit{fumC} gene. The predicted amino acid sequence of the FumC protein is also shown. A presumptive ribosome binding site upstream of the first ATG is underlined. Four possible translational starts are emphasized in bold letters. The Sph\textbf{I} sites used to construct the deletion are overscored.
Fig. 3. Amino acid sequence alignment of all available class II fumarases. The top sequence shows the consensus, in which the upper-case letters mean 100% conservation and the lower-case letters more than 50% conservation. The numbering system refers only to the amino acid arrangement in this figure and not to any of the sequences. The sequences were obtained from the following sources: EcFumC, *E. coli* FumC (Woods et al., 1986); BsCitG, B. subtilis CitG (Miles & Guest, 1988); ScFuml, *S. cerevisiae* Fum1 (Wu & Tzagoloff, 1987); HumFum, human liver fumarase (Kinsella & Doonan, 1986); RatFum, rat liver fumarase (Suzuki et al., 1989); PigFum, porcine heart fumarase (Sacchetti et al., 1988).
Table 2. Fumarase activity in B. japonicum strains

Fumarase activities are expressed in μmol fumarate min⁻¹ (mg protein)⁻¹. Heat treatment was carried out at 52 °C.

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Specific activity</th>
<th>Percentage of wild-type activity</th>
<th>Specific activity after heat treatment</th>
<th>Percentage of wild-type activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.16</td>
<td>100</td>
<td>0.08 (10 min)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 (50 min)</td>
<td></td>
</tr>
<tr>
<td>FC5</td>
<td>0.09</td>
<td>56</td>
<td>0.00 (10 min)</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type (pRJ2217)</td>
<td>0.79</td>
<td>493</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Wild-type (pRK290pol2)</td>
<td>0.14</td>
<td>88</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>FC5(pRJ2217)</td>
<td>0.37</td>
<td>230</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>FC5(pRK290pol2)</td>
<td>0.06</td>
<td>37</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested.

Phenotypic analysis of the B. japonicum fumC deletion mutant FC5

A B. japonicum fumC deletion mutant (strain FC5) was constructed by marker exchange as described in Methods. In the mutation two adjacent fumC-internal SphI fragments (1149 bp plus 19 bp) were replaced by a 1.2 kb SmaI fragment carrying a kanamycin resistance gene. The genotypic structure of the mutation is shown in Fig. 1. A plant infection test showed that the mutant FC5 had a Nod⁺ Fix⁺ phenotype in symbiosis with soybean. In free-living culture, strain FC5 was able to grow like the wild-type with all carbon sources tested (see Methods). Also, it grew anaerobically in YEM medium with nitrate as terminal electron acceptor.

The fumarase activities in crude extracts were determined for the wild-type and mutant FC5 grown aerobically in modified H-M medium with 20 mM succinate. The data obtained are summarized in Table 2. The fumarase activity in strain FC5 was 56% of that measured in the wild-type. This result suggested that fumC coded for a fumarase that contributed more than 40% of the total fumarase activity present in B. japonicum grown under these conditions.

The previous reports that E. coli possessed two biochemically distinct types of fumarases that differed also in their thermal stabilities (Woods et al., 1988b) prompted us to test the fumarase activities of the B. japonicum wild-type and the mutant FC5 after heat treatment. The crude extract of strain FC5 had no detectable fumarase activity after a 10 min heat treatment at 52 °C (Table 2). By contrast, the extract from the wild-type retained 50% of its fumarase activity after 10 min incubation at 52 °C, and even after 50 min incubation 31% fumarase activity was still detectable. This result provided evidence for the thermal stability of the B. japonicum FumC protein and the thermal instability of a second fumarase.

Mesaconase catalyses the interconversion of L-citramalate and mesaconate, but also has quite high fumarase activity due to its low substrate specificity. This enzyme was shown to function in the TCA cycle of Pseudomonas arvilla and Pseudomonas fluorescens (Oda et al., 1987). To test whether the postulated second fumarase activity observed in B. japonicum was to be ascribed to a mesaconase, B. japonicum was assayed for mesaconase activity (see Methods). No mesaconase activity, however, was detected in crude extracts of the wild-type.

Furthermore, the fumarase activity proved to be specific for L-malate, as no activity could be detected when D-malate was used as substrate in the assay. Pre-incubation of the crude extracts with 150 mM-D-malate resulted in a 50% inhibition of the fumarase activity (data not shown).

Complementation of the B. japonicum fumC deletion mutant FC5

To prove that the diminished fumarase activity observed in the fumC mutant was due to the mutation described, strain FC5 was complemented in trans with the wild-type fumC region cloned in the broad-host-range plasmid pRK290pol2. The strains carrying the complementing plasmid pRJ2217 (Fig. 1) showed about five-fold higher fumarase activities than the corresponding parent strains carrying either no plasmid or the vector alone (Table 2). This proved that the partial loss of fumarase activity observed in the mutant FC5 as compared to the wild-type was due to the fumC mutation described. The result also made it seem likely that the fumC promoter lay within the cloned fragment, thus allowing expression of the fumC gene.
Discussion

The *B. japonicum* ORF described in this paper encodes a functional fumarase which, by analogy with the homologous gene in *E. coli*, was designated *fumC*. The evidence for this was based on (i) the similarity of the predicted amino acid sequence of the FumC protein to fumarases from other organisms, (ii) the ability of the *fumC* gene to complement the fumarase deficiency of an *E. coli* mutant, and (iii) the reduced fumarase activity observed in a *B. japonicum* *fumC* deletion mutant.

Sequence comparisons

The high degree of amino acid sequence similarity between the *B. japonicum* FumC protein and other fumarases is typical for all class II fumarases. It is not clear whether the first 15 codons encoding the amino acids of the non-conserved N-terminus of the predicted *B. japonicum* FumC sequence are translated. The amino acid sequence alignment shows that only *B. japonicum* FumC and *S. cerevisiae* Fum1 have an extended N-terminus. However, the N-terminal sequence of Fuml was postulated to be involved in the transport of the protein from the cytoplasm into mitochondria and might not be present in the mature protein (Wu & Tzagoloff, 1987). In the *B. japonicum* FumC protein the first 30 amino acids, up to the first conserved residue, do not have the characteristics of a signal peptide for membrane transport and, above all, there is no reason to believe that this enzyme is not cytoplasmic.

All class II fumarases, including *B. japonicum* FumC, share a high degree of homology with aspartases (Takagi *et al.*, 1986a; Woods *et al.*, 1986). Fumarases and aspartases catalyse analogous reactions, i.e. a hydration and amination of fumarate, respectively. These structural and functional similarities suggest a possible conservation in the active site of both enzymes (Woods *et al.*, 1988b; Yumoto & Tokushige, 1988). Structural and functional correlations with fumarases, aspartases and also the biochemically related argininosuccinase have been discussed by Woods *et al.* (1986, 1988a). Class I and class II fumarases are only marginally related, as seen from primary sequence comparisons and their biochemical properties. They show less than 6% sequence identity (Woods *et al.*, 1988b), and their catalytic mechanisms are believed to be different (Yumoto & Tokushige, 1988). Woods *et al.* (1988a,b) reported the only significant homology between these two fumarase classes: the sequence motif GS--1M--K--N--Q (around position 444 in Fig. 3, and around position 456 in *E. coli* FumA) is found within the largest stretch (18 residues) of identical amino acids in class II fumarases (Fig. 3) and also belongs to a well-conserved domain in all aspartases.

Alternative fumarase activity and physiological role of the fumarases

The presence of fumarase activity in the crude extracts from the *B. japonicum* *fumC* deletion mutant FC5 led us to postulate the existence of a second fumarase, probably a class I type fumarase. *E. coli* possesses two of these fumarases: FumA and FumB. FumA accounts for about 80% of the fumarase activity when *E. coli* grows aerobically, whereas FumB is expressed only under anaerobic conditions and functions in the fermentative pathway that leads to the production of succinate (Yumoto & Tokushige, 1988; Woods & Guest, 1987). It seems unlikely that an obligate aerobe such as *B. japonicum* possesses a functional FumB homologue. Therefore, a FumA-homologous enzyme is more probably responsible for the observed thermolabile fumarase activity.

The existence of an alternative fumarase in *B. japonicum* explains why the *fumC* mutant could grow in minimal medium with fumarate as sole carbon source. Similarly, assuming that a functional TCA cycle is essential in symbiosis, the Fix+ phenotype of the FC5 mutant can now be explained by the existence of a second fumarase. In both physiological states this second fumarase was fully sufficient to phenotypically mask the *fumC* defect.

Prior to this study the only bacterium known to have more than one fumarase was *E. coli*. While the biological roles of the *E. coli* FumA and FumB proteins are now clear (see above), the role of the *E. coli* FumC protein has not yet been established. FumC accounts for up to 25% of the fumarase activity in aerobically grown *E. coli* cells. The respective gene is expressed under both aerobic and anaerobic conditions, and its weak promoter is not responsive to environmental changes (Woods & Guest, 1987). The substrate affinity of the *E. coli* FumC protein, like that of FumA, is higher for fumarate than for L-malate, suggesting a role in the TCA cycle (Woods *et al.*, 1988b).

The regulation of *B. japonicum* *fumC* expression also remains to be elucidated. The gene is expressed aerobically and contributes 40% or less (see above) of the total fumarase activity in aerobic cultures. The total fumarase specific activity measured here in free-living cultures of *B. japonicum* is equivalent to that reported for bacteroids isolated from root nodules of 18-d-old plants (Karr *et al.*, 1984). These authors showed that the fumarase activity in the bacteroids increased proportionally to the nitrogenase activity and reached a maximum of 0.35 μmol min⁻¹ mg⁻¹ at day 24 after infection. Similarly, *Rhizobium leguminosarum* MNF3841 bacteroids showed a threefold higher fumarase activity (0.96 μmol min⁻¹ mg⁻¹) as compared to chemostat
cultures grown in fumarate (0.29 µmol min⁻¹ mg⁻¹) (McKay et al., 1989). From these studies it is evident that at least one of the two fumarases plays an important role during symbiosis and its activity may be regulated.

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References


Bradyrhizobium japonicum fumarase gene (fumC) 999
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