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 (Gardioli *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 (*dct*) mutants of *Bradyrhizobium japonicum* (Humbeck & Werner, 1988), *R. meliloti* (Bolton *et al.*, 1986; Engelke *et al.*, 1989), *Rhizobium leguminosarum* biovar *viciae* (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.*, 1988*b*; 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.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant genotype or phenotype	Reference or origin
<i>E. coli</i>		
HB101	<i>hsdR hsdM recA13 strA</i>	Boyer & Roulland-Dussoix (1969)
JH400	$\Delta(dgs-fumCA-manA)$ D1 $\Delta(mel-fumB)$ <i>zdg-232::Tn10 metB1 spoT1 relA</i>	Henson <i>et al.</i> (1987)
JM101	$\Delta(lac-proAB)$ F'(traD36 <i>proAB</i> ⁺ <i>lacI</i> ^q Δ M15)	Messing (1983)
JM109	<i>hsdR17 recA1</i>	Messing (1983)
S17-1	<i>hsdR</i> RP4-2kan::Tn7 <i>tet</i> ::Mu (chromosomally located)	Simon <i>et al.</i> (1983)
<i>B. japonicum</i>		
110spc4	Spc ^R (referred to as wild-type)	Regensburger & Hennecke (1983)
110rif15	Rif ^R	Regensburger & Hennecke (1984)
259	Fix ⁻ Tn5 mutant of 110rif15; Km ^R Rif ^R	Regensburger <i>et al.</i> (1986)
FC5*	<i>fumC</i> deletion mutant (1.2 kb <i>SphI</i> fragment) of the wild-type; Km ^R Spc ^R	This work
Vectors and recombinant plasmids		
M13mp18	Sequencing vectors	Norrand <i>et al.</i> (1983)
M13mp19		
pBLS(KS)+	Ap ^R	Stratagene, La Jolla, CA, USA
pGS210	<i>E. coli fumC</i> in pUC12	Woods & Guest (1987)
pJF118EH	<i>lacI</i> ^q derivative of the expression vector pKK223-3; Ap ^R	Fürste <i>et al.</i> (1986)
pRK290pol2	pRK290 Tc ^R Mob ⁺ with the pBLS polylinker in the <i>EcoRI</i> site	P. Grob, ETH Zürich
pRK2013	Km ^R Tra ⁺ for bi-parental mating	Figurski & Helinski (1979)
pSUP202	Ap ^R Km ^R Cm ^R ; <i>oriT</i> from RP4	Simon <i>et al.</i> (1983)
pUC4-KIXX	Ap ^R Km ^R ; source of the Km ^R 1.2 kb <i>SmaI</i> fragment	Pharmacia, Uppsala Sweden
pL20-11H	Cosmid clone carrying the wild-type region corresponding to the Tn5 mutation of mutant 259	Regensburger (1986)
pRJ2034†	5.5 kb <i>BamHI</i> - <i>BglII</i> subclone of pL20-11H in pUC19	This work
pRJ2200†	3.7 kb <i>PstI</i> fragment of pRJ2034 in the <i>PstI</i> site of pBLS (KS)+	This work
pRJ2201†	1.2 kb <i>SphI</i> deletion of pRJ2200 replaced by 1.2 kb Km ^R <i>SmaI</i> fragment from pUC4-KIXX	This work
pRJ2202†	3.7 kb <i>EcoRI</i> - <i>BamHI</i> fragment of pRJ2201 in the <i>EcoRI/PstI</i> sites of pSUP202	This work
pRJ2204†	2.7 kb <i>XhoI</i> fragment of pRJ2200 in the <i>SalI</i> site of pJF118EH with <i>p_{tac}</i> opposite to <i>fumC</i>	This work
pRJ2205†	3.7 kb <i>BamHI</i> - <i>HindIII</i> fragment of pRJ2200 in the <i>BamHI/HindIII</i> sites of pJF118EH with <i>fumC</i> under the control of <i>p_{tac}</i>	This work
pRJ2217†	2.9 kb <i>PstI</i> - <i>SalI</i> fragment of pRJ2034 in the <i>PstI/XhoI</i> sites of pRK290pol2	This work

* The genomic structure of this mutant is shown in Fig. 1.

† The inserts of these recombinant plasmids are shown in Fig. 1.

are thermolabile homodimers of M_r 120 000 ($2 \times 60 000$) 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.*, 1988b). Class II fumarases are thermostable homotetramers of M_r 200 000 ($4 \times 50 000$). 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, 1986), 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 311b110 (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^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⁺).

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 (Km^R) 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, *Bam*HI; Bg, *Bgl*II; (Bg), *Bgl*II site lost after cloning; E, *Eco*RI; N, *Nsi*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I.

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 *Bam*HI–*Bgl*II fragment of the cosmid clone pL20-11H containing the Tn5 insertion site was cloned in the *Bam*HI site of pUC19, resulting in plasmid pRJ2034 (Fig. 1). For the purpose of constructing the *SphI* deletion in *fumC*, the *Pst*I fragment of pRJ2034 containing *fumC* was first subcloned in the *Pst*I 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 *Sph*I, treated with T4 DNA polymerase to generate blunt ends, and ligated to the *Sma*I fragment of pUC4-K1XX containing the Km^R gene. The orientation of the Km^R gene in the resulting plasmid, pRJ2201, is opposite to that of *fumC* (Fig. 1). The *Eco*RI–*Bam*HI fragment of pRJ2201 containing the mutated region was finally subcloned in pSUP202 digested with *Eco*RI and *Pst*I. To achieve this, the *Bam*HI site of the insert and the *Pst*I site of the vector were treated with Klenow and T4 DNA polymerases, respectively, to generate blunt ends. The final construct, pRJ2202 (Fig. 1), was transferred into *B. japonicum* by conjugation. Exconjugants in which a double crossover recombination event had occurred were selected for Km^R Cm^R , and screened for loss of Tc^R . 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 *Xho*I fragment of pRJ2034 (Fig. 1) in the *Sal*I site of the expression vector pJF118EH, which carried the *tac* promoter as well as the *lac*^R repressor gene. The orientation of the *fumC* gene in this construct was opposite to the *tac* promoter. In pRJ2205 (Fig. 1) the 3.7 kb *Bam*HI–*Hind*III fragment of pRJ2200 was cloned in the *Bam*HI/*Hind*III sites of pJF118EH so that *fumC* was under the control of the *tac* promoter. Plasmid pRJ2217 was constructed by cloning the *fumC*-containing *Pst*I–*Sal*I fragment of pRJ2034 in the vector pRK290pol2 digested with *Pst*I and *Xho*I. The orientation of *fumC* in this clone was the same as the Tc^R 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- KNO_3 (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 NaH_2PO_4 l^{-1} with 0.6 g NaH_2PO_4/K_2HPO_4 l^{-1} . 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 μ g methionine ml^{-1} , and using 40 mM-fumarate as carbon source. Ampicillin (200 μ g 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 pRK290pol2-based plasmid or the vector itself were transferred from *E. coli* HB101 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 $\mu\text{mol fumarate min}^{-1}$ (mg protein) $^{-1}$ using a value of 1479 $\text{M}^{-1}\text{cm}^{-1}$ for the absorption coefficient of fumarate at pH 7.3 and 250 nm (O'Hare & Doonan, 1985). Mesaconase 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_r* of 52 650. 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.

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1  ACTGCCTATATAGAGGAGCACATGAAGAGGCCGCGCGGCAATTCGCGCGGCGGAATGGAT  60
   . . . . . M K R P R G I S R G G M D
61  GTGCTCATGGCCAAATCTGCACGCACGAAGACCGCTCGCCCGCGACCCGACCGAGACC  120
   V L M A K S A R T K T A R P A T R T E T
121 GACAGTTTCGGTCCCATCGAGGTCCCCTCCGATCGCTATTGGGGCGCGCAGACCGAACGT  180
   D S F G P I E V P S D R Y W G A Q T E R
181 TCGCGGCAGAATTTCGCGATCGGCACGGATCGCATGCCGATCTCGCTCGTGCATGCCGCTC  240
   S R Q N F R I G T D R M P I S L V H A L
   SphI SphI
241 GGCATCGTCAAGCTCGCCGCGGCGCAGTCCAAACCGCGAGCTCGGCCTGCTCGACCGAGCGC  300
   G I V K L A A A Q S N R E L G L L D Q R
301 CGCGCCAGTGCCATATCCGCGCGCGCGCGAGGTATCGACGGCAGACCTCGACGATCAT  360
   R A S A I I R A A R E V S T A D L D D H
361 TTCCCGCTCGTGTGTGGCAGACCGGCTCGGGCAGCGAGACCAACATGAACCTCAACGAG  420
   F P L V V W Q T G S G T Q T N M N L N E
421 GTGATCGCCAACCGCGCCAATGAGCTGCTCGGCGGCGAGCTCGGCGCCAAGAAGCCGGTG  480
   V I A N R A N E L L G G E L G A K K P V
481 CATCCGAACGATCAGCTCAACATGAGCCAGTCGTCGAACGACTCTTTCCACCGCAATG  540
   H P N D H V N M S Q S S N D S F P T A M
541 CATATCGCGGCGGCTAGCCGCATCACCGCCGATCTCGTTCTCTGCCCTTGGCGAACTGCTC  600
   H I A A A S R I T A D L V P A L G E L L
601 CGCGCGCTGCGCAAGAAGGAGAAGGAGTTCCGCAAGATCGTCAAGATCGGCGCGACCCAC  660
   R A L R K K E K E F A K I V K I G R T H
661 ACCCAGGACGCGACGCGGCTGACGCTCGGCCAGGAATTCTCCGGCTATGCCGCGCAGGTC  720
   T Q D A T P L T L G Q E F S G Y A A Q V
721 GAACGCGGGATCGCGCGACTGAAGGTCGCGTGAAGGAGCTCTATCCGCTGGCGCAGGGC  780
   E R G I A R L K V A V K E L Y P L A Q G
781 GGCACCGCGCTCGGCACCGGCCCTCAACGCGAAGCCGCGCTTTGCAAGACTGTTGCGAAAAG  840
   G T A V G T G L N A K P R F A R L F A K
841 CACGTTGCCGGAATCACCAAACCTGCCCTTACCAGCGCCGCAACAAATTCGAGGCGCTG  900
   H V A G I T K L P F T S A A N K F E A L
901 GCCTCGAACGATGCTTATGTGTGTCGCGCACGGCCCATCAGTTCGGTCGCGACAGGCTG  960
   A S N D A Y V L A H G A I S S V A T G L
961 TTCAAGATCGCCAACGACATCCGCCTGCTCGGATCGGGTCCGCGCTCAGGGCTCGGCGAG  1020
   F K I A N D I R L L G S G P R S G L G E
1021 CTGATCTGCGGAGAACGAACCGGGCTCGTCGATCATGCCGGGCAAGGTCAATCCGACG  1080
   L I L P E N E P G S S I M P G K V N P T
1081 CAGTGCAGGGCGATGACCATGGTGTGCTGCCAGGTGTTGCGCAATCACACCGGATCACG  1140
   Q C E A M T M V C C Q V F G N H T A I T
1141 GTCGCCGCGAGCCAGGCCCATTTCGAGCTCAACGTCTACAAGCCCGTGCTGGCCTACAAC  1200
   V A G S Q G H F E L N V Y K P V L A Y N
1201 ATGCTGCACTCGATCCGCCTGATGGCGGATGCCGCGGCTCCTTACCGAACATTGCGTC  1260
   M L H S I R L M A D A A R S F T E H C V
1261 AGCGGCATCCGCGCCGACGAAAAGCGCATAAGTGAGCTGATGCAGCGCTCGCTGATGCTG  1320
   S G I R A D E K R I S E L M Q R S L M L
1321 GTGACCGCGCTCGCACCGAAGATCGGCTACGACAACGCCGCCAAGGTGCGGAAGACGGCG  1380
   V T A L A P K I G Y D N A A K V A K T A
   SphI
1381 CATGCCAACGGCACCACGCTGAAGGAGGAAGCGCTCGGGTTCGCTCACAGCGGAC  1440
   H A N G T T L K E E A L R L G F V T A D
1441 GAGTTCGACCGTTTGGTGCAGCCGGAAGATGACGAAACCGGGTAATTCCTAATTCCG  1500
   E F D R L V Q P E K M T K P
1501 ATAGTCATCCGTAATGATACGGAGCCAAAAAGCTCATAATTGTAAGGCTTAAAGGAGAGG  1560
1561 ATTTGATTACCGTCAATGTTCCGGAAAGACGGCGTCTACGAAGCTATTCTGCCCTTGGC  1620
1621 GGGGCGAAATTCATCGTTTGATGGCCA 1647

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Fig. 2. DNA sequence containing the ORF of the *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*I sites used to construct the deletion are overscored.

	1	50	100
Cons		..masqnsfr iE.DtfgEik VpAdhywGAQ TqRst.nFkI g..terMP.p ...AfgilKr aaAevN..lG gLdpkiasAI	
BjFumC	MKRPRGISRG	GMDVLMKSA RTKTARPATR TETDSFGPIE VPSDRYWGAQ TERSRQNFRI G..TDRMPIS LVHALGIVKL AAAQSNNREL LLDQRRASAI	
EcFumC		MNTVR SEKDSMGAI VPADKLWGAQ TQSRLEHFRI ..STEKMPTS LIHALALTKR AAAKVNEDLG LLSEEKASAI	
BsCitG		MEYR IERDTMGEVK VPADKFWGAQ TQSRKENFKI G..SEKMPMR VVKAFAILKR STALANKRLG NLDVEKAEAI	
ScFum1	MLRFTNCSC	KTFVKSSYL NIRRMNSSFR TETDAFGEIH VPADKYWGAQ TQSRFQNFKI GGARERMPLP LVHAFGLVK SAAIVNESLG GLDPKISKAI	
HumFum		..MASQNSFR IEYDTFGELK VPNDKYGAQ TVRSTMNFKI GGVTERMPTP VIKAFGILKR AAAEVNQDYG .LDPKIANAI	
RatFum		..ASQNSFR IEYDTFGELK VPTDKYGAQ TVRSTMNFKI GGATERMPIP VIKAFGILKR AAAEVNQEYG .LDPKIASAI	
PigFum		..ASQDSFR IEYDTFGELK VPNDKYGAQ TVRSTMNFKI GGVTERMPIP VLKAFGILKR AAAEVNQDYG LDPKIANAI	
	101	150	200
Cons	m.aadeVaeg kldDhFPLVv wQTGSGTQ.N MN.NEVi.NR AielLggelg skkpVHPNdH vNkSQSSNDT FPTAmHIAAA l.vhe.L.P. LqkLh.aLda		
BjFumC	IRAAREVSTA	DLDDHFPLV VQTGSGTQTN MNLNEVIANR ANELLGGELG AKKPVHPNDH VNMSQSSNDS FPTAMHIAAA SRITADLVPA LGELLRALRK	
EcFumC	RQAADEVLAG	QHDDFPLAI VQTGSGTQSN MNMNEVLANR ASELLGGVGR MERKVPHPND VNKSQSSNDV FPTAMHVAAL LALRKQLIPQ LKTLTQTLNE	
BsCitG	AAVCDVVLKG	KYDDNFPLV VQTGSGTQSN MNMNEVVANR ATALLKEKNS DQTI.HPND VNRQSSNDT FPTAMHVAAL LAVYEQLVPA LDQLRNTLDE	
ScFum1	QQAADAVASG	KLDDHFPLV VQTGSGTQSN MNANEVISNR AIELLGGKIG SKQV.HPNH CNQSQSSNDT FPTVHVHIAAS LQIQNELIPE LTNLKNALAE	
HumFum	MKAADAVAEG	KLNDHFPLV VQTGSGTQTN MNVNEVISNR AIEMLGGEIG SKIPVHPNDH VNKSQSSNDT FPTAMHIAAA IEVHEVLLPG LQKLHLDALDA	
RatFum	MKAADAVAEG	KLNDHFPLV VQTGSGTQTN MNVNEVISNR AIEMLGGEIG SKKPVHPNDH VNKSQSSNDT FPTAMHIAAA LEVHQVLLPG LQKLHLDALSA	
PigFum	MKAADAVAEG	KLNDHFPLV VQTGSGTQTN MNVNEVISNR AIEMLGGEIG SKKPVHPNDH VNKSQSSNDT FPTAMHIAAA VEVHEALLPG LQKLHLDALDA	
	201	250	300
Cons	KsKefa.ivK IGRTH.QDAT PLTLGQEFSG yvqqve...e rikaa.pr.y eLAqGGTAVG TGLNt.pgfa ekva..va.l TglpFvtapN kFeALashDa		
BjFumC	KEKEFAKIVK	IGRTHQDAT PLTLGQEFSG YAAQVERGIA RLKVAVKELY PLAQGGTAVG TGLNAKPRFA RLFAKHVAGI TKLPFTSAAN KFEALASNDA	
EcFumC	KSRADFADIVK	IGRTHLQDAT PLTLGQEFSG WVAMLEHNK HIEYSLPHVA ELALGGTAVG TGLNTHPEYA RRVADELAVI TCAPFVTAPN KFEALATCDA	
BsCitG	KAKAYNDIVK	IGRTHLQDAT PLTLGQEFSG WVHMLDRSKE MILEATDKMR ALAIGGTAVG TGINAHPEFG ELVSEETKL TQGTFFSSPN KFHALTSHDE	
ScFum1	KSKEFDHIVK	IGRTHLQDAT PLTLGQEFSG YVQVVENGIQ RVAHSLKTL FLAAGGTAVG TGLNTPKPGF VKIAEQISKE TGLKFQTPAP RFEALAAHDA	
HumFum	KSKEFAQIIK	IGRTHQDAV PLTLGQEFSG YVQVVKYAMT RIKAAMPRIY ELARGGTAVG TGLNTRIGFA EKLAAKVAAL TGLPFVTAPN KFEALAAHDA	
RatFum	KSKEFAQVIK	IGRTHQDAV PLTLGQEFSG YVQVQYAME RIKAAMPRIY ELAAGGTAVG TGLNTRIGFA EKVAAKVAAL TGLPFVTAPN KFEALAAHDA	
PigFum	KSREFAQIIK	IGRTHQDAV PLTLGQEFSG YVQVVKYAIT RIKAAMPRIY ELAAGGTAVG TGLNTRIGFA EKVAAKVAAL TGLPFVTAPN NFEALAAHDA	
	301	350	400
Cons	lvea.GAlnt lacsLmKIAN DiR.LgSGPR .GlGelilPE NEPGSSIMPG KVNPTQCEAL .Tmv.aQv.m GNhvaitv.g sqGhFELNVf kP.mi.N.Lh		
BjFumC	YVLAHGAISS	VATGLFKIAN DIRLLGSGPR SGLGELILPE NEPGSSIMPG KVNPTQCEA. MTMVCQV.F GNHTAITVAG SQGHFELNVY KPVLAYNMLH	
EcFumC	LVQAHGALKG	LAASLMKIAN DVRWLASGPR CGIGEISPE NEPGSSIMPG KVNPTQCEAL .TMLCCQV.M GNDVAINGMG ASGNFELNVF RPMVIHNFLQ	
BsCitG	ITYAHGALKA	LAADLMKIAN DVRWLASGPR CGIGEIVPE NEPGSSIMPG KVNPTQSEAL .TMIAAQI.M GNDATIGFAA SQGNFELNVF KPVIIYNFLQ	
ScFum1	IVECSGALNT	LACSLFKIAQ DIRYLGSGPR CGYHELMPE NEPGSSIMPG KVNPTQNEAL .TQVCVQV.M GNNAAITFAG SQGQFELNVF KPMIVNLLN	
HumFum	LVELSGAMNT	SSCSLMKIAN DIRFLGSGPR SGLGELILPE NEPGSSIMPG KVNPTQCEAA MTMVAAQV.M GNHVAITVGG SNGHFELNVF KPMMIKNVLH	
RatFum	LVELSGAMNT	TACSLMKIAN DIRFLGSGPR SGLGELILPE NEPGSSIMPG KVNPTQCEA. MTMVAAQVCM GNHVAITVGG SNGHFELNVF KPMMIKNVLH	
PigFum	LVEHSGAMNT	TACSLMKIAN DIRFLGSGPR SGLGELILPE NEPGSSIMPG KVNPTQCEAL TMVAAQV M GNHVAITVGG SNGHFELNVF KPMMIKNVLH	
	401	450	497
Cons	S.rLL.Da.v SFtehCvvGI .an.erInel .nesIMLVTA LnpHIGYdka akiAKTAHkn G.TLKe.Ale Lgylt.e.Fd ewVkpEdMlg p.K.gr		
BjFumC	SIRLMADAAR	SFTEHCVSGI RADEKRISL MQRSLMLVTA LAPKIGYDNA AKVAKTAHAN GTTLKEEALR LGFVTADEFD RLQVPEKMT. .KPG	
EcFumC	SVRLADGME	SFNKHCavgI EPNRERINQL LNESLMLVTA LNTHIGYDKA AEIAKKAHKE GLTLKAAALA LGYLSEAEFD SWVRPEQVMG SMKAGR	
BsCitG	SVQLSDGMN	SFHDCAVGI EPNKETIQEN LSNSLMLVTA LNPHIGYENA AKIAKLAHKE GLTLKEAALK LELLTEEQFN EMVKPEDMVK P.KA	
ScFum1	SIRLITDAAY	SFRVHCVEGI KANEPRIHEL LTKSLMLVTA LNPKIGYDAA SKVAKNAHKK GITLKESALE LGVLTEKEFD EWWVPEHMLG P.K	
HumFum	SARLLGDASV	SFTENCVVGI QANTERINKL MNESLMLVTA LNPHIGYDKA AKIAKTAHKN GSTLKETAIE LGYLTAEQFD EWWKPKDMLG P.K	
RatFum	SARLLGDASV	SFTENCVVGI QANTERINKL MNESLMLVTA LNPHIGYDKA AKIAKTAHKN GSTLKETAIE LGYLTAEQFD EWWKPKDMLG P.K	
PigFum	SARLLGDAV	SFTENCVVGI QANTERINKL MNESLMLVTA LNPHIGYDKA AKIAKTAHKN GSTLKATAVE LGYLTAEQFD EWWKPRDMLG P.K	

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, 1985); ScFum1, *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 (Sacchettini *et al.*, 1988).

Table 2. *Fumarase activity in B. japonicum strains*

Fumarase activities are expressed in $\mu\text{mol fumarate min}^{-1} (\text{mg protein})^{-1}$. Heat treatment was carried out at 52 °C.

Strain (plasmid)	Specific activity	Percentage of wild-type activity	Specific activity after heat treatment	Percentage of wild-type activity
Wild-type	0.16	100	0.08 (10 min)	50
			0.05 (50 min)	31
FC5	0.09	56	0.00 (10 min)	0
Wild-type (pRJ2217)	0.79	493	NT	NT
Wild-type (pRK290pol2)	0.14	88	NT	NT
FC5(pRJ2217)	0.37	230	NT	NT
FC5(pRK290pol2)	0.06	37	NT	NT

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-citraconate 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* FumI have an extended N-terminus. However, the N-terminal sequence of FumI 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--IM--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 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ at day 24 after infection. Similarly, *Rhizobium leguminosarum* MNF3841 bacteroids showed a threefold higher fumarase activity (0.96 $\mu\text{mol min}^{-1} \text{mg}^{-1}$) as compared to chemostat

cultures grown in fumarate ($0.29 \mu\text{mol min}^{-1} \text{mg}^{-1}$) (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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