Molecular and enzymological evidence for two classes of fumarase in *Bacillus stearothermophilus* (var. *non-diastaticus*)

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The gene (*fumA_{Bst}*) encoding an oxygen-labile fumarase of *Bacillus stearothermophilus* has been cloned and sequenced. The structural gene (1542 bp) encodes a product (FumA_{Bst}) of M_r 56788 containing 514 amino acid residues. The amino acid sequence is 23% identical (37% similar) to FumA and FumB, the labile [4Fe–4S]-containing fumarases (Class I enzymes) of *Escherichia coli*. It exhibits no significant similarity to FumC and CitG, the stable fumarases (Class II enzymes) of *E. coli* and *Bacillus subtilis* (respectively). Enzymological studies indicated that FumA_{Bst} resembles the iron–sulphur-containing fumarases in being dimeric (M_r 2×58500), oxygen labile and partially reactivated by Fe²⁺ plus DTT. The *fumA_{Bst}* gene is the first gene encoding a Class I fumarase to be characterized in any organism other than *E. coli*. Enzymological and DNA-hybridization studies further indicated that *B. stearothermophilus* resembles *E. coli* in containing an oxygen-stable fumarase (Class II enzyme). Sequence comparisons revealed significant similarities between the Class I fumarases and the products of adjacent open-reading frames (*orfZ1* and *orfZ2*) located upstream of the macromolecular synthesis operon (*rpsU–dnaG–rpoD*) at 67 min in the *E. coli* linkage map. Located downstream of *fumA_{Bst}*, there is an unidentified gene (*orf2*), which is homologous to the rhizobial *nodB* genes involved in the initiation of root nodule formation.

Introduction

Fumarase (fumarate hydratase: EC 4.2.1.2) catalyses the interconversion of fumarate and L-malate. It functions in the citric acid cycle during aerobic metabolism and in the reductive pathway of the branched form of the citric acid cycle which provides fumarate as an anaerobic electron acceptor during anaerobic metabolism (Guest *et al.*, 1990).

Two distinct classes of fumarase have been discovered, primarily by cloning and sequencing the fumarase genes of *Escherichia coli* K12 (Guest & Roberts, 1983; Miles & Guest, 1984; Guest *et al.*, 1985; Woods *et al.*, 1986, 1988a; Bell *et al.*, 1989). This organism contains at least three fumarase genes: *fumA* and *fumB* encoding two similar Class I enzymes, designated FumA and FumB; and *fumC*, encoding the unrelated Class II enzyme, designated FumC.

FumA and FumB (Class I) are oxygen-labile, iron–sulphur-containing homodimeric enzymes (subunit M_r 60000) exhibiting 90% sequence identity (Bell *et al.*, 1989). Aerobically inactivated FumA can be reactivated by anaerobic incubation with Fe²⁺ and thiols (Yumoto & Tokushige, 1988; Flint *et al.*, 1989; Ueda *et al.*, 1991). Recent EPR spectroscopy has shown that FumA resembles aconitase in containing a [3Fe–4S] cluster which is converted to a [4Fe–4S] cluster upon activation (Ueda *et al.*, 1991; Flint *et al.*, 1989, 1992). Other Class I fumarases include the immunologically cross-reacting and highly unstable enzyme of *Euglena gracilis* (Shibata *et al.*, 1985; Woods *et al.*, 1988a) and the oxygen-labile fumarase detected in *Bradyrhizobium japonicum* (Acuña *et al.*, 1991). In addition, these enzymes may belong to a wider group of hydratases and other enzymes which contain Fe–S centres but do not catalyse redox-reactions e.g. aconitase, L-(+)-tartrate dehydratase, maleate dehydratase, L-serine dehydratase, lactyl-CoA dehydratase, 2-hydroxyglutaryl-CoA dehydratase, dihydroxyacid dehydratase, DNA endonuclease III and glutamine PRPP amidotransferase (Switzer, 1989; Cammack, 1991; Buckel, 1992).

In contrast, FumC (Class II) is a stable homotetrameric

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enzyme (subunit M_r 50000) which is related to the products of the *citG* gene of *Bacillus subtilis* (Moir *et al.*, 1984; Miles & Guest, 1985), the *FUM1* gene of *Saccharomyces cerevisiae* (Wu & Tzagoloff, 1987) and the *fumC* gene of *Bradyrhizobium japonicum* (Acuña *et al.*, 1991), and to the mammalian mitochondrial fumarases (Kinsella & Doonan, 1986; Suzuki *et al.*, 1989; Sachettini *et al.*, 1988). The Class II fumarases from diverse sources exhibit a remarkable degree of amino acid sequence conservation (50–65% identity). It is also clear that the Class II fumarases belong to a family of structurally related homotetrameric enzymes including aspartase, argininosuccinase (Woods *et al.*, 1988b) and adenylo-succinase (He *et al.*, 1992), which are 38%, 15% and 19% identical to FumC, respectively.

In previous studies with *citG* mutants of *B. subtilis* there was no evidence for multiple fumarases (Rutberg & Hoch, 1970), suggesting that the obligately aerobic bacilli (Gram-positive) may differ from the facultatively anaerobic enterobacteria (Gram-negative) in containing a single Class II fumarase rather than representatives of both classes. A fumarase gene of *B. stearothermophilus* was therefore sought for use in amplifying a thermostable and oxygen-insensitive enzyme for structural and mutagenic studies. This paper reports the characterization of a gene (*fumA_{Bst}*) encoding a Class I (oxygen-labile) fumarase, as well as preliminary evidence for the presence

of a Class II fumarase in *B. stearothermophilus*. An adjacent gene related to the *nodB* genes of nodulating bacteria was also characterized, and a novel relationship between the Class I fumarases and a pair of unidentified *E. coli* genes was detected.

Methods

Bacterial strains, plasmids and phagemids. The bacterial strains, plasmids and phagemids are listed in Table 1. *E. coli* JH400 lacks all three *fum* genes and was used in the cloning and complementation studies; other strains used for the isolation and propagation of phages and phagemids were: C600 (λ ZAPII derivatives), JM101 (M13 derivatives), and XL1-Blue (phagemids). The gene bank of *B. stearothermophilus* var. *non-diastaticus* (DSM 2334) contained products of complete and partial *EcoRI* digestion (2 to 9 kb) ligated in the Stratagene vector λ ZAPII (Mallinder *et al.*, 1992). Phagemids were excised from λ ZAPII derivatives by the *in vivo* procedure provided by the supplier.

Media and growth conditions. The rich medium used for routine subculture was L-broth. A weak peptone medium (BBL) was used to detect λ fum phages as described previously (Guest & Roberts, 1983). Transformants of *E. coli* JH400 were tested for complementation of the Fum[−] phenotype on plates of citrate-free minimal medium (Cole & Guest, 1980) containing glucose (11 mM), sodium L-malate (40 mM) or sodium fumarate (40 mM) as sole carbon source and supplemented with methionine (40 μ g ml^{−1}) and ampicillin (100 μ g ml^{−1}) as required. All strains were grown aerobically at either 30 °C (λ -infected cultures), 55 °C (thermophiles) or 37 °C (other strains).

Table 1. *Bacterial strains, phagemids and plasmids*

Bacteria, phagemids and plasmids	Relevant genotype or phenotype	Source or reference
<i>Bacterium</i>		
<i>E. coli</i>		
W3110	Wild-type	
JM101	$\Delta(lac-proAB)$ F' (<i>traD36 proA⁺B⁺ lacI^qZAM15</i>)	Messing (1983)
JH400	$\Delta(dgs-fumCA-manA)D1 \Delta(mel-fumB) zdg-232::Tn10$ <i>metB1 spoT1 relA</i>	Henson <i>et al.</i> (1987)
C600	<i>thr leuB6 thi-1 supE44 tonA21 lacY1</i>	Guest & Roberts (1983)
XL1-Blue	<i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1</i> F' (<i>proA⁺B⁺ lacI^q lacZAM15 Tn10</i>)	Bullock <i>et al.</i> (1987)
<i>B. stearothermophilus</i> var. <i>non-diastaticus</i> DSM 2334	Bio [−]	Mallinder <i>et al.</i> (1992)
<i>B. stearothermophilus</i> NRRL 1174	Undefined auxotrophy	Liao <i>et al.</i> (1986)
<i>B. subtilis</i> 1604	<i>trpC2</i>	Moir <i>et al.</i> (1979)
<i>B. caldotenax</i> DSM 406		Sharp <i>et al.</i> (1986)
<i>Phagemid</i>		
pBluescript	Ap ^R	Stratagene
pGS507 } pGS509 } pGS511 }	pBluescript derivative, Ap ^R }	This study
<i>Plasmid</i>		
pGS96	<i>citG_{Bsu}</i> in pHV33, Ap ^R	Moir <i>et al.</i> (1984)
pGS54	<i>fumAC_{Eco}</i> in pBR322, Ap ^R	Guest & Roberts (1983)
pGS210	<i>fumC_{Eco}</i> in pUC12, Ap ^R	Woods & Guest (1987)

Enzymology. Cultures were grown in L-broth to early stationary phase, harvested, and disrupted by ultrasonic treatment (6 periods of 30 s, frequency 10 MHz, at 0 °C with 30 s intervals) in 40 mM-potassium phosphate buffer, pH 7.8. Cell debris was removed by centrifuging at 11 600 *g* for 30 min and supernatants (cell-free extracts) were assayed the same day for fumarase by following the formation of fumarate from L-malate at 240 nm and at 55 °C (unless otherwise stated): activities are expressed in units (μmol fumarate formed min^{-1}) per mg protein using an extinction coefficient of $2.44 \text{ l mmol}^{-1} \text{ cm}^{-1}$ at 240 nm (Hill & Bradshaw, 1969). For heat treatments, samples (100 μl) of the extracts were incubated at 50 °C for specified times either in air (aerobic samples) or degassed and held in an anaerobic cabinet (0 °C for 30 min) under an atmosphere of $\text{H}_2/\text{N}_2/\text{CO}_2$ (5:85:10, by vol.) and sealed prior to incubation (anaerobic samples); the residual activities were assayed under aerobic conditions. Protein was assayed according to Bradford (1976) using Bio-Rad reagent. Native M_r values were determined by gel-filtration with crude extracts (0.5 ml) of *B. stearothermophilus* (1.2 units) and plasmid (pGS509)-amplified JH400 (46 units), using a Pharmacia XK26 column containing a Sephacryl 200HR matrix, equilibrated and eluted (1.5 ml min^{-1}) under aerobic conditions with 40 mM-potassium phosphate buffer, pH 7.8. The column was calibrated using standard proteins (M_r): β -amylase (200 000); alcohol dehydrogenase (150 000); carbonic anhydrase (29 000); cytochrome *c* (12 300). Fractions (5 ml) were collected and assayed for fumarase activity. SDS-PAGE was performed by the method of Laemmli (1970).

DNA manipulation and sequencing strategy. Standard methods were used for cloning, restriction endonuclease digestion, plasmid isolation and transformation (Sambrook *et al.*, 1989). The sequencing strategy involved cloning the 0.9 kb, 0.6 kb and 1.4 kb *Eco*RI fragments of pGS509 into the corresponding site of M13mp18 and sequencing with universal primer (Fig. 1). Deleting *Hind*III and *Sa*I fragments from appropriate restriction fragment derivatives allowed further sequence to be obtained. Synthetic primers were used to complete the sequence with pGS509 as the double-stranded template. Single-stranded and double-stranded templates were sequenced by the dideoxy chain termination method, using Sequenase (Sequenase protocols, United States Biologicals). Oligonucleotides were synthesized using an Applied Biosystems 381 DNA synthesizer.

Nucleotide sequences were compiled and analysed using the Staden computer programs (Staden, 1980, 1982, 1984; Staden & McLachlan, 1982). Multiple sequence alignments were generated initially by the CLUSTAL microcomputer programs (Higgins & Sharp, 1988, 1989) and refined by DIAGON and visual inspection. For other computer analyses, including database searching with TFasta and FASTA, the University of Wisconsin Genetics Computer Group (UWGCG) package (Devereux *et al.*, 1984) was accessed through the SEQUENT VAX 3600 on node DLVH (Daresbury Laboratory, Warrington, UK).

In vitro transcription-translation and Southern blotting. The prokaryotic DNA-directed translation kit containing an *E. coli* S-30 extract (Amersham) was used with intact phagemid DNA and L-[^{35}S]-

Fig. 1. Restriction maps of phagemids (pGS507, pGS509 and pGS511) containing the *fumA_{Bst}* gene and summary of sequencing strategy. The unlabelled arrows denote the polarity of *lacZ* transcription in pBluescript. The enlarged region shows the 3 kb fragment cloned in pGS509, the subfragments cloned in M13mp18 and the *Hind*III or *Sa*I deletion derivatives. The positions and polarities of coding regions are indicated by filled arrows and the hybridization probes by broken arrows. In the sequencing strategy, thin arrows denote directions and extents of sequence obtained from M13 derivatives or double stranded pGS509 using universal primer (I) or specifically designed primers (◆). Relevant restriction sites are as follows: E, *Eco*RI; H, *Hind*III; and S, *Sa*I.

methionine, according to the manufacturer's instructions. For Southern blotting, bacterial DNA was digested with *Eco*RI, fractionated by electrophoresis (0.8% agarose, Tris/acetate/EDTA gel) and transferred to nitrocellulose membrane (Sambrook *et al.*, 1989). The blots were hybridized for 18 h at 60 °C with 6 × SSC (low stringency) and washed (2 × 15 min) with 2 × SSC plus 0.1% SDS (w/v) at 65 °C [2 × SSC contains NaCl (0.3 M) and sodium citrate (0.03 M, pH 7)]. Probes containing specific regions of four fumarase genes were derived from M13 templates by primer-extension labelling with digoxigenin (Boehringer Mannheim): *citG*, 0.8 kb *Bcl*I–*Hind*III fragment of pGS96 in M13mp10 (Miles & Guest, 1985); *fumC*, 0.8 kb *Xho*I–*Bcl*I fragment in M13mp10 (Woods *et al.*, 1986); *fumA*, 0.7 kb *Pst*I–*Bcl*I fragment in M13mp8 (Miles & Guest, 1984); *fumA_{Bst}*, M13mp18 derivatives F14LH and F6R, see Fig. 1. The corresponding restriction fragments were isolated by electrophoresis in a low melting point agarose gel for use in hybridization and detection was according to the manufacturer's instructions.

Materials. Restriction endonucleases and DNA ligase were purchased from Northumbria Biologicals, the Sequenase kit was from Cambridge Bioscience, the non-radioactive (digoxigenin) DNA-

labelling and detection kit was from Boehringer Mannheim and the prokaryotic DNA-directed translation kit, L-[³⁵S]methionine (> 37 TBq mmol⁻¹) and [α -³⁵S]thio-dATP (> 37 TBq mmol⁻¹) were purchased from Amersham. *M_r* markers for SDS-PAGE and gel-filtration were obtained from BDH and Sigma, respectively.

Results and Discussion

Cloning a fumarase gene of B. stearotherophilus

A λ ZAPII gene bank of *B. stearotherophilus* was screened for λ *fum* phages by their ability to produce transduction plaques on *E. coli* JH400 (Δ *fumAC* Δ *fumB*). Two distinct and stably inherited plaque morphologies were detected. Those designated smoke rings were turbid rings of 3–6 mm diameter having clear centres that eventually filled with diffuse micro-colonies, whereas the galaxies consisted of compact clusters of micro-colonies.

Fig. 2 Aerobic and ammonium persulphate inactivation and molecular properties of FumA_{Bst}. (a) Extracts were incubated aerobically and anaerobically at 50 °C for different periods prior to assaying for residual fumarase activity (%). Strains and initial specific activities are: JH400(pGS509), aerobic (■), anaerobic (□), 42.3 units (mg protein)⁻¹; *B. stearotherophilus*, aerobic (▲), anaerobic (△), 2.2 units (mg protein)⁻¹. (b) Extracts were treated with ammonium persulphate (1 mM) at 0 °C in air and assayed for residual fumarase activity (%). Strains and initial specific activities are: JH400(pGS509), ammonium-persulphate-treated (■), untreated (□), 14.2 units (mg protein)⁻¹; *B. stearotherophilus*, ammonium-persulphate-treated (▲), untreated (△), 1.2 units (mg protein)⁻¹. (c) Gel-filtration profiles for fumarase activities (units ml⁻¹) with extracts of JH400(pGS509) (■), and *B. stearotherophilus* (△). The vertical arrows denote *M_r* deduced from the elution of standard proteins. (d) Coomassie-blue-stained SDS-PAGE gel (15%) of cell-free extracts of JH400 (lane 1) and JH400(pGS509) (lane 2). FumA_{Bst} (arrowed) and the positions of *M_r* markers are indicated.

The aetiology of these morphologies is unknown, but neither was exactly like the dense transduction plaques obtained with λ *fumA* and λ *fumB* or the lysogen-filled plaques obtained with the *B. subtilis* counterpart, λ *citG* (Guest & Roberts, 1983; Moir, 1983). This is probably because λ ZAPII contains a *ColE1* replicon which would prevent stable prophage integration. Studies with the corresponding lysogens were frustrated by instability and irreproducibility, possibly for the same reason.

The complementing phages were purified, converted to pBluescript phagemids, and studies with double-stranded phagemid DNA from nine independent phages showed that they contain a nested set of three to five *EcoRI* fragments inserted with the same polarity. Four phagemids (represented by pGS509) contained the common 3 kb insert consisting of three *EcoRI* fragments (0.92, 0.64 and 1.40 kb), whereas three (represented by pGS511) contained an extra 1.16 kb *EcoRI* fragment, and two (represented by pGS507) contained a further 1.50 kb *EcoRI* fragment (Fig. 1). There was no correlation between plaque morphology and inserted DNA.

Characterization of the phagemid-encoded fumarase

The fumarase-deficient strain JH400 grows on malate minimal medium but not with fumarate as the sole

carbon source. This nutritional lesion was complemented by transformation with pGS507, pGS509 and pGS511, as with pGS54 (*fumAC*), pGS96 (*citG*) and pGS210 (*fumC*), but not with pBluescript. The fumarase activity [units (mg protein)⁻¹] in cell-free extracts of JH400 (0.006) was likewise increased in transformants containing pGS507 (45.3), pGS509 (31.8), pGS511 (8.4), pGS54 (91.0), pGS96 (7.4) and pGS210 (40.3). Further studies with JH400(pGS509) indicated that a polypeptide of M_r 58 000 is amplified to approximately 40% of soluble cell protein in freshly transformed cells (Fig. 2). In gel-filtration, the fumarase activity eluted with an apparent M_r of 117 000 indicating that the enzyme is homodimeric. The fumarase activity was unstable in air and rapidly inactivated by ammonium persulphate (Fig. 2) but the rate of inactivation could be lowered by fumarate (2 mM) and partial reactivation was obtained by incubating with 0.5 mM-Fe²⁺ plus 50 mM-dithiothreitol. These results indicate that the common 3 kb inserts of pGS507, pGS509 and pGS511 express an oxygen-labile homodimeric (Class I) fumarase, FumA_{Bst}. When amplified in *E. coli*, the enzyme had a thermostability consistent with its source. The temperature optimum was 55–60 °C and, when incubated under anaerobic conditions, the half-lives (min) at different temperatures were: > 100 (50 °C), 20 (60 °C) and < 5

Fig. 3. *In vitro* transcription-translation analysis. Autoradiogram of ³⁵S-labelled polypeptides expressed from pGS507 (lane 1), pGS509 (lane 2), pGS511 (lane 3), pBluescript (lane 4), no DNA (lane 5) and pAT153 (lane 6). Relevant labelled products and the positions of M_r markers are indicated.

GAATTCGCAAGCTGAATCTTTTAACTGCTGCAATGAATGCTGAAACACTTCCCTATCCCCCTAAAAAATGATCAGCACGTGAATGC
 EcoRI 10 20 30 40 50 60 70 80 90
 -35 -10 +1 RBS *fumA_{Bst}*
 CTTTCCAATCAAATTAAGAAGTATAATAAAAAATTGTGTTAAGTGGGAGTTTTTTGAAGTGAATGTAAGGGGGATAAGCGGGATGAAAA
 100 110 120 130 140 150 160 170 180
 K L Y E S M Y Q L I V E T S T K L P K D V R R A I L K A K Q
 AATTATATGAGAGTATGTATCAATTAATGTGGAACGCTACAAAATTGCCGAAGGATGTCCGCCGCTATTTTGAAGCGAAGCAGC
 190 200 210 220 230 240 250 260 270
 R E N A G T R A A M S L A T I T E N I K M A D E N V S P I C
 GTGAAAATGCTGGTACAGAGCTGCGATGTGCTTGCCACGATAACCGAAAAATTTAAAATGGCAGATGAAAATGTATCGCGGATCTGCC
 280 290 300 310 320 330 340 350 360
 Q D T G G L P T F K I K V P V G I N Q I Q I K E T I K K A I A
 AAGATACCGGATTGCCGACATTTAAAATTAAAGTGCCAGTCGGTATCAATCAAATCAAATAAAGGAAACATAAAAAACGATCGCTC
 370 380 390 400 410 420 430 440 450
 Q A T K D G K L R P N S V D S L T G E N S G D N L G E G L P
 AAGCGACGAAGGACGGGAAGCTTCGCCCCGAACCTCGGTCGATTCACTCACTGGAGAAAATTCGGCGGATAACTTGGGCGAAGGGCTTCAG
 460 470 480 490 500 510 520 530 540
 V V K F E Q W E K D Y M D V R L I L K G G G C E N K N I Q Y
 TCGTAAATTTGAACAATGGGAAAAAGATTATATGGATGTTGCTTAAATATTAAGGCGGCGCTGCGAAAACAAAAATATCAATACA
 550 560 570 580 590 600 610 620 630
 S L P C E L E G L G R A G R D L D G I R K C I L H A V Y Q A
 GCCTGCCGTGCCAACTAGAAGGATTGGGAAGAGCCGGCAGAGATTGGATGGAATCCGCAAATGTATTTGCATGCGGTATATCAAGCAC
 640 650 660 670 680 690 700 710 720
 Q G Q G C S A G F I G V G I G G D R S A G Y D L A K E Q L F
 AGGGCCAAGGATGCAGCGCAGGATTCATTGGAGTTGGAATCGGTGGAGACCGTTCAGCGGGGTACGATTTAGCGAAAGAACAATTATTC
 730 740 750 760 770 780 790 800 810
 R E V D D V N P N E E L R Q L E E Y I M E N A N K L G I G T
 GCGAAGTCGATGACGTAAATCCGAACGAAGAATTGCGGCAATTTGGAAGAATATATTATGGAATAAGCAAGCTTGGCATTGGAACGA
 820 830 840 850 860 870 880 890 900
 M G F G G E T T L L G C K V G A M H R I P A S F F V S V A Y
 TGGGATTGGCGGGGAAACCACTTTATTAGGCTGCAAAGTCGGCGCAATGCACAGAAATTCCTGCGAGCTTTTGTCTGTGCGCATACA
 910 920 930 940 950 EcoRI 970 980 990
 N C W A F R R L G V H I D P N T G E I I K W L Y Q D G E D V
 ACTGCTGGGCATTCGCCCTTTAGGTGTGCATATCGATCCAAATACGGGTGAAATCATAAAATGGCTTTATCAAGATGGAGAAGATGTTG
 1000 1010 1020 1030 1040 1050 1060 1070 1080
 D F Q E N A A Q E E H L A S T D S E R R V I T L Q A P I T E
 ATTTTCAAGAAATGCTGCACAAGAAGACATTTAGCATCAACGGACTCTGAAAGACGTGTATCAGCTGCAAGCGCAATTACAGAAG
 1090 1100 1110 1120 1130 1140 1150 1160 1170
 E Q I R E L K V G D V V R I N G I I Y T G R D A I H K Y L M
 AACAAATCCGCGAGCTGAAAGTCGCGGACGTTGTTCCGATCAACGGAATCATTTACACAGGAAGGGATGCCATCCATAAATATTTAATGG
 1180 1190 1200 1210 1220 1230 1240 1250 1260
 D H D A P V T D L N G Q I I Y H C G P V M L K D E N G N W E V
 ATCATGTGCGCGGTTGATTAAACGGTCAAATTATTTACCCTGCGGACCTGTATGTTAAAGATGAAAACGGCAATTGGGAAGTGA
 1270 1280 1290 1300 1310 1320 1330 1340 1350
 K A A G P T T S I R E E P Y Q G D I M K K F G I R A V M G K
 AAGCGGCGGGCCGACCACTTCCATTCGCGAAGAGCCTTATCAAGGGGATATTATGAAAAAATTTGGCATCCGTGCGGTAATGGGCAAG
 1360 1370 1380 1390 1400 1410 1420 1430 1440
 G G M G Q K T L Q A L K E H G G V Y L N A I G G A A Q Y Y A
 GCGGCATGGGGCAAAAACGCTGCAAGCTTTAAAAGAGCATGGCGGAGTATACTGAATGCGATTGGCGGTGCTGCTCAATATTATGCGG
 1450 1460 1470 1480 1490 1500 1510 1520 1530
 D C I E A V E G V D L L E F G I P E A M W H L R V K D F T A
 ATTGCATCGAAGCGGTTGAGGTGTTGACTTGCTGGAGTTTGAATTCAGAAAGCTATGTGGCATTGCGCGTAAAGACTTCACGGCCG
 1540 1550 1560 1570 EcoRI 1590 1600 1610 1620
 V V T M D S H G N S L H E D I E K S S L E K L S Q F K E P V
 TTGTACAAATGGATTACACGGCAACAGCCTGCATGAAGACATTGAAAAATCGTCATTAGAAAAGCTTTCTCAATTTAAAGAGCCGGTAT
 1630 1640 1650 1660 1670 1680 1690 1700 1710
 514
 F S *
 TTAGCTAAATATTAAGTGAATGCCATAGCCAGATGATTGGCTATGGTATTTTGTGAAATCAAGATGGAGCATAATTCCTAAAAACGGA
 1720 1730 1740 1750 1760 1770 1780 1790 1800

Fig. 4. For legend see facing page.

An *in vitro* transcription-translation analysis (Fig. 3) showed that the three phagemids express a major labelled product (M_r 58 000), presumed to be the fumarase subunit, two minor products (M_r 43 000 and 27 000) and β -lactamase (M_r 30 000). The novel products exceed the

The complete nucleotide sequence of the common 3 kb fragment was determined by the strategy summarized in Fig. 1. The sequence (Fig. 4) was fully overlapped and 98% was derived from both strands. Two complete coding regions were detected using the FRAMESCAN and POSITIONAL BASE PREFERENCES options of ANALYSEO

(Staden, 1984) in conjunction with a codon usage table compiled from the open reading frames detected by Mallinder *et al.* (1992). The first (coordinates 174 to 1715) encodes a product of M_r 56 788 (514 amino acid residues including formyl-methionine), which corresponds to the major *in-vitro*-synthesized product. It could be identified as the Class I fumarase of *B. stearothermophilus*, FumA_{Bst}, by virtue of its sequence similarity with FumA and FumB. The second coding region (*orf2*, coordinates 1842 to 2636) encodes a product of M_r 30 563 (*orf2*, 265 amino acids), which probably corresponds to the *in-vitro*-synthesized product of M_r 27 000 (Fig. 3) and resembles the NodB proteins (see below). There is also an incomplete coding region (*orf3*) starting at coordinate 2699 and extending for 263 bp to the end sequenced region, but this could not be identified by database searching.

The *fumA*_{Bst} coding region is preceded by a putative promoter sequence resembling the σ^{70} and $E\sigma^{43}$ promoters of *E. coli* and *B. subtilis* (respectively) and a potential ribosome-binding site (Fig. 4). It is followed by a potential stem-loop structure ($\Delta G = -89.9 \text{ kJ mol}^{-1}$) which could serve as a *rho*-independent terminator. The start codon (TTG) predicted for *orf2* is not uncommon in Gram-positive bacilli – e.g. *B. subtilis argC* (Smith *et al.*, 1986) and *gerA* (Feavers *et al.*, 1990), and *B. pumilis cat86* (Harwood *et al.*, 1983) – and it is preceded by a well-placed ribosome-binding site. Neighbouring ATG codons at coordinates 1777, 1822, 1972, 1990 and 2184 (Fig. 4) are not associated with a ribosome-binding site, and in the latter case are situated well downstream of a region of similarity with the NodB proteins (see below). No potential terminator sequences for *orf2*, or ribosome-binding sites for *orf3*, were detected.

Multiple sequence alignment and detection of two *fumA*-related genes in *E. coli*

In addition to the observed similarities between FumA_{Bst} and the FumA and FumB proteins, a database search revealed that OrfZ1 and OrfZ2, the products of adjacent open reading frames located upstream of the *rpsU-dnaG-rpoD* operon at 67 min in the *E. coli* linkage map (Nesin *et al.*, 1987) are similar to the respective N- and C-terminal moieties of the FumA-related proteins. This suggests that OrfZ1 and OrfZ2 combine in forming an iron-sulphur-containing hydratase-dehydratase with an $\alpha_2\beta_2$ structure like the L-tartrate dehydratase of *Pseudomonas putida* (Kelly & Scopes, 1986) or the $(\alpha\beta)_2$ structure like 2-hydroxyglutaryl-CoA dehydratase of *Acetaminococcus fermentans* (Schweiger *et al.*, 1987), rather than the homodimeric (α_2) arrangement in the Class I fumarases.

A multiple sequence alignment for the FumA family is

shown in Fig. 5 and an analysis by DISTANCES showed that FumA_{Bst} is slightly closer to the combined OrfZ1/Z2 sequence (25% identity, 45% similarity) than to FumA (23%, 38%) and FumB (23%, 37%), which are themselves remarkably close (90%, 98%; Bell *et al.*, 1989). The Fum_{Bst} and OrfZ1/Z2 sequences widen the family of FumA-related proteins and draw attention to potentially important residues and motifs which could perform common structural and catalytic roles. Previous comparisons could only be made between the Class I fumarases of *E. coli* (FumA/B) and the potentially analogous iron-sulphur proteins (aconitase, ACN; the iron-responsive-element binding protein, IREBP; and isopropylmalate isomerase, IPMI), and the Class II fumarases and their relatives [aspartase, ASP; argininosuccinase, ARS; and adenylosuccinase, ASL – see Prodromou *et al.* (1992); Woods *et al.* (1988a, b); Aimi *et al.* (1990)]. The alignment shows that 58 (12.7%) of 457 equivalenced residues are conserved in all of the sequences, including 3 of the 9 or 10 cysteine residues, only 1 of the 11 to 14 histidine residues, and as many as 16 glycine plus proline residues (possibly because the latter residues flank regions of conserved secondary structure).

Cysteine residues generally provide ligands for iron atoms in iron-sulphur proteins via motifs such as CxxCxxC in bacterial ferredoxins or CxxC in ACN, where the [4Fe-4S] cluster has three cysteine ligands (Robbins & Stout, 1989a, b). The three conserved cysteine residues, C₆₂, C₁₈₆ and C₂₇₄ at positions 108, 238 and 334 in the alignment (Fig. 5), could presumably perform this function in FumA. Previously, residue C₃₁₇ (position 334) and two residues in the CxxC motif near the C-terminus of FumA were proposed as the three iron ligands (Prodromou *et al.*, 1992) but it is now clear that the CxxC motif is not conserved in the other FumA-related proteins, FumA_{Bst} and OrfZ1/Z2 (Fig. 5).

Only one histidine residue (H₃₉₆ in FumA, position 420) is conserved in the alignment (Fig. 5). This residue was identified previously as a potential active-site residue because its context resembles that of an active-site histidine residue in ACN (Prodromou *et al.*, 1992). The similarity extends to aligned sequences in related enzymes, ACN, IREBP and IPMI (Fig. 6a). However, these sequences show no similarity to those containing the sole conserved histidine in the Class II fumarases, ARS and ASL, where there is a GxTHxxA consensus (Woods *et al.*, 1988b; Aimi *et al.*, 1990).

There are three conserved lysine residues and two conserved arginine residues in the multiple alignment (Fig. 5) which could provide carboxyl-binding sites. One of the lysine residues (K₄₆₂ in FumA at position 489) is in the only significant sequence motif that is shared by both the FumA (Class I) and FumC (Class II) ASP, ARS and

FumA .Bst MEKL YESMYQLIVE TSTKLPKDVR RAILKAKQRE NAGTRAAMSL ATITENIKMA 51
Orfz1.Eco MSE SNKQALNKL TEIVANFTAM ISTRMPDDVV DK.LKQLKDA ETSSMGKIY HTWFDNMOKA 62
FumB .Eco SNKPFYIQAP FPMGKDNTFY YLLTSDYVSV ADFDGETILK VEPEALTLVA QOAFHDASF LRPQHOKQVA AI....LHDP EASENDKYVA LOFLRNSEIA 96
FumA .Eco SNKPFHYQAP FPLKKDDTEY YLLTSEHVSU SEFEGQEILK VAPEALTLA ROAFHDASF LRPHQQQQVA DI....LRDP EASENDKYVA LOFLRNSDIA 96
N A
101 ↓
FumA .Bst DENVSPICQD TGLPTFKIKV PVGI.NQIQI KETIKKAIQA ATKDGKLR.. PN.SVDSL TG ENSGNLGE LRVKPEQWE .KDYMDVRLI LKGGGCENKN 149
Orfz1.Eco IDLNRPAQD TGEIMFFVKV GSRPPLLGE QSILKQAVE ATVKAPLR.. HN.AVEIFDE VNTGKNTSG VPWVTWDIIP DNDDAEIEVY MAGGC.... 155
FumB .Eco AKGVLPICQD TGTAILVGGK GQRVWTGGGD EAALARGVYN TYIEDNLAYS QNAPLDMYKE VNTGTLNPAQ IDLYAVDGD E YKFLC....I AKGGGSANKT 192
FumA .Eco AKGVLPICQD TGTAILVGGK GQRVWTGGGD EAALARGVYN TYIEDNLAYS QNAPLDMYKE VNTGTLNPAQ IDLYAVDGD E YKFLC....I AKGGGSANKT 192
P CQD TG K LR N N G N GGG
201 ↓
FumA .Bst IQYSLPCELE GLGRAGRDLD GIRKCILHAV YQAQGCQCSA GFIVGIGGD RSA....GYD LAKEQLFREV DDVNPNEELR QLEBYIMENA NKLG....I 240
Orfz1.Eco ...TLPGRSK VL.MPSEGYE GVVKFVFNENI STLAVNACPP VLVGVASHLG ETA....AV. LSRKAILRPI GRPPSQSKSG RTGRTPGRRT QPSGDMWSTRA 246
FumB .Eco YLYQ...ETK ALLTPGK... .LKNYLVEKM RTLGTAAACPP YHIAFVIGGT SAETNLKTKV LASAKVYDEL ..PTEGNEHG QAFRDVELEK ELLIEAQNLG 283
FumA .Eco YLYQ...ETK ALLTPGK... .LKNYLVEKM RTLGTAAACPP YHIAFVIGGT SAETNLKTKV LASAKVYDEL ..PTEGNEHG QAFRDVELEK ELLIEAQNLG 283
L C L
301 ↓
FumA .Bst GTMGFGGETT LLGCKVGAMH RIPASFFVSU AYNCWAFRRL GVHIDPNTGE IIKWLYQDGE DVDFQENAAQ EEHLASTDSE RRVITLQAPI TE...EQIREL 338
Orfz1.Z2 DRQOFSQDRT YRICRPPSVN HR..... .RCCLYRLL GASSRHAAGS CRSHL 291 **Orfz2** MKKILTTPI KA...EDLQDI 17
FumB .Eco LGAQFGGKYF AHDIRVIRLP RHGASCPVGM GVSCSADRNI KAKINREGIW IEKLEHNP..GQYIP QELRQAGEGE AVKVDLNRPM KEILAQLSQY 376
FumA .Eco LGAQFGGKYF AHDIRVIRLP RHGASCPVGM GVSCSADRNI KAKINREGIW IEKLEHNP..GKYIP BELRKAGEGE AVRVDLNRPM KEILAQLSQY 376
F C R L P
401 V
FumA .Bst KVGDVVRING IITGRDAIH KYLMD.... HDAPVDLNGQ IYHCGPVML KDENGNEWEV AAGPTTSIRE EPYQGDIMKK FGIRAVMGKG GMGQKTLQAL 433
Orfz2.Eco RVGDVIYLTG TLVTCRDVCH RRLIELK... RPIPYDLNGK AIFHAGPIVR KNGDK.WEMV SVGPTTQYAY GKFEREFIEQ TGVKLVVKGK GMGPLETEGC 113
FumB .Eco PVSTRLSLTG TIIVGRDIAH AKKLELIDAG KELPOYIKDH PIYAGPA.. KTPAG.YPSG SLGPTTAGRM DSYVDLLQSH GGSWIMLAKG NRSQOVTDAC 473
FumA .Eco PVSTRLSLTG TIIVGRDIAH AKKLERMDNG EGLPOYIKDH PIYAGPA.. KTEG.YASG SLGPTTAGRM DSYVDLQAAQ GGSWIMLAKG NRSQOVTDAC 473
V G RD H L P GPTT G KG
501
FumA .Bst KEHGGVYLNA IGGAAQYYAD .CIEAVEGVD LLEFGIPBAM WHLRVKDFTA VVT.MDSHGN SLHEDIKSS LEKLSQKPEP VFS 514
Orfz2.Eco QKFKALHWIF PAGCAVLAAT Q.VEEIEEVH WTELGMPESL WTCRVKFEFP LIASIDTHGN NLIAENKLF AERRDPIVEE ICEHVHYIK 201
FumB .Eco KHGGFYLS IGGPAVLAQ QSIKHLECYA YPELGM.EAI WKIEVEDFPA FIL.VDDKGN DFFQ..... .QIVNK QCANCTK 547
FumA .Eco KHGGFYLS IGGPAVLAQ GSIKSLECYA YPELGM.EAI WKIEVEDFPA FIL.VDDKGN DFFQ..... .QIQLT QCTRCVK 547
G A E V E G E W V F D GN

Fig. 5. A multiple amino acid sequence alignment for three Class I fumarases and the unidentified OrfZ1 and OrfZ2 proteins. Conserved residues are shown below the alignment. The aligned sequences are: fumarases A (FumA_Eco) and B (FumB_Eco) of *E. coli*; fumarase A of *B. stearothermophilus* (FumA_Bst); and OrfZ1 (OrfZ1_Eco) and OrfZ2 (OrfZ2_Eco) of *E. coli*. Conserved cysteine residues (C), a putative active-site residue (D), and insertion-deletions (—), are indicated.

Fig. 6. Sequence similarities between the Fe-S-containing fumarases and related enzymes. (a) Sequences resembling an active-site histidinyI peptide of porcine aconitase. (b) Sequences containing a motif which is conserved between Class I and Class II fumarases and related enzymes that catalyse β -elimination reactions yielding fumarate. The sequence sources and abbreviations are as follows: FumA_{Bst}, FumA.Bst; FumA and FumB of *E. coli*, FumAB.Eco (Miles & Guest, 1984; Bell *et al.*, 1989); OrfZ2, OrfZ2.Eco (Nesin *et al.*, 1987); FumC, FumC.Eco (Woods *et al.*, 1986); aspartase, AspA.Eco (Woods *et al.*, 1986); adenylosuccinase, ASL.Eco (He *et al.*, 1992); aconitase, ACN.Eco (Prodromou *et al.*, 1992); porcine heart aconitase, ACN.Por (Zheng *et al.*, 1990); isopropylmalate isomerase, IPMI.Sty (Rosenthal & Calvo, 1990); human iron-responsive-element binding protein, IREBP.Hum (Rouault *et al.*, 1990); *B. subtilis* fumarase, CitG.Bsu (Miles & Guest, 1985); human argininosuccinase, ARS.Hum (Aimi *et al.*, 1990).

ASL protein families (Fig. 6*b*). This motif was originally discovered by Woods *et al.* (1988*a, b*) and later designated as the 'signature sequence' for enzymes which catalyse β -elimination reactions (Aimi *et al.*, 1990). However, with the inclusion of further sequences, the original GSxxMxxKxN consensus has degenerated to GxxxxxxKxx, but it still draws attention to a potentially important lysine residue (Fig. 6*b*). It may also be significant that the leucine and glutamate dehydrogenases contain a conserved lysine residue in a KGGG motif (McPherson *et al.*, 1988; Nagata *et al.*, 1988; Teller *et al.*, 1992) and such a motif occurs at positions 192–195 in the three Class I fumarase sequences (Fig. 5). This motif is not retained in OrfZ1/Z2, where the substrate is unknown, but there are other lysine residues within conserved KG and Kx^{G/P} motifs at positions 489–490 and 119–121, respectively.

Distribution of the Class I and Class II fumarase genes

Hybridization probes containing segments of the *fumA*, *fumA_{Bst}*, *fumC* and *citG* genes were used with *EcoR* digests of bacterial DNA to assess the distribution of the two classes of fumarase in different species by Southern blotting. Identical results were obtained with the two *fumA_{Bst}* probes shown in Fig. 1, specific hybridization being detected with *B. stearothersophilus* NRRL1174 (23 kb) and *B. caldotenax* (9.5 kb) but not with *B. subtilis* 1604 or *E. coli* (Fig. 7a). No hybridization was detected

between the *E. coli* *fumA* probe and any of the *Bacillus* species. Using the cross-hybridizing *citG* or *fumC* probes (Guest *et al.*, 1985), hybridization was detected with *B. stearothermophilus* NRRL1174 (8.8 kb), *B. caldotenax* (10 kb) and *B. stearothermophilus* var. *non-diastaticus* (4.1 kb) (see Fig. 7*b* for results with the *citG* probe).

These results indicate that the thermophilic bacilli contain a Class I fumarase gene (analogous to *fumA_{Bst}*) and a Class II fumarase gene (analogous to the *fumC* and *citG* genes of *E. coli* and *B. subtilis*). The *B. stearothermophilus* gene bank is now being screened by hybridization for phages containing *fumC_{Bst}* DNA since none was recovered by complementation. Despite the absence of hybridization with *B. subtilis* DNA and the lack of fumarase activity in *citG* mutants (Rutberg & Hoch, 1970), it cannot be concluded that *B. subtilis* has no Class I fumarase, because the corresponding *B. subtilis* gene may not hybridize with the *fumA* probes and an oxygen-labile product may have escaped detection in previous work.

Features of Orf2

Database searches revealed a significant sequence relationship (21–39% identity, 39–52% similarity) between the 211-residue C-terminal segment of Orf2 and the NodB proteins of *Rhizobium* and *Bradyrhizobium* (Fig. 8). The precise function of the *nodB* gene has not been defined but it forms part of the *nodABC* operon.

(a)

FumA .Bst	352	T G	R D	A I	H	K Y	L	M D
FumAB.Eco	390	V G	R D	I A	H	A K	L	K E
OrfZ2.Eco	31	T C	R D	V C	H	R R	L	I E
ACN .Eco	176	P	G T	G I	C	H	Q V	N L
ACN .Por	141	P	G S	G I	I	H	Q I	I L
TREBP.Hum	120	P	G S	G I	I	H	Q V	N L
IPMI .Sty	102	P	Y Q	G I	V	H	V M	G P

(b)				*				*								
FumA .Bst	415	G	I	R	A	V	M	G	K	G	G	M	G	Q	K	T
OrfZ2.Eco	95	G	V	K	L	V	V	G	K	G	G	M	G	P	L	T
FumAB.Eco	455	G	S	M	I	M	L	A	K	G	N	R	S	Q	Q	V
CitG .Bsu	314	G	S	S	I	M	P	G	K	V	N	P	T	Q	S	E
FumC .Eco	316	G	S	S	I	M	P	A	K	V	N	P	V	V	P	E
AspA .Eco	319	G	S	S	I	M	P	A	K	V	N	P	V	V	P	E
ASL .Eco	294	G	S	S	T	M	P	H	K	V	N	P	I	D	F	E
ARS .Hum	280	G	S	S	L	M	P	Q	K	K	N	P	D	S	L	E

Fig. 7. Southern hybridization of *fumA_{Bst}* and *citG* DNA probes to *EcoRI* fragments of total DNA from other bacteria. The digoxigenin-labelled probes were (a) *fumA_{Bst}*, F14LH (Fig. 1) and (b) *citG* from *B. subtilis* (see Methods), the sizes (kb) of the hybridizing fragments are shown for digests of the following bacteria: 1, *B. subtilis* 1604; 2, *E. coli* W3110; 3, *B. stearothermophilus* NRL1174; 4, *B. caldotenax* DSM406; and 5, *B. stearothermophilus* var. *non-diastaticus*.

which is involved in the initiation of root nodulation, possibly by generating signalling molecules that stimulate plant cell mitosis (Downie, 1991; Schmidt *et al.*, 1991; Fisher & Long, 1992). Compared with the NodB proteins, Orf2 has a 54-residue N-terminal extension possessing some features of a signal peptide over the first 23 residues. It is not clear why *B. stearothermophilus* might express such a protein but further studies might help to define a common role for Orf2 and the NodB proteins in *Bacillus* and *Rhizobium*.

Conclusions

Molecular genetic and biochemical studies have shown that *B. stearothermophilus* joins *E. coli* and *Bradyrhizobium japonicum* (Acuña *et al.*, 1991) in containing two types of fumarase. The *fumA_{Bst}* gene encoding a Class I fumarase of *B. stearothermophilus* was cloned by nutritional complementation of a fumarase-deficient mutant of *E. coli*, sequenced, and identified by its ability to express an oxygen-labile and thermostable fumarase and by the sequence similarity between FumA_{Bst} and the FumA and FumB enzymes of *E. coli*. Evidence for a Class II fumarase (FumC_{Bst}) was obtained from enzyme studies and DNA hybridization. The reason why these

bacteria possess two classes of fumarase is not clear but recent studies with *E. coli* indicate that *fumC* belongs to the *soxR* modulon (Liochev & Fridowich, 1992). The *soxR* regulatory system operates during oxidative stress to maintain cellular redox balance and normal metabolic flux, and this would be favoured by replacing the unstable FumA by FumC. In contrast, *fumA* is a member of the *arcA* and *crp* modulons, i.e. subject to aerobic respiratory control and catabolite repression, whereas *fumB* is subject to anaerobic repression and belongs to the *fnr* modulon (Woods & Guest, 1987; Guest & Russell, 1992).

Database searches revealed significant similarity between the Class I fumarases and the products, OrfZ1 and OrfZ2, of two adjacent *E. coli* genes, indicating that the latter combine to form an oxygen-labile, iron-sulphur-containing hydratase-dehydratase. Work is now in progress to isolate the *fumC_{Bst}* gene and to characterize the OrfZ1/Z2 enzyme. Putative active-site residues and structural motifs were detected by sequence comparisons with aconitase, isopropylmalate isomerase and other related enzymes. *B. stearothermophilus* was also shown to contain a gene that resembles the *nodB* genes of *Rhizobium* and *Bradyrhizobium* where it is essential for the initiation of root nodulation.

1	50	100
NodB.Rm4		MKHLDY IHEPNSCDY GTEDRSIYLT FDDGNPHCT PEILDVLA
NodB.Rm1		MKHLDY IHEPNSCDY GTEDRSIYLT FDDGNPHCT PEILDVLA
NodB.Rle		MKRPAY MSEPVPNHTS GQEARCVYLT FDDGNPHCT FQILDVLA
NodB.Rlu		MKRPAY IGEVPPNHTS GQEARCVYLT FDDGNPHCT FQILDVLA
NodB.Rtr		MKRRAY ISEVPFDEA. GSDRNIIYLT FDDGNPHCT GQILDVLA
NodB.Brs		VTEFIP LFAVRNRYGD VSGTRSVYLT FDDGNPHCT PDVLDVLTQH
NodB.Aca		MSVLGQAARI TQNGSIYIT FDDGNPHSVT PAVCEILREH
Orf2.Bst	LNFRILYCS VLLFFSFFSC TSAAVSNHPI HWGFQNNNH QPPDAGKEWN	ELLNKYGAFY LGD.PSKKE.IYLT FDNQYNGYT SKILDVLT
		Y T FD G T L
101	150	200
NodB.Rm4	GVPATFFVIG TYTKSQPELI RRIVAEHEV ANHTMTHPDL STCGPHEVER	EIVEASEAII AACPQAAVRR IRAPYGVWSE EALTRSASAG LTAIHW
NodB.Rm1	GVPATFFVIG TYAKSQPELI RRIVAEHEV ANHTMTHPDL STCGPHEVER	EIVEASEAII ALVLRPRSDT YEAPYGVWSE EALTRSASAG LTAIHW
NodB.Rle	RVPTFFFAIG SYVKDHPDL RRLVAEGHDV ANHTMTHPDL ATCDPKDKR	EIDEAHOAIV SACPQALVRH LRAPYGVWTE DVLASVRAG LGAVHWS
NodB.Rlu	RVPTFFFAIG SYVKDHPDL RRLVAEGHDV ANHTMTHPDL ATCDPKDKR	EIDEAHOAIV SACPQALVRH LRAPYGVWTE DVLASVRAG LGAVHWS
NodB.Rtr	RVPTFFFAIG SYVKDHPDL RRLVAEGHDV ANHTMTHPDL ATCDPKDKR	EIDEAHOAIV SACPQALVRH LRAPYGVWTE DVLASVRAG LGAVHWS
NodB.Brs	RVPTFFFAIG SYVKDHPDL RRLVAEGHDV ANHTMTHPDL ATCDPKDKR	EIDEAHOAIV SACPQALVRH LRAPYGVWTE DVLASVRAG LGAVHWS
NodB.Aca	SALTAFQIG RFTKEYPIS RQCOLDGHAH GNHTFDHNL QDRAGEVEY	QISSAQKCLE HICRGFVRH FRAPYGAWST QILNVNKGIG LRPVSWP
Orf2.Bst	DVHATFFVTG HYLKTAFLDV KRMVKEGHV GNHSWHPDM TTISADKIKK	ELDAVSDKVK ELTGQEGTVY VRPRGIFSE RTLASEKYG YRNIFWSLAF
	FF G P GH NH HP	G L G W
201	250	
NodB.Rm4	RDWSRPGANA ...IVDAVLD SVRPGAIVLL HDGCPDDESG ..ALTGLRDQ	TMALSRIIVP ALHERGFAIR PLPPHH 217
NodB.Rm1	RDWSRPGANA ...IVDAVLD SVRPGAIVLL HDGCPDDESG ..ALTGLRDQ	TMALSRIIVP ALHERGFAIR PLPPHH 217
NodB.Rle	RDWSCPVDV ...IVDEVLA AARPGAIVLL HDGCPDDEVE QCSLAGLRDQ	TLIALSRIIP ALHSRGFEIR SLP 216
NodB.Rlu	RDWSCPVDV ...IVDEVLA AARPGAIVLL HDGCPDDEVE QCSLAGLRDQ	TLIALSRIIP ALHSRGFEIR SLP 216
NodB.Rtr	RDWSRPGVDV ...IVDAVLA AARPGAIVLL HDGCPDDEIG NCKLTGLRDQ	TVRALSILIP ALQRRGFSLR SLPQ 215
NodB.Brs	RDWSRPGVDV ...IVSSVLA AIRPGAIVLL HDGYPPEER SCTDATSRDQ	TVRALSILIP ALQRRGFSLR SLPQ 219
NodB.Aca	RDWEAPRIEN ...LINEILD NARPSSIILL HDGCPDDEAA MWDVRGGRAQ	TLAALRYVWP ALQARGFALQ PLP 210
Orf2.Bst	VDWKVNEQKG WRYAYDNIIN QIHPGAIILL H.....TVSKD	NADALDEAIK TLKQQGYTFK SLDDLVMKRM FESPFLY 265
	DW I LL H	AL L G L

Fig. 8. Multiple amino acid sequence alignment for NodB proteins and Orf2. Conserved residues are shown below the alignment and coordinates are given for the alignment and for the C-terminal residues of individual segments. The sequences and sources are: NodB.Rm4, *Rhizobium meliloti* 41 (Torok *et al.*, 1984); NodB.Rm1, *Rhizobium meliloti* 1021 (Egelhoff *et al.*, 1985); NodB.Rle, *Rhizobium leguminosarum* plasmid pRL1J1 (Rosen *et al.*, 1984); NodB.Rlu, *Rhizobium leguminosarum* (sequence unverified, submitted to EMBL Data Library, accession number S10232, by J. A. Downie); NodB.Rtr, *Rhizobium leguminosarum* bv. *trifolii* (Schofield & Watson, 1986); NodB.Brs, *Bradyrhizobium* sp. strain ANU289 (Scott, 1986); NodB.Aca, *Azorhizobium caulinodans* (Goethals *et al.*, 1989).

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