ELSEVIER

FEBS Letters 337 (1994) 93–98

FEBS 13500

FEBS LETTERS

Molecular cloning, nucleotide sequence and expression of a *Sulfolobus solfataricus* gene encoding a class II fumarase

Sonia Colombo, Margareth Grisa, Paolo Tortora*, Marco Vanoni

Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano, Via Celoria 26, I-20133 Milano, Italy

Received 29 October 1993; revised version received 29 November 1993

Abstract

Fumarase catalyzes the interconversion of L-malate and fumarate. A *Sulfolobus solfataricus* fumarase gene (*fumC*) was cloned and sequenced. Typical archaebacterial regulatory sites were identified in the region flanking the *fumC* open reading frame. The *fumC* gene encodes a protein of 438 amino acids (47,899 Da) which shows several significant similarities with class II fumarases from both eubacterial and eukariotic sources as well as with aspartases. *S. solfataricus* fumarase expressed in *Escherichia coli* retains enzymatic activity and its thermostability is comparable to that of *S. solfataricus* purified enzyme despite a 11 amino acid C-terminal deletion.

Key words: Fumarate hydratase; Archaebacteria; Thermostable enzyme; Gene cloning; *Solfolobus solfataricus*

1. Introduction

Fumarase (fumarate hydratase; EC 4.2.1.2), an enzyme catalyzing the reversible hydration of fumarate into L-malate, is widely distributed in plants, animals and bacteria [1,2]. Two classes of fumarase have been so far described [1]: the class I enzymes include thermolabile homodimers of M_r 120,000 (2 × 60,000) containing a Fe–S cluster, whereas class II enzymes are thermostable homotetramers of M_r 200,000 (4 × 50,000). *Escherichia coli* possesses two class I fumarase genes, namely *fumA* [3] and *fumB* [4] and one class II gene, *fumC* [5]. The gene products of *fumA* and *fumB* are thought to function respectively under aerobic conditions in the citric acid cycle (*fumA*) and in the generation of fumarate for use as an anaerobic electron acceptor (*fumB*) as supported both by their differential gene regulation and by their relative affinities for fumarate and malate (reviewed in [6]). In spite of these functional differences, the class I enzymes display strong sequence similarity and immunological relatedness [1].

The role of *fumC* is yet not completely understood. Based on its substrate affinities, which resemble those of *fumA*, it has been suggested that it might function as an auxiliary aerobic enzyme in the citric acid cycle [1,4].

There is evidence for the existence of two different classes of fumarases also in *Bacillus stearothermophilus* and *Bradyrhizobium japonicum*. *B. stearothermophilus* fumABst protein [7], a fumarase of *Euglena gracilis* [8] and the oxygen-labile fumarase detected in *Bradyr. japonicum* [9], belong to the first class. Class II fumarases include *Bacillus subtilis* CitG protein [10], *Bradyr. japonicum* FumC protein [9], *Saccharomyces cerevisiae* FUM1 protein [11], the mammalian fumarases [12,14] and probably also an oxygen-stable fumarase of *B. stearothermophilus* [7]. Fumarase have also been successfully used industrially for continuous production of L-malate [15].

In recent years enzymes from thermophilic organisms have been studied with increasing interest. In fact, clarification of the molecular basis of protein thermostability will widen our knowledge of structure/function relationship and aid in engineering enzymes with improved catalytic performance to be used in biotechnological processes. Proteins from archaebacteria are particularly interesting also from an evolutionary point of view. It has long been reported that archaebacteria represent a third kingdom besides eubacteria and eukariotes, but – depending upon which protein is chosen as phylogenetic probe – different relationships between kingdoms have been inferred [16]. The availability of sequences of proteins playing key metabolic or structural roles may thus contribute to clarify this point.

Here we report the first complete nucleotide sequence of an archaebacterial fumarase gene (*fumC*). The cloned enzyme shows several significant similarities with class

*Corresponding author. Fax: (39) (2) 236-2451.

SSDI 0014-5793(93)E1417-K

II fumarases from both eubacterial and eukaryotic sources and with aspartases. The *E. coli*-expressed enzyme is enzymatically active and retains a thermostability comparable to that of *S. solfataricus* purified enzyme despite a 11 amino acid C-terminal deletion.

2. Materials and methods

2.1. Enzymology

Fumarase was purified essentially according to Puchegger et al. [15], except that the last purification step was non-denaturing preparative polyacrylamide gel electrophoresis (PAGE), performed as described [18]. Fumarase activity was assayed at 60°C in 50 mM Tris/acetate, pH 8.0, 10 mM malate; 1 U enzyme activity is defined as the amount of the enzyme which converts 1 μmol substrate/min in the standard assay conditions.

Antibodies were raised in rabbits according to standard protocols [19]. Specific anti-fumarase antibodies were affinity-purified essentially as described in [20].

SDS-PAGE was performed by the method of Laemmli [21]. Immunoblotting was performed according to standard techniques. N-terminal sequence analysis of purified fumarase was performed after transfer of the protein to a poly(vinylidene difluoride) membrane (Immobilon, Millipore, Bedford, MA, USA) using an automated protein sequencer [22].

2.2. Enzymes, radioactive biochemicals and synthetic oligonucleotides

Restriction enzymes were purchased from Promega (Madison, WI, USA) and Boehringer-Mannheim (Mannheim, Germany). Radioactive biochemicals were obtained from Amersham (Amersham, UK). Oligonucleotides were obtained from Primm s.r.l. (Milano. Italy).

2.3. Bacteria strains, genomic library and cloning vectors

Cells of *S. solfataricus* MT-4 (ATCC 49155) were kindly supplied by *Servizio di Fermentazione dell'Istituto per la Chimica di Molecole di Interesse Biologico del CNR*, Arco Felice, Italy. The expression library constructed in the λgt11 vector from *S. solfataricus* genomic DNA was kindly provided by Prof. M. Rossi, University of Napoli, Italy. *E. coli* strain Y1090 (*Δlac$_{U169}$ Δ(lon) araD139 strA supF trpC22*::Tn*10 mcrA*/ pMC9) and Y1089 (*Δlac$_{U169}$ Δ(lon) araD139 strA hflA150 chr*::Tn*10*/ pMC9) were used for plating λgt11 phages and obtaining lysogens, respectively. Plasmids pUC19 and pGEM-3Z were used for subcloning and DNA sequencing.

2.4. Isolation and characterization of phage clones

Standard recombinant DNA techniques were according to Sambrook et al. [23]. The library was screened with affinity-purified antibodies using an adaptation of the method of Young and Davis [24]. The inserts of the positive clones were analyzed by restriction mapping, subcloned and sequenced by the chain-termination method using the Pharmacia T7 sequencing kit as suggested by the manufacterer.

2.5. Computer analysis

The amino acid sequence of fumarase was compared to other proteins in the SwissProt protein sequence database using FASTA program [25]. Multiple alignments were obtained using PileUp program form the GCG package (Genetics Computer Group Inc.).

3. Results and discussion

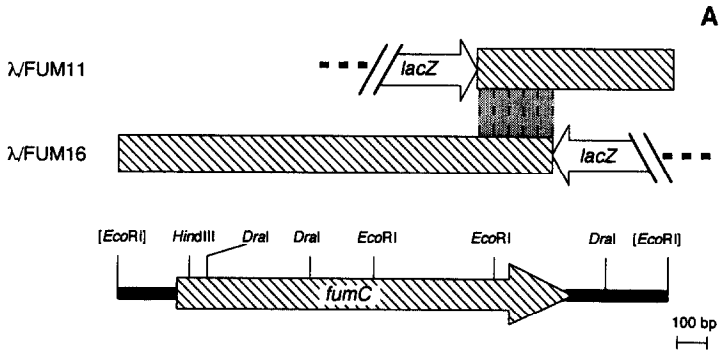
3.1. Molecular cloning and nucleotide sequencing of fumC, a fumarase-encoding gene

A fumarase-encoding gene was cloned using an expression library constructed in the λgt11 vector from *S. solfataricus* genomic DNA. About 2×10^5 phage clones were screened with affinity-purified anti-fumarase antibodies. Four positive phages (out of eight interacting with the antibodies) were isolated by repeated plaque purification. Antibodies affinity-purified from the proteins expressed by two λgt11 recombinant lysogens recognized a protein comigrating with fumarase in a *S. solfataricus* crude extract, suggesting that the isolated inserts, named FUM11 and FUM16, code for fumarase from *S. solfataricus* (data not shown). These phages were analyzed in more detail. After removal of the phage arms by *Kpn–Sac*I digestion, *Kpn*I–*Sac*I fragments were subcloned in pUC19, generating plasmids pUC19/FUM11 and pUC19/FUM16 characterized by restriction mapping and nucleotide sequence. The complete sequence of these DNA fragments was determined after subcloning in plasmid pGEM-3Z using proper restriction sites and three synthetic oligonucleotides as primers. FUM11 and FUM16 sequences overlap for 245 bp and altogether cover a region of 1802 bp (Fig. 1A). An open reading frame extending 1314 bp was found (Fig. 1B) encoding a protein of 438 amino acids with a predicted molecular mass of 47,899 in good agreement with the value directly measured for the enzyme (45 kDa per subunit). Two in frame ATG codons – shown in bold in Fig. 1B – were found at positions 150 and 171. The predicted amino acid sequence encoded starting from nucleotide 171 is identical to the experimentally determined N-terminal sequence of purified *S. solfataricus* fumarase – double underlined in Fig. 1B – suggesting that translation starts at position 171 and confirming the identity of the cloned gene. Because the gene encodes a class II fumarase homologous to *E. coli fumC* protein, the gene has been called *fumC*. FUM11 fragment does not contain the complete sequence of the gene, since a stop codon – TGA – is found only on fragment FUM16 in a position extending 33 bp downstream the last nucleotide of fragment FUM11.

The codon usage of *fumC* shows a high preference towards A and T , reflecting a low G–C content of the

→

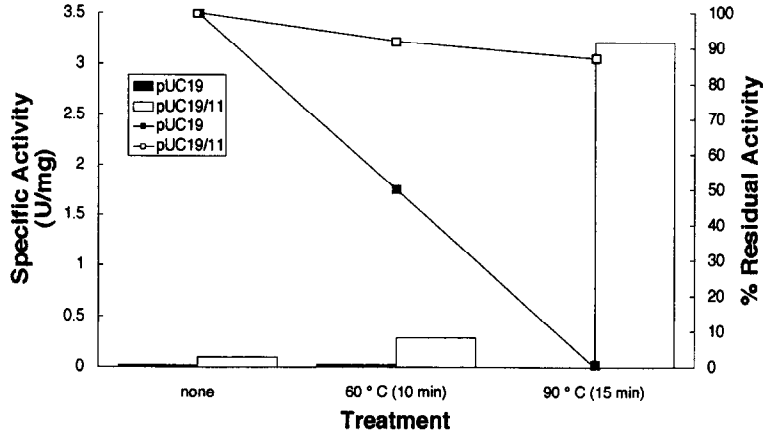
Fig. 1. (A) Restriction map of *S. solfataricus fumC* gene. The white and striped arrows indicate the *lacZ* and *fumC* open reading frames, respectively. (B) Nucleotide and deduced amino acid sequence of *fumC* . Two in frame ATGs are shown in bold character. A putative bipartite promoter is underlined. The experimentally determined N-terminal sequence is double underlined. The nucleotide sequence was numbered in the 5′ to 3′ direction beginning with the first sequenced nucleotide. The ATG at position 171 was assigned as the first amino acid by comparison with the experimentally determined N-terminal sequence (double underlined). A putative transcription terminator is underlined by a dotted line. The sequence has been deposited in EMBL Data Library under the accession number X75402.



B

```
                GG GGT TTA TAT CTC TAA GTG CAA TAG TCT TAA CAT TCC CCT CAT AGA TCT        50
TGG TAA CAT TTT CAA GTC TTA TTA CGG ACA TAT ATT GGG TAT TAC GTA AAG TGG GTT AAA        110
GAT TTT TTC AAA GAT AAT TCC TTG TAA AGA TTT AAT ATA ATG GAT TAC GTT CAA TTA ATT        170

ATG AAA TAT ACC GAT ACT GCG CCC AAG CTT TTT ATG AAT ACT GGA ACC AAA TTT CCT AGA        230
Met Lys Tyr Thr Asp Thr Ala Pro Lys Leu Phe Met Asn Thr Gly Thr Lys Phe Pro Arg        20

AGA ATT ATC TGG TCT ATG GGA GTT TTA AAG AAG TCC TGT GCT AAA GTC AAC GCA GAT CTT        290
Arg Ile Ile Trp Ser Met Gly Val Leu Lys Lys Ser Cys Ala Lys Val Asn Ala Asp Leu        40

GGA TTA TTA GAT AAA AAA ATT GCG GAT TCA ATT ATT AAG GCA TCT GAC GAT TTA ATT GAT        350
Gly Leu Leu Asp Lys Lys Ile Ala Asp Ser Ile Ile Lys Ala Ser Asp Asp Leu Ile Asp        60

GGA AAA TTA GAT GAT AAG ATA GTG CTT GAT GTA TTT CAA ACG GGT TCA GGG ACC GGA CTT        410
Gly Lys Leu Asp Asp Lys Ile Val Leu Asp Val Phe Gln Thr Gly Ser Gly Thr Gly Leu        80

AAT ATG AAC GTA AAT GAG GTT ATA GCA GAA GTA GCT TCT AGC TAT TCT AAT CTT AAA GTA        470
Asn Met Asn Val Asn Glu Val Ile Ala Glu Val Ala Ser Ser Tyr Ser Asn Leu Lys Val        100

CAT CCA AAT GAT CAT GTA AAT TTT GGT CAG TCC TCA AAC GAT ACT GTG CCA ACA GCT ATA        530
His Pro Asn Asp His Val Asn Phe Gly Gln Ser Ser Asn Asp Thr Val Pro Thr Ala Ile        120

AGA ATT GCA GCA GTA GCT GAG GTA ACA AAT AGG CTA CTG CCT GCA TTA CAG CAA ATA ATA        590
Arg Ile Ala Ala Val Ala Glu Val Thr Asn Arg Leu Leu Pro Ala Leu Gln Gln Ile Ile        140

TCC TCT TTA AAT AAG AAG GCT GAG GAG TAC AAG GAT GTT ATA AAG GCT GGT AGA ACC CAT        650
Ser Ser Leu Asn Lys Lys Ala Glu Glu Tyr Lys Asp Val Ile Lys Ala Gly Arg Thr His        160

TTA AGA GAC GCA TTA CCA GTA ACT TTA GGT CAA GAA CTT TCA GCC TAC GCA GAC GCT TTC        710
Leu Arg Asp Ala Leu Pro Val Thr Leu Gly Gln Glu Leu Ser Ala Tyr Ala Asp Ala Phe        180

CAG CAT GAA CAT GAA CAA GTT ATG AAT ATT TTG GAA TAT GTG AAG GAA TTG CCA ATT GGA        770
Gln His Glu His Glu Gln Val Met Asn Ile Leu Glu Tyr Val Lys Glu Leu Pro Ile Gly        200

GGT ACT GCG ACT GGT ACT GGG CTA AAT AGC CAC CCA GAA TTC CAA GAA AGA GTT ATA AAC        830
Gly Thr Ala Thr Gly Thr Gly Leu Asn Ser His Pro Glu Phe Gln Glu Arg Val Ile Asn        220

GAA ATA AAC AGA ATT ACC GGT TTA GGA TTT AAG CCA GCT AAT AGG TTT AGA GCA ATG AGA        890
Glu Ile Asn Arg Ile Thr Gly Leu Gly Phe Lys Pro Ala Asn Arg Phe Arg Ala Met Arg        240

TTG CTC ACC GAT CTC TTA TTA TTA AGC GGA GCA CTG AGG AAT ATT GCA GTA GAC CTA TAT        950
Leu Leu Thr Asp Leu Leu Leu Leu Ser Gly Ala Leu Arg Asn Ile Ala Val Asp Leu Tyr        260

AGA TTA GGG CAA GAT ATA AGG TTA ATG TTT TCC GGT CCC TTA ACT GGG TTA AAT GAA ATT        1010
Arg Leu Gly Gln Asp Ile Arg Leu Met Phe Ser Gly Pro Leu Thr Gly Leu Asn Glu Ile        280

GAT TTA CCT ACA CAA GAG GAG ATA GCT GGT AGC TCA ATA ATG CCT GGT AAA ACT AAT CCA        1070
Asp Leu Pro Thr Gln Glu Glu Ile Ala Gly Ser Ser Ile Met Pro Gly Lys Thr Asn Pro        300

GTT ACG GTT GAG GCT ACT TTA CTA ATC TCA GCA CAA GTT GTG GGA TTA GAT CAT GCA AAC        1130
Val Thr Val Glu Ala Thr Leu Leu Ile Ser Ala Gln Val Val Gly Leu Asp His Ala Asn        320

CAA TTC GCA TCA ATG TTA GGC GAA TTT GAG TTA TCA ATG GGA ATT CCA TTA GTT GGT TAT        1190
Gln Phe Ala Ser Met Leu Gly Glu Phe Glu Leu Ser Met Gly Ile Pro Leu Val Gly Tyr        340

AAT ATC GTA ACC CAA GTT AAT TTC ATC TCA GAG GCT TTA GAG AAG ATG TCA AGA TTA GTG        1250
Asn Ile Val Thr Gln Val Asn Phe Ile Ser Glu Ala Leu Glu Lys Met Ser Arg Leu Val        360

ATT GAT GGA ATG GTA GCA AAT GTA GAG AAG ATG AAG AGA TAT GCT GAA TCC TCT CCT TCA        1310
Ile Asp Gly Met Val Ala Asn Val Glu Lys Met Lys Arg Tyr Ala Glu Ser Ser Pro Ser        380

CTT ATA ACC ATA GTA TCT CCA GTA ATA GGC TAT GAT AAA GCA ACA GAA ATA GGA AAG AAG        1370
Leu Ile Thr Ile Val Ser Pro Val Ile Gly Tyr Asp Lys Ala Thr Glu Ile Gly Lys Lys        400

TTA AAT AAG GGA ATG TCC ATA CGT GAA GCA TTA AGC GAA TTA GGA TAT AGC GAT AAT GAG        1430
Leu Asn Lys Gly Met Ser Ile Arg Glu Ala Leu Arg Glu Leu Gly Tyr Ser Asp Asn Glu        420

ATA AAT AAA ATA TTA GAC TTA AGC AAA CTA GTT AAA CCA GGG TTC ACA GCT AAA TGA GAA        1490
Ile Asn Lys Ile Leu Asp Leu Ser Lys Leu Val Lys Pro Gly Phe Thr Ala Lys TER           438
AAG ATA AAC GTC CCA AGG GTA GGT TAC CTC TTT TTA ACA CCT TCT ATT ATC CAA AAT CCT        1550
TCC TTC TCC TTT ACC ATG GCC ACT TTA AAT TTA CTG GAC AGC TTT CTT AAA CCT TCT AAT        1610
GGA TCA ACT CCG CCA AAT AAC TTA TCA CGT AAG ATC ATG TAA ACC TTA GAG CCG CTT TTA        1670
ACT ACC CTA TAT ACC TCT CTA ATT ATA TCT AGA TCA AGA ACC TCA AAA ATT ATT GCA GAA        1730
TTA AAC GAT GAA TCT CTA AAA GGA AAT GGA TAT GTG AAA ATA ACA TCC TTA AAA GAT ATA        1790
CCA GAA CAG TCC                                                                    1802
```

C



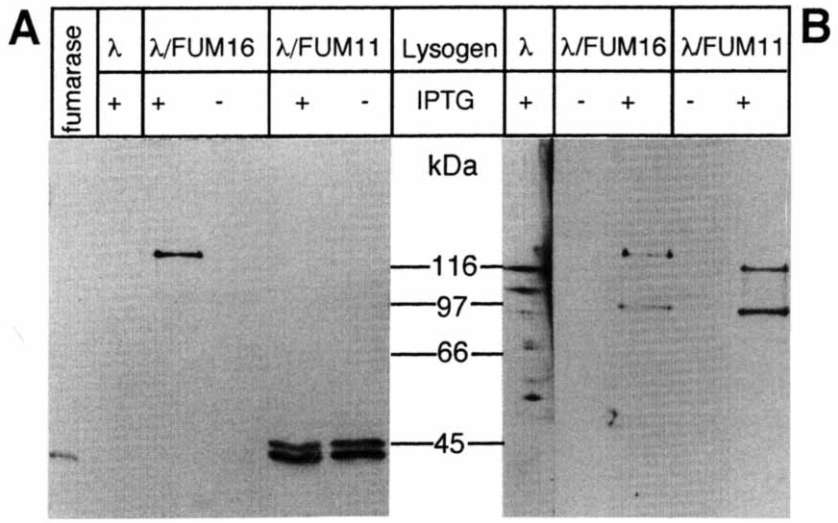


Fig. 2. Expression of *S. solfataricus* fumarase in *E. coli*. Western blot analysis of Y1089 lysogens – grown either in the absence (−) or in the presence (+) of 1 mM IPTG – probed with either anti-fumarase (A) or anti-β-galactosidase (B) antibodies. Panel C shows specific activities (bars) and residual activities (squares) after different heat treatments of cell-free extracts from *E. coli* JM109 transformed with pUC19 or pUC19/FUM16. Fumarase activity was assayed as described in section 2.

DNA from this organism, as reported for other *S. solfataricus* protein-encoding genes [16,26–28]. The overall A + T content of the *fumC* gene is 63%, rising to 74.5%, when only the third position is considered, Trp and Met excluded. Exclusively AGG and AGA codons, that are very rare in *E. coli* but common in eukaryotes, are used for arginine in agreement with data previously reported [26,27].

Comparing the sequence around the starting ATG codon with the 3′ end of the *S. solfataricus* 16S rRNA [29] two potential ribosome binding sites (RBSs) were identified. The first RBS overlaps the ATG codon, as reported for some other archaebacterial genes [30,31]; the second RBS is located upstream, as found in the genes for *S. solfataricus* β-galactosidase and 'Docking α' proteins. The identified potential promoter resembles the consensus bipartite sequence drawn for archaebacterial promoters [32], consisting of the TTTAAT (box A) and the ATGA (box B) motifs. The motifs are underlined in Fig. 1B. They closely match the proposed consensus sequence (box A, 5/6 nucleotides; box B 4/4 nucleotides, spacing 24 nucleotides) proposed for archaebacterial promoters [32]. A putative terminator signal TCTTTTT, matching in 6 out of 7 residues the consensus (TTTTTYY) proposed by by Reiter et al. [31], was localized 34 bp downstream of the termination codon.

3.2. *Expression of* fumC *in E. coli*

Lysogens for the two sequenced positive phages were obtained using *E. coli* Y1089 as host and proteins expressed analyzed by immunoblotting with anti-fumarase (Fig. 2A) and anti-β-galactosidase antibodies (Fig. 2B). The anti-fumarase antibody did not recognize any protein in the λgt11 lysogen. As expected the anti-β-galactosidase antibody recognized a 116 kDa protein – and some degradation products – in the λgt11 lysogen. Strain Y1089/λFUM16 expressed a ca. 138 kDa IPTG-inducible fusion protein recognized by both antibodies, in keeping with the observation that the *S. solfataricus* fragment is in frame with *lacZ*. Strain Y1089/λFUM11 expressed constitutively two proteins (ca. 44 kDa and ca. 45 kDa) recognized only by anti-fumarase antibodies, while anti-β-galactosidase antibodies recognized an IPTG-inducible protein comigrating with β-galactosidase. The 45 kDa protein comigrates with *S. solfataricus* fumarase. This observation suggested that the encoding gene was inserted in the opposite direction with respect to the *lacZ* promoter in λgt11 and was in fact confirmed by direct sequencing of the insert. The *fumC* promoter may be functional in *E. coli*, as previously reported for other archaebacterial promoters [27], or some other upstream promoter on the −1 strand may be responsible for transcription [33]. Fumarase-activity was assayed in *E. coli* JM109 transformed with plasmid pUC19 and plasmid pUC19/FUM11. In crude extracts of JM109 transformed with pUC19/FUM11 fumarase specific activity was five-fold higher than in JM109 transformed with pUC19. Extracts were heated at 60°C (10 min) and 90°C (15 min) and denatured proteins were eliminated by centrifugation. Fig. 2C shows that, even after the 90°C treatment, as much as 87% of the activity is retained with a 30-fold purification. It is also interesting to note that, despite a 11 amino acid C-terminal deletion, the protein encoded by JM109 transformed with pUC19/FUM11 retains enzymatic activity, and its thermostability is comparable to that of *S. solfataricus* purified enzyme.

3.3. *Sequence homology*

Comparing the amino acid sequence of *S. solfataricus* fumarase with proteins in the SwissProt data bank we

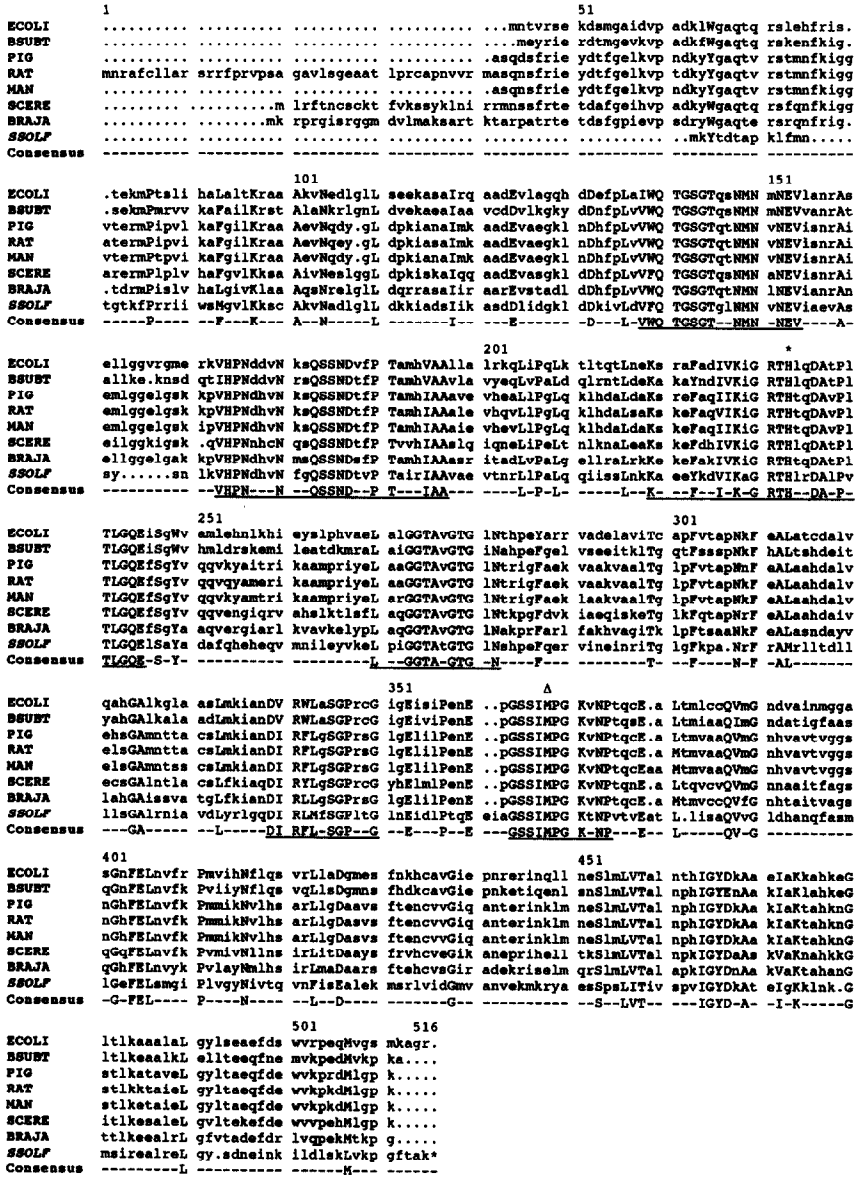


Fig. 3. Multiple alignment of class II fumarases. Proteins were aligned using the PILEUP program from GCG. Residue pairs with a score higher than 1.0 in all proteins are shown in capital LETTERS. Gaps are indicated by dots. The calculated consensus sequence is also shown. The position of the six more conserved regions are shown as underlined characters in the consensus sequence. Conserved histidine and methionine residues – which have been postulated to be involved in the catalytic mechanism – are indicated by an asterisk and an open triangle, respectively. SSOLF = *S. solfataricus*; SCERE = *Sacch. cerevisiae*; ECOLI = *E. coli*; BSUBT = *B. subtilis*; BRJAP = *Bradyr. japonicum*.

found several significant similarities with fumarases from both eubacterial and eukaryotic sources and with aspartases. Identity scores with class II fumarases are reported in Table 1. A low degree of similarity (ca. 28% identity in ca. 50 amino acids) was found with *E. coli* FumA and FumB proteins, belonging to the class I fumarases. Therefore, *S. solfataricus* fumarase not only resembles class II fumarases in its size (native M_r 200,000) and tetrameric structure, but also in its primary structure. The amino acid sequence alignment of all available class II fumarases is reported in Fig. 3. Six highly conserved regions, underlined in Fig. 3, are apparent: Val-138 to Val-152, Val-173 to Ala-197, Lys-228 to Glu-245, Gly.364 to Pro-374, Asp-338 to Gly-350 and Gly-364 to Pro-374. The last mentioned sequence contains the motif GS--M--K-N, which is conserved among class I and class II fumarases, aspartases, arginosuccinases and adenylosuccinases, all enzymes belonging to the lyase class for which fumarate is a substate. This short conserved sequence, the only significant region of similarity between the class I and class II fumarases, includes a methionine which is probably involved in the catalytic activity of this type of enzymes (marked with an open triangle). A histidine residue has also been proposed to be involved in the catalytic mechanism of fumarase [5]. Interestingly, a histidine residue (indicated

Table 1
Identity scores among class II fumarases

	SSOLF	SCERE	ECOLI	PIG	BSUBT	RAT	MAN	BRAJA
SSOLF	–	39.5	39.1	41.5	39.7	42.2	41.6	37.5
SCERE		–	57.9	67.1	56.4	66.9	66.3	63.9
ECOLI			–	60.3	64.0	60.3	59.9	58.0
PIG				–	57.4	95.3	95.7	65.0
BSUBT					–	57.1	57.2	53.7
RAT						–	96.1	65.4
MAN							–	64.6
BRAJA								–

Sequences were aligned in all possible pairwise combinations using the FASTA program. SSOLF = *S. solfataricus*; SCERE = *Sacch. cerevisiae*; ECOLI = *E. coli*; BSUBT = *B. subtilis*; BRAJA = *Bradyr. japonicum*.

by an asterisk) within the third homology box is conserved among all fumarases.

The biotechnological potential of fumarases has long been exploited for continuous production of L-malate [15,34]. The *Sulofolbus solfataricus* enzyme, in being more thermostable (Fig. 2C) and chemostable [17] than its mesophilic counterparts, might prove to be expecially suitable for use in such biotechnological processes. In particular the operation at high temperature might allow the utilization of magnesium or calcium fumarate – which are poorly soluble at room temperature – which offers the advantage of increased enzymatic conversion [15]. The availability of the *fumC* gene, allowing easy purification of the recombinant enzyme, might make the process even more attractive. Experiments to improve enzyme expression are currently underway in our laboratory.

Acknowledgements: The authors wish to thank Prof. M. Rossi for the generous gift of the *S. solfataricus* gene bank, Dr. A. Gambacorta, V. Calandrelli, E. Esposito and E. Romano for supplying *S. solfataricus* and Dr. G. Orsini for performing N-terminal sequencing of purified fumarase. This work has been supported by grants from the Ministero dell'Universitê e della Ricerca Scientifica e Tecnologica (40% program).

References

[1] Woods, S.A., Schwartzbach, S.D. and Guest, J.R. (1988) Biochem. Biophys. Acta 954, 14–26.
[2] Guest, J.R., Green, J., Spiro, S., Prodromou, C. and Sharrocks, A.D. (1990) in: The Molecular Basis of Bacterial Metabolism (Hauska, G. and Thauer, R. eds.) pp. 134–145, Springer-Verlag, Berlin.
[3] Miles, J.S. and Guest, J.R. (1984) Nucleic Acids Res. 12, 3631–3642.
[4] Bell, P.J., Andrews, S.C., Sivak, M.N. and Guest, J.R. (1989) J. Bacteriol. 171, 3494–3503.
[5] Woods, S.A., Miles, J.S., Roberts, R.E. and Guest, J.R. (1986) Biochem. J. 237, 547–557.
[6] Kroger, A., Geisler, V., Lemma, E., Theis, F. and Lenger, R. (1992) Arch. Microbiol. 158, 311–314.
[7] Reaney, S.K., Bungard, S.J.and Guest J.R. (1993) J. Gen. Microbiol. 139, 403–416.
[8] Shibata, H., Gardiner, W.E. and Schwartzbach, S.D. (1985) J. Bacteriol. 164, 762–768.
[9] Acuna, G., Ebeling, S. and Hennecke, H. (1991) J. Gen. Microbiol. 137, 991–1000.
[10] Miles, J.S. and Guest, J.R. (1985) Nucleic Acids Res. 13, 131–140.
[11] Wu, M. and Tzagoloff, A. (1987) J. Biol. Chem. 262, 12275–12282.
[12] Suzuki, T., Sato, M., Yoshida, T. and Tuboi, S. (1989) J. Biol. Chem. 264, 2581–2586.
[13] Kinsella, B.T. and Doonan, S. (1986) Biosci. Rep. 6, 921–929.
[14] Sacchettini J.C., Frazier, M.W., Chiara, D.C., Banaszak, L.J. and Grant, G.A. (1988) Biochem. Biophys. Res. Commun. 153, 435–440.
[15] Chibata, I., Tosa, T. and Sato, T. (1987) in: Biotechnology Vol. 7a (Rehm, H.J. and Reed, G. eds.) pp. 653–684, Verlag Chemie, Weinheim.
[16] Sanangelantoni, A.M., Barbarini D., Di Pasquale, G., Cammarano, P. and Tiboni, O. (1990) Mol. Gen. Genet. 221, 187–194.
[17] Puchegger, S., Redl, B. and Stoffler, G. (1990) J. Gen. Microbiol. 136, 1537–1541.
[18] Colombo, S., D'Auria, S., Fusi, P., Zecca, L., Raia, C.A. and Tortora, P. (1992) Eur. J. Biochem. 206, 349–357.
[19] Harlow, E. and Lane, D. (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Smith, D.E. and Fisher, P.A. (1984) J. Cell Biol. 99, 20–28.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Matsudara, P. (1987) J. Biol. Chem. 262, 10035–10038.
[23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[24] Young, R.A. and Davis, R.W. (1983) Proc. Natl. Acad. Sci. USA 80, 1194–1198.
[25] Lipman, W.J. and Pearson, W.R. (1985) Science 227, 1435–1441.
[26] Cubellis, M.V., Rozzo, C., Nitti, G., Arnone, M.I., Marino, G. and Sannia, G. (1989) Eur. J. Biochem. 186, 375–381.
[27] Cubellis, M.V., Rozzo, C., Montecucchi, P. and Rossi, M. (1990) Gene 89–94.
[28] Ramirez, C. and Matheson, A.T. (1991) Mol. Microbiol. 5, 1687–1693.
[29] Olsen, G.J., Pace, N.R., Nuell, M., Kaine, B.P., Gupta, R. and Woese, C.R. (1985) J. Mol. Evol. 22, 301–307.
[30] Pisani, F.M., De Martino, C. and Rossi, M. (1992) Nucleic Acids Res. 20, 2711–2716.
[31] Reiter, W.D., Palm, P., Henschen, A., Lottspeich, F., Zillig, W. and Grampp, B. (1987) Mol. Gen. Genet. 206, 144–153.
[32] Reiter, W.D., Palm, P. and Zillig, W. (1988) Nucleic Acids Res. 16, 1–19.
[33] Chiraia, S.S. (1986) Nucleic Acids Res. 14, 5935.
[34] Yamamoto, K., Tosa, T., Yamashita, K. and Chibata, I. (1977) Biotechnol. Bioeng. 19, 1104–1114.