Identification of the L-tartrate dehydratase genes (*ttdA* and *ttdB*) of *Escherichia coli* and evolutionary relationship with the Class I fumarase genes

SUSAN K. REANEY,¹ CARRIE BEGG,¹ STEPHEN J. BUNGARD² and JOHN R. GUEST^{1*}

¹The Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, PO Box 594, Firth Court, Western Bank, Sheffield S10 2UH, UK

²ICI Bio Products & Fine Chemicals, PO Box 1, Billingham, Cleveland TS23 1LB, UK

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The genes encoding an oxygen-labile stereospecific L-tartrate dehydratase (L-Ttd, EC 4.2.1.32) have been identified as the *orfZ1* and *orfZ2* genes located upstream of the *rpsU-dnaG-rpoD* operon at 67 min in the *Escherichia coli* linkage map. They were previously cloned and sequenced by M. Nesin and others (*Gene* 51, 149–161, 1987) and have now been independently cloned, partially resequenced, and designated as an operon (*ttdAB*) containing two translationally coupled genes. The enzyme behaves as a tetramer (M_r 105 000) containing two pairs of non-identical subunits, TtdA (M_r 32 589) and TtdB (M_r 22 641), which otherwise resembles the homodimeric iron-sulphur-containing Class I fumarases of *E. coli* and *Bacillus stearothermophilus*. The amino acid sequences of the TtdA–TtdB subunits are colinearly related to a single fumarase subunit, indicating a common evolutionary ancestry. *E. coli* can use L-, D- and *meso*-tartrates as aerobic growth substrates and as reducible substrates for supporting anaerobic growth on glycerol. L-Ttd was induced during anaerobic growth on glycerol plus L- and *meso*-tartrates, and a stereospecific D-tartrate dehydratase was induced by all three stereoisomers under comparable conditions. No *meso*-tartrate dehydratase was detected, nor were any dehydratases detected after aerobic growth on tartrate minimal media suggesting that different catabolic routes operate under aerobic conditions.

Introduction

There are three stereoisomers of tartaric acid, L-tartrate being the most widely distributed isomer (Fig. 1). They can be degraded under aerobic or anaerobic conditions by a variety of bacteria, e.g. *Pseudomonas* (Shilo & Stanier, 1957), *Aerobacter* (Barker, 1936), *Clostridium* (Mercer & Vaughn, 1951), *Rhodopseudomonas* (van Niel, 1944), *Bacteroides* (Wagener & Pfennig, 1987), *Lactobacillus* (Radler & Yannissis, 1972), and coliform bacteria including *Escherichia coli* (Vaughn *et al.*, 1946). Two major pathways of bacterial tartrate metabolism have been identified. One involves the conversion of tartrate to oxaloacetate by stereospecific dehydratases (Barker, 1936; La Rivière, 1956; Shilo, 1957; Krampitz & Lynen, 1964; Rode & Giffhorn, 1982*a,b*; Schink, 1984; Janssen, 1991). The other involves the conversion of tartrate to D-glycerate via oxaloglycolate, either by

tartrate dehydrogenase (EC 1.1.1.93) and oxaloglycolate reductase (EC 1.1.1.92) (Kohn *et al.*, 1968; Kohn & Jakoby, 1968), or by L-tartrate decarboxylase (Furuyoshi *et al.*, 1991). A bifunctional L-tartrate dehydrogenase–D-malate dehydrogenase, which catalyses the decarboxylation of *meso*-tartrate as a side reaction, has also been described in *Rhodopseudomonas sphaeroides*, but its role in tartrate metabolism is unknown (Giffhorn & Kuhn, 1983; Ebbighausen & Giffhorn, 1984). The route(s) used by *E. coli* have not been defined, but DL-tartrate is known to be taken up by the *dctB*-encoded C₄-dicarboxylic acid transport system (Kay & Kornberg, 1971).

Stereospecific tartrate dehydratases from various sources have been characterized. The L-tartrate dehydratase of *Pseudomonas putida* (EC 4.2.1.32) is an oxygen-sensitive, iron-sulphur-containing tetramer, M_r 2 × 23 000 plus 2 × 27 000 (Kelly & Scopes, 1986). In contrast, the D-tartrate dehydratase of *Pseudomonas* sp. is an oxygen-stable homodimer (M_r 2 × 36 000) that requires Fe²⁺ or Co²⁺ (Rode & Giffhorn, 1982*a*), and the

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* Author for correspondence. Tel. (0742) 768555 ext. 4406; fax (0742) 728697.

Fig. 1. Comparative structures of the stereoisomers of tartrate, oxaloacetate and L-malate.

R. sphaeroides enzyme is an oxygen-stable homotetramer ($M_r 4 \times 39\,000$) requiring Mg^{2+} (Rode & Giffhorn, 1982*b*). Little is known about *meso*-tartrate dehydratases except that *P. putida* contains a highly unstable (but oxygen-insensitive) enzyme which appears not to require a metal cofactor (Furuyoshi *et al.*, 1987). This diversity in subunit structure, presence or absence of an iron-sulphur centre and metal cofactor requirements, resembles that observed with other hydratase-dehydratases including the fumarases (Cammack, 1992; Switzer, 1989; Woods *et al.*, 1988).

During the characterization of a *Bacillus stearothermophilus* fumarase gene (*fumA_{Bst}*), it was observed that the products of two cotranscribed genes, *orfZ1* and *orfZ2*, located upstream of the *rpsU-dnaG-rpoD* macromolecular-synthesis operon at 67 min in the *E. coli* linkage map (Nesin *et al.*, 1987), are related to the Class I fumarases (Reaney *et al.*, 1993). The Class I fumarases are homodimeric, oxygen-labile, iron-sulphur-containing enzymes, typified by fumarase A and B (FumA and B) of *E. coli* and FumA_{Bst}. OrfZ1 and OrfZ2 resembled the respective N- and C-terminal segments of these Class I fumarases, the overall identity for 446 equivalent residues being 23–25%, which rises to 37–45% similarity when conservative substitutions are included. This suggests that OrfZ1 and OrfZ2 might combine to form a FumA-related iron-sulphur-containing hydratase-dehydratase having an $\alpha_2\beta_2$ structure like the L-tartrate dehydratase of *P. putida* (Kelly & Scopes, 1986). Alternatively, if a single reading frame had been interrupted by mutation, DNA damage or by sequencing errors, a fused OrfZ1–Z2 peptide might form a homodimeric enzyme (α_2) analogous to the Class I fumarases.

In this paper, the *orfZ1-orfZ2* operon of *E. coli* was cloned from an independent source and the existence of two distinct translation products was confirmed. The two products were shown to form a tetrameric L-tartrate dehydratase (L-Ttd) and the open reading frames (revised by partial resequencing) were redesignated *ttdA* and *ttdB* to denote that they encode non-identical subunits of an L-Ttd. The sequences show that the tetrameric L-Ttd is evolutionarily related to the homodimeric fumarases. Studies on the aerobic and anaerobic utilization of tartrate stereoisomers further suggested that *E. coli* possesses more than one route of tartrate metabolism.

Methods

Bacterial strains, bacteriophages, phagemids and molecular-genetic methods. The strains of *E. coli* K12 were: W3110 (wild-type); DH5 α (*supE44* Δ *lacU169* ϕ 80*lacZ* Δ *M15* *hsdR17* *recA1* *gyrA96* *endA1* *thi-1* *relA1*) the routine transformation host; and NM621 (*leu pro thi supE* *hsdR* *mcrB*⁺ *A*⁺ *recD1009* *ton tsx*) kindly provided by Dr M. Masters (University of Edinburgh) for propagating λ phages. A λ EMBL4 derivative, λ 19F2, from the *E. coli* W3110 gene library (Kohara *et al.*, 1987) was used as the source of DNA for subcloning the *orfZ1* and *orfZ2* genes into the phagemid vector pUC118. Bacteriophage and phagemid DNA was prepared, manipulated and sequenced as described by Sambrook *et al.* (1989) and Reaney *et al.* (1993). Nucleotide and amino acid sequences were analysed and compared using the FRAMESCAN and DIAGON programs (Staden, 1982, 1984).

Media and growth conditions. The rich media used for routine subculture were L broth and L agar, supplemented with ampicillin (100 mg l⁻¹) as required, and with glucose (11 mM) for anaerobic growth. The citrate-free minimal medium of Cole & Guest (1980) supplemented with thiamin.HCl (5 mg l⁻¹) and Casamino acids (0.5 g l⁻¹), was used with glucose (11 mM) or potassium L-, D- or *meso*-tartrate (40 mM) for aerobic growth, and with glucose (11 mM) or with the tartrates (40 mM) plus glycerol (20 mM) for anaerobic growth. Cultures (50 ml) were grown at 37 °C, aerobically in shaking 250 ml conical flasks or anaerobically in stationary bottles filled to the neck. The inocula were prepared from cultures grown on the same solid media, added to give a starting OD₆₅₀ of 0.05. Anaerobic cultures on solid media were incubated in an atmosphere of N₂/CO₂/H₂ (85:10:5, by vol.) at 34 °C.

Enzymology. Extracts of cultures grown to early stationary phase were prepared in TTD buffer (30 mM-potassium phosphate, pH 7.0; 5 mM-potassium succinate; 5%, w/v sucrose; 0.1 mM-Fe²⁺; 5 mM-cysteine; 5 mM-glutathione; 10 mM-ascorbate; and 5 mM-MgCl₂; Kelly & Scopes, 1986) flushed with N₂ before use, by disruption in a French press or ultrasonic treatment (Reaney *et al.*, 1993). The extracts were flushed with N₂ and stored anaerobically at –20 °C for up to 2 weeks. The general assay for hydratase-dehydratase activity was to follow the increase or decrease in absorbance at 240 nm upon addition of the hydrated or unsaturated substrate (20 mM) in a reaction mixture containing potassium phosphate (33 mM, pH 7.3) at 30 °C according to the assay for fumarase (Hill & Bradshaw, 1969). Tartrate dehydratase was assayed by following the formation of oxaloacetate in Tris/HCl (67 mM, pH 7.5) with potassium L-, D- or *meso*-tartrate (20 mM) under aerobic conditions at 30 °C. The discontinuous assay involved detecting oxaloacetate as the 2,4-dinitrophenylhydrazone derivative at 540 nm according to Hurlbert & Jakoby (1965). The continuous assay involved converting oxaloacetate to malate with malate dehydrogenase (1 unit) and measuring the disappearance of NADH (0.13 mM) at 365 nm using an extinction coefficient of 3.4 l mmol⁻¹ cm⁻¹ (Rode & Giffhorn, 1982*b*). Tartrate dehydratase activities are expressed as units (μmol oxaloacetate formed or NADH oxidized min⁻¹) per mg protein. Protein was assayed according to Bradford (1976) using the Bio-Rad reagent.

Fig. 2. Restriction map of the segment of the *E. coli* chromosome at 67 min cloned in λ 19F2, and strategy for subcloning the *orfZ1* and *orfZ2* genes in pGS581. The positions and polarities of coding regions (arrowed), relevant promoters and a terminator, are derived from Nesin *et al.* (1987). Restriction sites are abbreviated as follows: B, *Bam*HI; H, *Hind*III; P, *Pst*I; G, *Bgl*II; and S, *Sac*I.

Native and subunit M_r values were determined for L-tartrate dehydratase in extracts of amplified strains, by gel-filtration in TTD buffer with a calibrated Sephacryl 200HR column and SDS-PAGE (Reaney *et al.*, 1993; Laemmli, 1970).

In vitro transcription-translation. The prokaryotic-DNA-directed translation kit (Amersham) was used with intact phagemid DNA, an *E. coli* S-30 extract and L-[^{35} S]methionine, according to the manufacturer's instructions. Samples were fractionated by SDS-PAGE and analysed autoradiographically.

Materials. Restriction endonucleases and DNA ligase were purchased from Northumbria Biologicals. The prokaryotic-DNA-directed translation kit and L-[^{35} S]methionine (> 37 TBq mmol $^{-1}$) were from Amersham. Molecular mass markers for gel-filtration were from Sigma and those for SDS-PAGE were from BDH. The sources of substrates were as follows: trisodium isocitrate, dipotassium L-tartrate, D- and meso-tartaric acids, and oxaloacetic acid (Sigma); (+)- and (-)-citramalic and D- and L-malic acids (Aldrich); and fumaric and maleic acids (BDH).

Results

Subcloning and expression of the *orfZ1-orfZ2* (*ttdA-ttdB*) genes of *E. coli*

The *orfZ1-orfZ2-rpsU-dnaG-rpoD* region of the *E. coli* chromosome (Fig. 2) that had been characterized by Nesin *et al.* (1987) was independently isolated by starting with λ 19F2 (Kohara *et al.*, 1987). The purified 4.8 kb

*Bam*HI–*Hind*III fragment was treated with *Pst*I, and the 2.7 kb *Bam*HI–*Pst*I fragment was ligated into the corresponding sites of pUC118 without purification (Fig. 2). The resulting phagemid (pGS581) contains the *orfZ1-orfZ2* genes and their upstream promoter inserted with the same polarity as the flanking *lac* promoter of the vector.

The presence of two cloned genes encoding products of M_r 35 000 and 23 500, was confirmed by transcription-translation analysis with intact pGS581 DNA (Fig. 3a). The sizes are in reasonable agreement with the DNA-derived values: 31 935, OrfZ1 (revised below to 32 589); and 22 641, OrfZ2 (not 18 130, quoted in error by Nesin *et al.*, 1987). The results agree with the tentative conclusions drawn from maxicell studies by Nesin *et al.* (1987) in which a weakly labelled polypeptide of M_r 32 000 was detected, but one of M_r 22 000 was largely obscured by the product of the vector *cat* gene.

The same polypeptides were amplified in cell supernatant extracts of aerobically grown pGS581 transformants of DH5 α (Fig. 3b). Interestingly, the polypeptides were not significantly amplified during anaerobic growth, nor did IPTG (0.1 to 1 mM) induce their aerobic or anaerobic synthesis, despite the presence of an upstream *lac* promoter. The lack of induction by IPTG is presumably due to the presence of a

Fig. 3. *In vitro* transcription-translation analysis of pGS581 and amplification of pGS581-encoded polypeptides in transformed strains. (a) Autoradiogram of [³⁵S]methionine-labelled polypeptides expressed from: pGS581 (lane 1); pUC118 (lane 2); pAT153 (lane 3); and no DNA (lane 4). (b) Coomassie-blue-stained SDS-PAGE gel (15 %) of cell supernatant extracts of DH5 α containing: pUC118 (lane 5); pGS581 (lane 6); pGS581 induced with 0.1 mM-IPTG (lane 7). Cultures were grown aerobically in L broth or anaerobically in L broth plus 0.2 % glucose. Relevant polypeptides including β -lactamase (Bla) and the positions of M_r markers are indicated.

transcriptional terminator between the *lac* and *orfZ1*–*Z2* promoters.

Enzymological studies and identification of the *orfZ1*–*orfZ2* (*ttdA*–*ttdB*) gene products

Cell extracts of DH5 α (pGS581) and DH5 α (pUC118) were compared using a variety of potential substrates in the general spectrophotometric assay for hydratase-dehydratases. No significant activity or enrichment of activity, was observed for DH5 α (pGS581) extracts with fumarate, L-malate, D-malate, maleate, (+)- and (–)-citramalate or isocitrate (each at 20 mM). However, using two procedures specifically designed for assaying tartrate dehydratases, with different stereoisomers, extracts of aerobically grown DH5 α (pGS581) proved to be highly enriched for L-Ttd. The specific activities ranged from 1.2 to 4.8 units (mg protein)⁻¹ for different extracts using the discontinuous assay, the higher values being associated with freshly transformed strains. The activities observed with the continuous assay were 40 % lower. Anaerobic cultures of DH5 α (pGS581) contained 10 % of the aerobic activities, despite the apparent absence of polypeptide amplification (Fig. 3b), whereas no activity [<0.001 unit (mg protein)⁻¹] could be detected in DH5 α (pUC118) extracts. The visible absorption spectrum of the dinitrophenylhydrazine derivative formed in the discontinuous assay was identical to that of authentic oxaloacetate. These observations strongly indicate that one or both of the *orfZ1* and *orfZ2*

genes encodes an L-Ttd, which catalyses the dehydration of L-tartrate to oxaloacetate via an unstable tautomer of the latter.

L-Ttd was shown to be stereospecific because no activity could be detected with the D- or *meso*-isomers in both assays. The K_m for L-tartrate was 0.76 ± 0.2 mM, when determined with crude extracts of DH5 α (pGS581) using a range of substrate concentrations (0.04 to 4 mM) in the continuous assay. The Wilkinson computational method in the Enzpack3 program (P. A. Williams & B. N. Zaba, distributed by BIOSOFT, Cambridge, UK) was used.

The L-Ttd activity was very unstable when cell extracts were stored at -20°C in air, 90 % being lost in <10 h. Furthermore, no activity could be detected unless the protective TTD buffer was used in preparing the extracts. Preliminary attempts to reactivate unprotected extracts with 0.5 mM-Fe²⁺ and 50 mM-DTT were unsuccessful, although this procedure was successful with FumA_{Bst}.

In quantitative gel-filtration studies, the L-Ttd activity co-eluted with the amplified polypeptides at a position corresponding to a native M_r of 105000 (Fig. 4). Assuming that both polypeptides contribute to L-Ttd activity, the size most closely conforms to an $\alpha_2\beta_2$ or $(\alpha\beta)_2$ subunit organization with an overall M_r of 110460 (2×32589 plus 2×22641), based on the predicted sizes of OrfZ1 and OrfZ2. These subunits were accordingly redesignated TtdA and TtdB (respectively) and the *orfZ1*–*orfZ2* operon was assigned a *ttdA*–*ttdB* designation. It would thus appear that the quaternary

Fig. 4. Gel filtration analysis of L-tartrate dehydratase. (a) Elution profile of activity of a French press extract of DH5 α (pGS581) containing 43 mg protein using a Sephacryl 200HR column (90 cm $\times$ 7 cm²). The activity (μ mol min⁻¹ ml⁻¹) in fractions (5 ml) was measured by the continuous method. The vertical arrow corresponding to M_r 105 000 was deduced from the elution of standard proteins. (b) Coomassie-blue-stained SDS-PAGE (15% gel) of combined fractions from gel-filtration (60 μ l total). The two subunits of L-tartrate dehydratase (TtdA and TtdB) and the positions of M_r markers are indicated.

Table 1. Tartrate dehydratase activities in *E. coli* grown anaerobically with glycerol and different stereoisomers of tartrate.

Ultrasonic extracts of duplicate cultures were protected from aerobic inactivation and assayed by the continuous method. Specific activities are expressed as munits (mg protein)⁻¹; values for anaerobic glucose-grown organisms were < 1 with all three stereoisomers.

Growth substrates	Tartrate dehydratase specific activities with:		
	L-tartrate	D-tartrate	meso-tartrate
Glycerol + L-tartrate	130 $\pm$ 18	11 $\pm$ 1	< 1
Glycerol + D-tartrate	< 2	16 $\pm$ 1	< 2
Glycerol + meso-tartrate	8 $\pm$ 2	8 $\pm$ 0.2	< 1

structure of *E. coli* L-Ttd resembles that of the *P. putida* enzyme, rather than the homodimeric arrangement exhibited by the oxygen-labile (Class I) fumarases.

Tartrate utilization by *E. coli*

The above observations prompted studies on the ability of *E. coli* K12 to use tartrates as substrates for supporting aerobic and anaerobic growth. Growth tests with W3110 showed that L-, D- and meso-tartrate could each serve as the major carbon and energy source for aerobic growth, but no growth was observed under anaerobic conditions. However, in the presence of glycerol, the tartrates could be used as reducible substrates for supporting anaerobic growth. This growth was relatively poor, but variant

strains capable of good anaerobic growth on glycerol plus L-tartrate, D-tartrate, and weak but better growth on glycerol plus meso-tartrate, were readily selected as faster growing colonies. Anaerobic selection on the L-isomer promoted better anaerobic growth with the D-isomer, but a culture of W3110(pGS581) behaved exactly like the parental strain.

Cell extracts of W3110 and the derivatives exhibiting better anaerobic growth, were prepared in TTD buffer, and maintained at 0 °C under N₂ for no more than 1 h before assaying for tartrate dehydratase activity with all three isomers. No activity was detected in aerobic cultures grown on any of the tartrate isomers, or after aerobic or anaerobic growth on glucose. In contrast, L-Ttd was detected after anaerobic growth with glycerol

plus L- or *meso*-tartrate, and D-Ttd activity was detected in cultures grown with glycerol plus each of the three isomers (Table 1). However, no *meso*-Ttd activity could be detected in any of the extracts. Anaerobic growth was most vigorous with the L-isomer and the L-Ttd activity was correspondingly high. The *ttdA*–*ttdB* operon would thus appear to be induced by L- and *meso*-tartrates under anaerobic conditions. Also, since L-Ttd is stereospecific, it was concluded that the D-Ttd activity is probably due to a genetically distinct stereospecific enzyme induced by all three isomers under anaerobic conditions.

Discussion

The most plausible interpretation of the results is that the *orfZ1*–*orfZ2* operon of *E. coli*, now redesignated as the *ttdA*–*ttdB* operon, encodes a stereospecific L-tartrate dehydratase, which resembles the oxygen-labile, iron–sulphur-containing L-Ttd of *Pseudomonas putida* (Kelly & Scopes, 1986). It also follows that the *E. coli* *ttd* genes are the first such genes to be cloned and sequenced (Nesin *et al.*, 1987). The enzyme is induced by L- and *meso*-tartrates during anaerobic growth with glycerol, and appears to be repressed by glucose and aerobiosis. However, with the multicopy *ttdAB* plasmid, the need for substrate and anaerobiosis was overcome. This could be due to the titration of specific repressors or simply to the amplification of *ttdAB* operons expressing at a basal level, as observed with the fumarate reductase operon (*frdABCD*) under analogous conditions (Guest, 1981). The lower anaerobic expression observed with the plasmid-containing strain is ascribed to the presence of glucose or the absence of tartrate. In this context, there is a core motif (TTGAT) for a potential FNR half-site at about –55 in the putative *ttdAB* promoter region (Nesin *et al.*, 1987), and an analogous CRP half-site motif (GTGTA) at –130. These motifs could place the *ttdAB* operon in the FNR modulon, and possibly in the CRP modulon.

The anaerobic utilization of D-tartrate likewise seems to involve a stereospecific D-Ttd, which is induced, albeit to a lesser extent, by all three tartrate isomers. The stability of this enzyme was not investigated, but it could resemble the oxygen-stable enzymes of *Pseudomonas* and *Rhodospseudomonas* (Rode & Griffithorn, 1982*a,b*) and thus be analogous to the stable Class II fumarases of *E. coli* (FumC), *B. subtilis* (CitG) and mitochondria (Woods *et al.*, 1988). In contrast, no specific *meso*-Ttd could be detected, and unless it is rapidly inactivated, the presence of L- and D-Ttd in anaerobic cultures grown with the *meso*-isomer suggests that the first step in *meso*-tartrate metabolism may involve its conversion to the L- and/or D-isomer. Such a route involving a tartrate epimerase (EC 5.1.2.5) is found in plants (Ranjan *et al.*, 1961;

Gupta & Nigam, 1970). No tartrate dehydratase activities could be detected after aerobic growth on the tartrate isomers, suggesting that enzymes analogous to the tartrate dehydrogenase or decarboxylase of *Pseudomonas* (Kohn & Jakoby, 1968; Furuyoshi *et al.*, 1991) might be operating in *E. coli*.

The L-Ttd of *E. coli* is a tetrameric enzyme ($\alpha_2\beta_2$) like the L-tartrate dehydratase of *P. putida* (Kelly & Scopes, 1986). Previous comparisons with the homodimeric fumarases of *E. coli* and *B. stearothermophilus* (FumA/B and FumA_{Bst}) showed that in combination, the TtdA and TtdB subunits of L-Ttd are colinear with, and have approximately the same size as a single fumarase subunit (Reaney *et al.*, 1993). This suggests that L-Ttd may assemble as a dimer of heterodimers ($\alpha\beta$)₂, in which each heterodimer corresponds to a fumarase subunit. When investigating possible mechanisms of evolutionary transition between the two types of subunit organization, a good coding region was detected by FRAMESCAN in an alternative reading frame near the 3' end of the *ttdA* gene. Further studies with DIAGON showed that the corresponding amino acid sequence matches FumA_{Bst} better than the original TtdA sequence. An M13mp18 template containing the relevant 1.28 kb *Bam*HI–*Bgl*III fragment (Fig. 2) was accordingly sequenced with the aid of universal and specific primers. This revealed four discrepancies in the 480 bp sequence immediately upstream of the *Bgl*III site, relative to the sequence of Nesin *et al.* (1987). Three insertions (after the following coordinates), G (921), C (927) and G (936), lead to the replacement of a five-residue segment of TtdA (197–202) by six different residues, and a deletion at 986 (G) replaces 74 C-terminal residues of TtdA (218–291 in the original sequence) by an 84-residue segment. The latter is encoded by the good coding region detected by FRAMESCAN and it extends to a stop codon (TGA) which overlaps the *ttdB* start codon (ATG), indicating that two *ttd* genes are translationally coupled. These changes increase the predicted size for the TtdA subunit to 302 amino acid residues, *M_r* 32589. The alterations are included in a partial sequence alignment (Fig. 5) based on an earlier alignment for L-Ttd and three fumarases (Reaney *et al.*, 1993).

The relationship between L-Ttd and FumA resembles that between the heterodimeric isopropylmalate isomerase, encoded by the adjacent *leuC* and *leuD* genes in *Salmonella typhimurium*, and the monomeric isomerases of fungi and yeast or the monomeric aconitases, which contain equivalent sequences in a single polypeptide, as discussed by Prodromou *et al.* (1992). Indeed, it would be interesting to engineer a fusion of the *ttdA* and *ttdB* genes, and to create two functional *fumA* sub-genes by cleaving at the corresponding site, to see whether the products express active

Fig. 5. Partial amino acid sequence alignment of the C- and the N-terminal regions of TtdA and TtdB with the corresponding regions of three Class I fumarases. The alignment is based on that of Reaney *et al.* (1993), the sequences being: L-Ttd of *E. coli* (TtdA.Eco and TtdB.Eco) and the fumarases of *E. coli* (FumA.Eco and FumB.Eco) and *B. stearotheophilus* (FumA.Bst). The alterations to the TtdA sequence (underlined), conserved cysteine residues ($\downarrow$), and insertion-deletions (.) are indicated.

enzymes having the alternate quaternary structures. It is possible that an interdomain linker peptide may be needed to allow the two components (domains or former subunits) of the *ttdA-ttdB* fusion product to assemble in an active conformation. It is also interesting that the equivalent segments of FumA_{Bst} and FumB (but not FumA) in the multiple sequence alignment (Fig. 5) resemble Q-linkers, the interdomain sequences found in bacterial regulatory proteins (Wootton & Drummond, 1989).

It is abundantly clear that the *E. coli* L-Ttd belongs to the family of enzymes with non-redox iron-sulphur centres, which includes aconitase, isopropylmalate isomerase, maleate dehydratase, dihydroxyacid dehydratase, L-serine dehydratase, 2-hydroxyglutaryl-CoA dehydratase, endonuclease III and glutaminy-PRPP amidotransferase (Switzer, 1989; Cammack, 1992).

The relationship between L-Ttd and the Class I fumarases was discussed prior to the identification of the *ttdA-ttdB* operon (Reaney *et al.*, 1993) and even after revision (Fig. 5), the overall sequence identity and similarity increase by only 3% and 4% (respectively), and none of the putative active-site residues is affected. Three cysteine residues (C70, C194 and C276 in TtdA), which could provide ligands for iron, and one potential active-site histidine residue (H37 in TtdB) in a context similar to the active-site histidine of aconitase, are conserved at equivalent positions in the Class I fumarases

and L-Ttd. There are also three conserved lysine residues and two conserved arginine residues, which could function as carboxyl binding sites. One of the former, K102 in TtdB, is in the only sequence motif that is common to the Class I fumarases and L-Ttd, the Class II fumarases and other enzymes which catalyse β -elimination reactions (aspartase, argininosuccinase and adenylosuccinase), although the consensus degenerates to GxxxxxxKxx (Reaney *et al.*, 1993).

The results demonstrate that tartrate can be converted to oxaloacetate and fumarate for use as electron acceptors in supporting anaerobic growth on a non-fermentable substrate. It is also apparent that there are multiple routes for tartrate utilization in *E. coli* depending on the isomer and the presence or absence of oxygen. Only two of the relevant enzymes (L-Ttd and D-Ttd) have been detected. It would now be of interest to characterize the latter enzyme so as to determine its relationship to L-Ttd and to other dehydratases with different quaternary structures, and different oxygen sensitivities, dependent on the presence or absence of an iron-sulphur centre. It would also be of interest to define the unknown routes of aerobic tartrate metabolism in *E. coli*.

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