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A novel prokaryotic *trans*-2-enoyl-CoA reductase from the spirochete *Treponema denticola*

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Abstract **An NADH-dependent *trans*-2-enoyl-CoA reductase (EC1.1.1.36) from the Gram negative spirochete *Treponema denticola* was identified, expressed and biochemically characterized. The recombinant protein is a monomeric enzyme with a molecular mass of 44 kDa with a specific activity of 43 ± 4.8 U/mg (μmol mg^{-1} min^{-1}) and K_m value of 2.7 μM for crotonoyl-CoA. This NADH-dependent *trans*-2-enoyl-CoA reductase represents the first enzymatically characterized member of a prokaryotic protein family involved in a fatty acid synthesis pathway that is distinct from the familiar fatty acid synthase system.**

Keywords: *trans*-2-enoyl-CoA reductase; NADH-dependent; Crotonoyl-CoA; Fatty acid biosynthesis; *Treponema denticola*

1. Introduction

Treponema denticola is a spirochete that is commonly found in the human oral cavity and is responsible for the pathogenesis of human periodontal diseases and acute necrotizing ulcerative gingivitis [1,2], which results from the inflammatory response of the host to the presence of subgingival plaque bacteria [3]. *T. denticola* is a facultative anaerobe, growing in partially aerobic or anaerobic environments [4], that typically produces lactate, succinate, and acetate as major end products. Whereas *T. denticola* is closely related to the syphilis-causing obligate human pathogen *Treponema pallidum* sub. *pallidum*, their genomes show distinct metabolic differences due to the greater biosynthetic capabilities of *T. denticola* [5]. The *T. denticola* genome encodes 2786 proteins, of which 352 are currently annotated as unidentified open reading frames [5].

TER is an enzyme involved in the mitochondrial fatty acid synthesis with a net gain of ATP in the absence of oxygen.

Recently, Hoffmeister et al. characterized a novel *trans*-2-enoyl-CoA reductase (TER; EC1.3.1.44) from *Euglena gracilis* [6] that catalyzes the reduction of enoyl-CoA to acyl-CoA in the wax ester fermentation pathway of the facultatively anaerobic *E. gracilis* mitochondrion [6–9]. *Euglena* TER identified a new family of enzymes that is widely distributed among prokaryotic genomes, but without functionally characterized prokaryotic homologues. *Euglena* TER showed sequence similarity to *T. denticola* open reading frame TDE0597, annotated, like all other prokaryotic members of the family, with unknown function. Members of this protein family in prokaryotes are often organized in gene cluster that suggest involvement in fatty acid or related synthesis operating with CoA esters instead of acyl-carrier-protein (ACP)-esters [6]. The function of prokaryotic TER homologues defined by the *Euglena* enzyme has not been investigated so far. Here we report cloning, heterologous expression and biochemical characterization of the *T. denticola* *trans*-2-enoyl-CoA reductase, thereby assigning a function to a member of this previously uncharacterized prokaryotic protein family.

2. Materials and methods

2.1. Cloning and heterologous expression of TdenTER

A freeze dried genomic DNA sample of *T. denticola* ATCC 35405 was kindly provided by Prof. H.F. Jenkinson (University of Bristol, UK). PCR was performed, after initial denaturation for 10 min at 98 °C, 30 cycles with 94 °C for 15 s, 64 °C for 30 s and 72 °C for 90 s, final extension at 72 °C for 7 min in 20 mM Tris–HCl pH 8.0, 50 mM KCl, 0.5 mM betaine, 1.5 mM magnesium sulfate, 0.1 μM of each dNTP, 1 mM of each primer, 1 unit of Triple Master polymerase (Eppendorf), and 50–100 ng of DNA sample of *T. denticola* ATCC 35405 as template. A 1194-bp fragment [GenBank Accession No. AE017248] was amplified with the following primers: Tden1Nde for, 5′-TATACATATGATTGTAAAACCAATGGTTAGG-3′; Tden1Bgl for, 5′-TATAGATCTTATGATTGTAAAACCAATGGTTAG-3′; and Tden1Xho rev, 5′-CTCGAGTTAAATCCTGTCGAACCTTTC-TACC-3′ to introduce 5′-*Nde*I, respectively, 5′-*Bgl*II and 3′-*Xho*I restriction sites. The amplification products were cloned into pET28a or pET32a vectors (Novagen) which provide N-terminal His-tag fusion proteins. For expression, *E. coli* strain BL21(DE3) (Novagen) was used. Expression was induced by addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 4 h. Expressed protein was purified by affinity chromatography using nickel–nitrilotriacetic acid (Ni–NTA) agarose, eluted as recommended by the manufacturer (Qiagen, Germany) and diluted with 70% glycerine to a final concentration of 0.5 μg/μl.

2.2. Enzyme assay

The assay was performed as described [6]. The standard assay mixture contained potassium phosphate buffer pH 6.2, 0.4 mM NADH, 2 μM FAD and 500 μM crotonoyl-CoA (Sigma). The reaction mixture was preincubated for 10 min at 30 °C before addition of the substrate. Activity was determined on a GENios microplate reader (Tecan Instruments) by measuring decrease of absorbance at 340 nm. Kinetic parameters of TdenTER were determined using substrate concentrations from 0.1 to 1.5 mM of crotonoyl-CoA (*trans*-2-butenoyl-CoA, Sigma) for determination of K_m value. The standard concentration of 500 μM crotonoyl-CoA was used to determine the K_m value for NADH. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenylether, Irgasan, Sigma) and Isoniazid (Sigma) were added to the assay mixture to final concentrations from 5 to 1000 μM. A stock solution of triclosan was

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prepared in 98% ethanol. Appropriate amounts of ethanol were tested in control assay to check possible inhibitory effects on the enzymatic activity.

2.3. Phylogenetic analysis

TER homologues were retrieved from GenBank and aligned with ClustalW [10]. NeighborNet graph was constructed and visualized with Splitstree [11]. The analysis and the comparison of the genomic region surrounding TdenTER and the TER homologues from sequenced genomes was performed using the Region View Search of the CMR resource at TIGR (tigr.org/tdb/).

3. Results and discussion

3.1. Expression and catalytic properties of TdenTER

The 1194-bp fragment from the gene sequence TDE0597 [GenBank Accession No. AE017248] of *T. denticola* ATCC 35405, hitherto annotated as a hypothetical reading frame, was amplified by PCR, cloned into the expression vector pET28a and transformed into *E. coli* BL21(DE3). The TdenTER ORF expressed in pET28a with BL21(DE3) resulted in soluble (Fig. 1) and active protein. The expressed enzyme showed NADH-dependent activity, but was not active when NADPH was used as cofactor. This is in contrast to the *Euglena* TER, which accepts both NADH and NADPH [6]. The specific activity of TdenTER was determined as 43.4 ± 4.8 U/mg ($\mu mol\, mg^{-1}\, min^{-1}$) using crotonoyl-CoA as substrate, 10-fold higher than *E. gracilis* TER activity (3.9 U/mg, Table 1). In contrast to *Euglena* TER, TdenTER did not accept *trans*-hexenoyl-CoA as a substrate. TdenTER also possesses a much higher affinity for crotonoyl-CoA (K_m = 2.7 μM) than *Euglena* TER (Table 1) and a lower substrate inhibition (K_i = 0,198 μM for crotonoyl-CoA) using NADH as cofactor. As also reported for the *Euglena* TER, no oxidizing activity was detectable in TdenTER in the reverse reaction when butyryl-CoA was used as substrate. Multiple alignment of the TdenTER amino acid sequence (Fig. 3) with its closest homologues revealed the presence of a fingerprint motif typical for short-chain dehydrogenase/reductase enzyme family [12]. The N-terminal part of the consensus sequence displayed the motif $G^{53}XXXGXG^{59}$ characteristic for NAD(P)H binding site (GXXXGXG) [11]. Similarly, the $YX_{3-7}K$ motif containing tyrosine and lysine usually present in the catalytic center of this enzyme family was also found in TdenTER at residues 241–248 and 290–296 with the motif YX_6K and YX_5K, respectively [13]. Additionally, a putative FAD binding site (GXGXXG) at the C-terminal of the amino acid sequence was missing (Fig. 3) [14]. Accordingly, addition of FAD in the enzyme assay (0–10 μM) had no effects on enzyme activity.

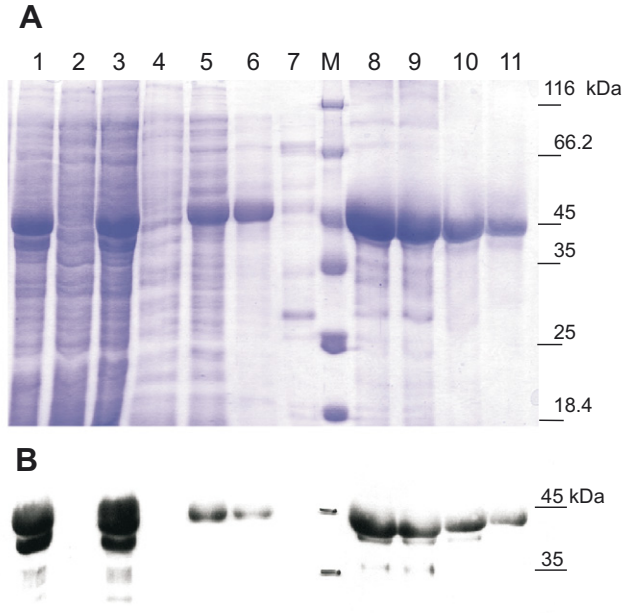

Fig. 1. (A) Coomassie-stained 12% SDS–Page of the fractions after Ni–NTA purification and (B). Western blot analysis with immunodetection of the TdenTER ORF in pET28a expressed in BL21(DE). Detection was carried out with anti-His antibodies (monoclonal mouse IgG, Novagen). Anti-mouse secondary antibody horseradish peroxidase conjugate from goat was used and the signal was visualised with the ECL Western blotting Analysis System (Amersham Biosciences). Lane 1: soluble bacterial fraction. Lanes 2–11: different fractions after Ni–NTA purification. Lane 2: flow-through pET28a; lane 3: flow-through pET28a-TdenTER; lane 4: first wash pET28a; lanes 5 and 6: first and second wash pET28a-TdenTER; lane 7: first elution pET28a; lanes 8–11: respectively, first, second, third and fourth elution pET28a-TdenTER.

The activity of TdenTER was measured in the presence of triclosan, an antibacterial agent, known to inhibit enoyl-CoA/ACP reductase (*fabI*) in *E. coli* and other organisms through the formation of a *fabI*-NAD^+-triclosan ternary complex [15,16]. However, TdenTER showed no inhibition by triclosan in a wide range of concentration (5–1000 μM) (data not shown). Sequence analysis revealed the absence in TdenTER of amino acids such as Leu100, Tyr146, Tyr156, Met159, Ala196, Ala197 (*fabI*, *E. coli* numbering) which play a key role in the *fabI*-NAD^+-triclosan ternary complex [17]. Isoniazid, a clinically used antituberculosis drug, was also added to the assay (5–1000 μM) with no effects on TdenTER activity, as well.

3.2. Metabolism of T. denticola

T. denticola possesses a genome considerably larger in size than the genomes of other spirochaetes, possibly reflecting niche adaptation in the oral biofilm environment [5,18]. The genome analysis revealed the existence of genes encoding enzymes for important metabolic and biosynthetic pathways. The presence of glycolysis, gluconeogenesis, and a pentose phosphate pathway in *T. denticola* with the absence of tricarboxylic acid cycle (TCA) and an electron transport chain (ETC) suggests that ATP is generated mainly through sugar fermentation and substrate level phosphorylation [5]. Additionally, *T. denticola* seems to be able to perform a de novo fatty acid synthesis in contrast to *T. pallidum* and *Borrelia burgdorferi*, which are completely dependent on the fatty acids present as substrates in the culture medium [5,19,20]. Spirochetes like *T. hyodysenteriae*, *T. innocens* and *T. vincentii* are able to produce short chain fatty acid (SCFA) as result of pyruvate fermentation [21]. Acetate and butyrate are, in those organisms, the main fermentation products from pyruvate and are directly associated with ATP production. In the butyrate pathway, the reduction from crotonoyl-CoA to butyryl-CoA is catalyzed by an enoyl-reductase (EC1.3.1.44) [22–24], that is, however distinct from TdenTER. *T. denticola*, on the other hand, is able to ferment cysteine, serine, alanine, and glycine to acetate and smaller amounts of lactate, succinate, formate and pyruvate. It is also able to ferment glucose, but far less efficiently than amino acids, and butyrate is present only in trace amounts [21–25].

Table 1
Specific activity, K_m and K_i values for *Treponema denticola* and *Euglena gracilis* with different cofactors and substrates

		E. gracilis	*T. denticola*	
		K_m	K_m	K_i
NADH	Crotonoyl-CoA	68 μM	2.7 μM	0.198 μM
	Hexenoyl-CoA	91 μM	–	–
NADPH	Crotonoyl-CoA	109 μM	–	–
	Hexenoyl-CoA	119 μM	–	–
Specific activity				
NADH	Crotonoyl-CoA	3.9 U/mg	43 ± 4.8 U/mg	
	Hexenoyl-CoA	1.4 ± 0.02 U/mg		
NADPH	Crotonoyl-CoA	0.7 ± 0.09 U/mg		
	Hexenoyl-CoA	0.4 ± 0.10 U/mg		

Specific activities and the K_m values for *E. gracilis* are obtained from Hoffmeister et al. [6].

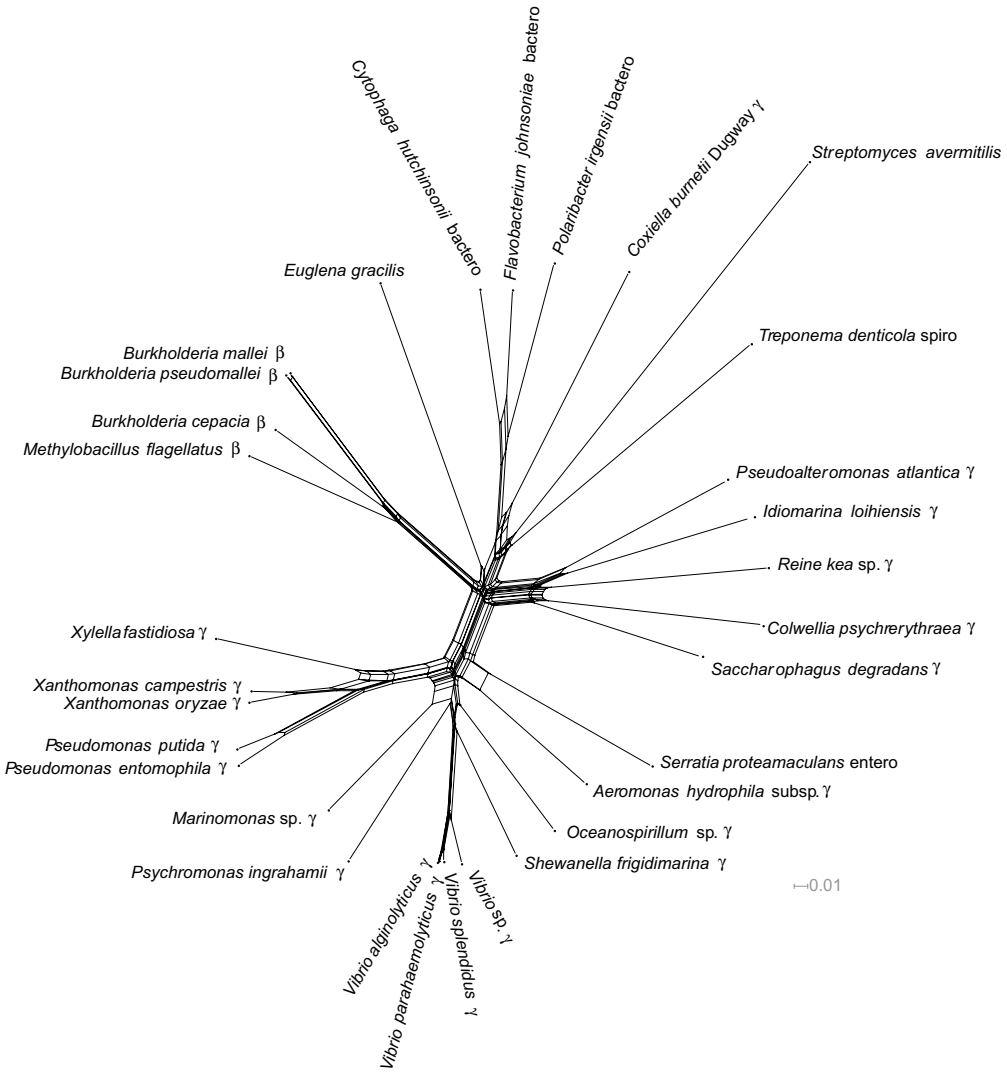

Fig. 2. NeighborNet graph of TdenTER homologous sequences. The scale bar at the lower right side indicates estimated substitutions per site. Abbreviations are as follows: β and γ, proteobacteria; *bactero*, bacteroides; *entero*, enterobacteria, *spiro*, spirochete.

3.3. TdenTER: a novel fatty acid biosynthesis route?

The phylogenetic distribution of TER homologues among prokaryotes reveals no archaebacteria homologues and a prevalence among β and γ-proteobacteria (Fig. 2). It occurs in the genome of just one Gram positive organism characterized so far, *Streptomyces avermitilis*. The TER homologue SAV2368

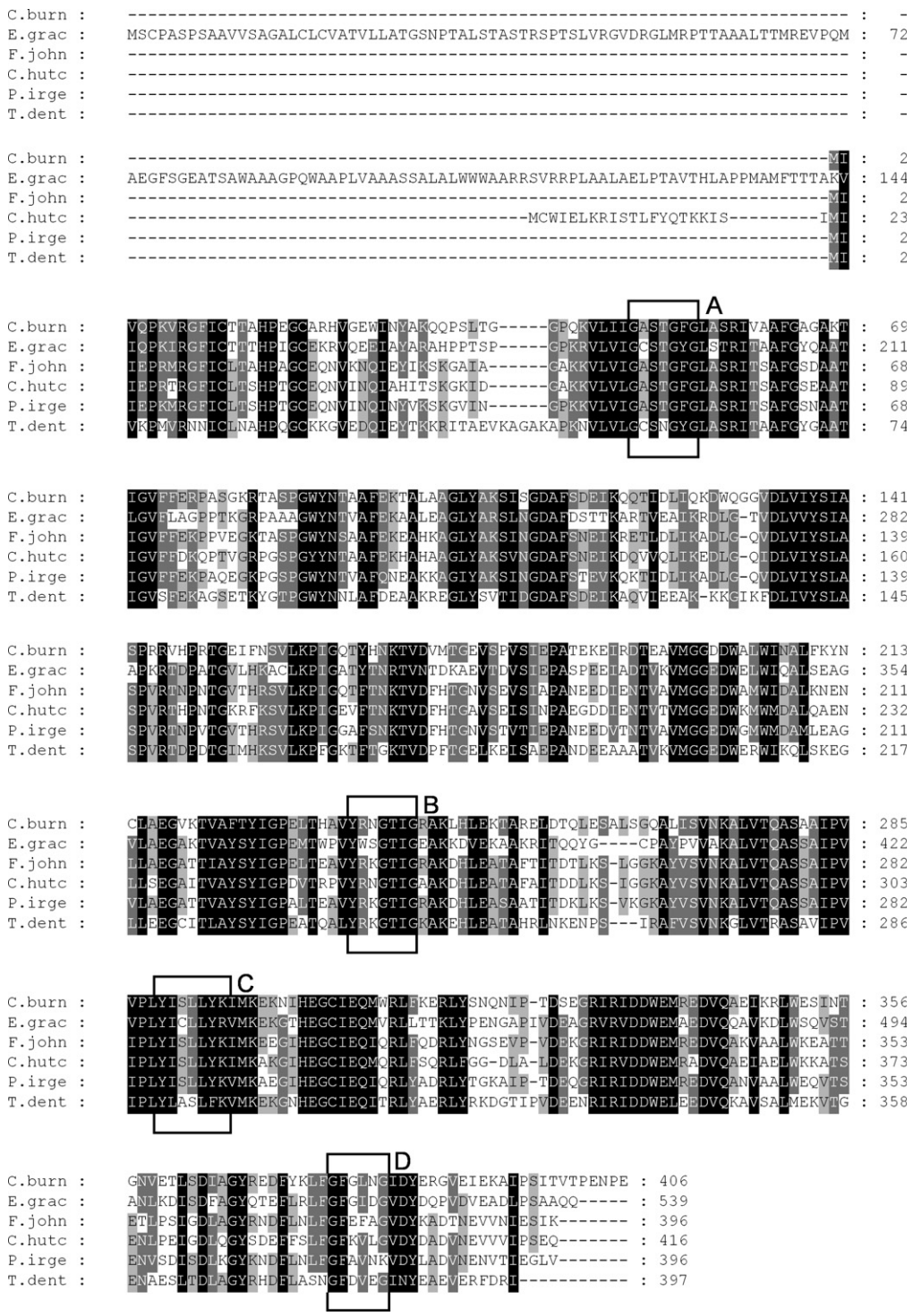

Fig. 3. Multiple sequence alignment of TdenTER with hypothetical *trans*-2-enoyl-CoA reductases. (A) Represents the putative binding site for NAD(P)H; (B and C) for the substrate and (D) for FAD. Organisms and accession numbers are given as follows: *Treponema denticola* AE017248; *Euglena gracilis* Q5EU90; *Flavobacterium johnsoniae* ZP01243065; *Cytophaga hutchinsonii* YP677688; *Polaribacter irgensii* ZP01118954; *Coxiella burnetii* ZP01298067. The protein sequences retrieved from GenBank were aligned with ClustalW [10].

[GenBank Accession No. AB070934] from the *S. avermitilis* polyketide biosynthetic cluster 5 (*pks5*) was expressed in *E. coli* and *Saccharomyces cerevisiae*, but the recombinant protein was insoluble and inactive with either crotonoyl-CoA and *trans*-2-hexenoyl-CoA as substrate (data not shown).

Many proteobacteria, such as *B. mallei* ATCC 23344, *B. pseudomallei* K96243, *C. psychrerythraea*34H, *P. aeruginosa* PAO1, and *P. putida* KT2440 perform polyhydroxyalcanoate (PHA) biosynthesis. PHAs are a class of biodegradable polyesters produced mostly under oxygen limitation as carbon and energy reserve [26–28]. The most well-studied type of PHA is poly-hydroxybutyrate (PHB), synthesized from acetyl-CoA by a sequence of three reactions catalyzed by β-ketothiolase (EC2.3.1.9), acetoacetyl-CoA reductase (EC1.1.1.36) and

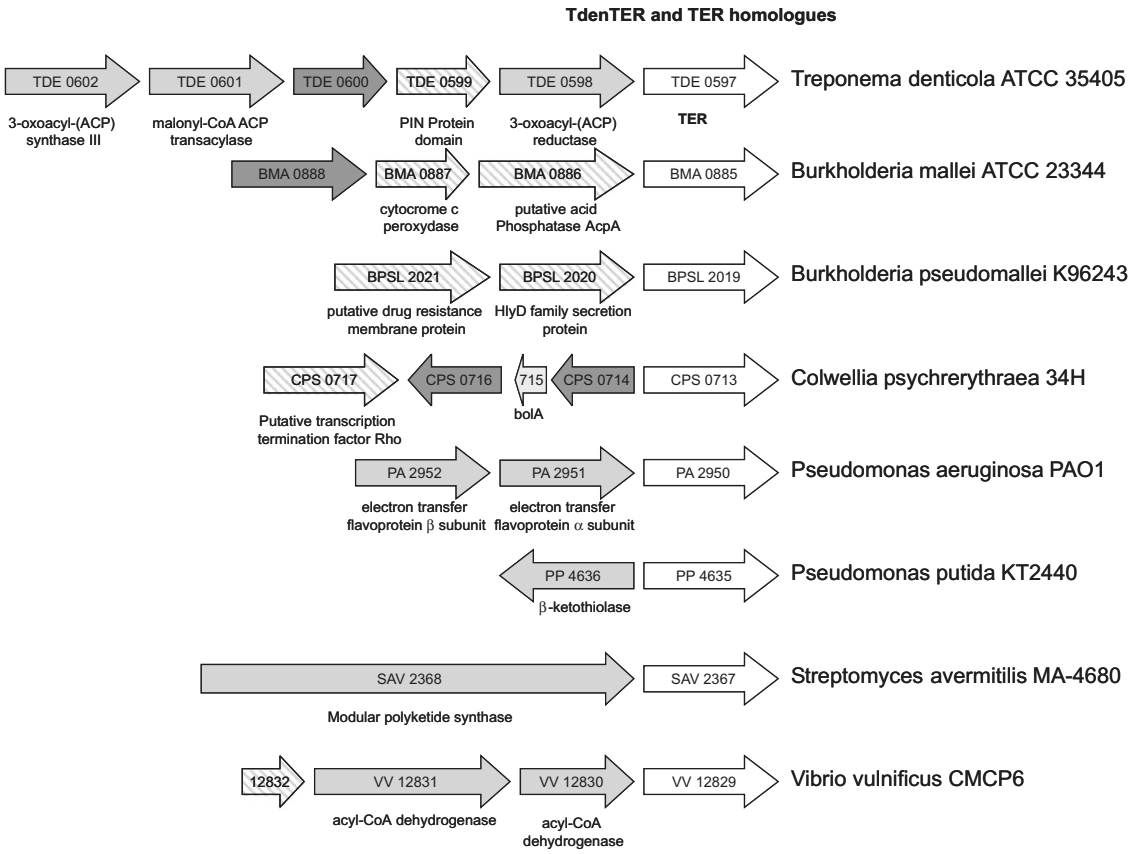

Fig. 4. Operon analysis of TdenTER and TER homologues through the CMR resource at TIGR. White arrows show TER homologues of TdenTER, grey arrows indicate hypothetical reading frames and hatched arrows proteins with distinct functions.

poly(β-hydroxybutyrate) synthase [28,29]. However, in this pathway the presence of TdenTER is not necessary as the product of the first reduction step, β-hydroxybutyryl-CoA, is required for the synthesis of PHB. *Treponema* has not been reported to synthesize PHAs and lack the fatty acid biosynthesis pathway. *T. denticola*'s ability to synthesize fatty acids and the position of the gene sequence encoding TdenTER on the downstream of fatty acid biosynthesis as shown in Fig. 4 suggest that this enzyme could be involved in a novel fatty acid synthesis pathway, possibly similar to PHA biosynthesis and possibly independent of malonyl-CoA as starter unit and operating with CoA rather than ACP esters. The use of acetyl-CoA directly avoids ATP consumption at the malonyl-CoA activation step catalyzed by the acetyl-CoA carboxylase [30]. Based upon their distribution among various gene clusters (Fig. 4), the family of prokaryotic TER enzymes described here would appear to belong to the more versatile and flexible components of metabolism, rather than to dedicated core pathways of energy metabolism in these organisms, in contrast to the situation in *Euglena*.

Finally, some herbicides, such as flufenacet, have been shown to inhibit the TER-dependent mitochondrial fatty acid biosynthesis pathway in *Euglena* [31]. These appear to inhibit the elongation step, which distinguishes *Euglena*'s mitochondrial fatty acid biosynthesis from the FAS-pathways in yeast or plants [32]. The effect of flufenacet or structurally related elongase-inhibitor herbicides on *Treponema* has not been reported.

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