Characterization of *Treponema denticola pyrF* encoding orotidine-5′-monophosphate decarboxylase

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Abstract

The *Treponema denticola* ATCC 35405 genome annotation contains most of the genes for *de novo* pyrimidine biosynthesis. To initiate characterization of pyrimidine synthesis in *Treponema*, we focused on TDE2110 (the putative *pyrF*, encoding orotidine-5′-monophosphate decarboxlyase). Unlike the parent strain, an isogenic *pyrF* mutant was resistant to 5-fluoroorotic acid. In complex medium, growth of the *pyrF* mutant was independent of added uracil, indicating activity of a uracil uptake/salvage pathway. Transcription of *pyrF* was greatly reduced in *T. denticola* grown in excess uracil, demonstrating that *de novo* pyrimidine synthesis is regulated and suggesting a feedback mechanism. *Treponema denticola* PyrF complemented uracil auxotrophy in an *Escherichia coli pyrF* mutant. This study provides biochemical confirmation of *T. denticola* genome predictions of *de novo* and salvage pyrimidine pathways and provides proof of concept that *pyrF* has potential as a selectable marker in *T. denticola*.

Introduction

Understanding of the basic biology, biochemistry and genetics of spirochetes has lagged behind other more widely studied pathogens, commensals and environmental microorganisms that are more readily cultivated. The advent of whole genome sequencing has permitted characterization of spirochete proteins and biochemical pathways including both those that are universal or widespread in most organisms (Figge & Cerff, 2001) as well as those that may be unique to certain spirochetes (Kent *et al.*, 2004). The information revealed by genome sequencing provides a huge amount of potentially important information that must at some point be confirmed to both biochemical and biological function. This requires generation of specific mutant strains in which predicted biochemical pathways can be demonstrated (White, 2006).

A survey of the *Treponema denticola* ATCC 35405 genome identifies TDE2110 as *pyrF*/URA3, present in a single copy in the genome. *pyrF*/URA3 genes encode orotidine-5′-phosphate (OMP) decarboxylase, which catalyzes a key step in *de novo* pyrimidine biosynthesis, producing uridine monophosphate (UMP). This pathway, highly conserved from bacteria to humans, is a key source of pyrimidines as DNA and RNA precursors, and also as precursors for the synthesis of certain phospholipids, complex carbohydrates, vitamins and glycoproteins. In addition to or in place of *de novo* synthesis, many organisms also rely on exogenous uracil in a salvage pathway for pyrimidine synthesis. *Treponema denticola* genes in the salvage pathway were recently annotated in the genome sequence and include TDE1612 (*upp*, encoding uracil phosphoribosyltransferase), TDE2251 (*uraA*, uracil permease) and TDE1360 (xanthine/uracil permease family protein). Interestingly, *Treponema pallidum* has the genes for the salvage pathway including *upp* and *pyrH*, but lacks *pyrB-F* required for *de novo* synthesis.

Regulation of pyrimidine synthesis has been studied extensively for many years (reviewed in O'Donovan & Neuhard, 1970), and different organisms show a range of mechanisms regulation. In *Escherichia coli*, expression of PyrC, D, E, F and G are repressed by uracil, while PyrA is feedback-inhibited by UMP. In contrast, pyrimidine synthesis in *Pseudomonas aeruginosa* appears to be constitutive. In yeast, uridine triphosphate simultaneously inhibits the activities of PyrA- and PyrB-like enzymes by a feedback inhibition mechanism. While a study by Ishihara *et al.* (1992) identified a *T. denticola* gene encoding PyrB and demonstrated its enzymatic activity in *E. coli*, there is no information available on the rest of the *de novo* pyrimidine synthesis pathway in this organism or on its regulation.

In *T. denticola*, defined mutants are currently made by allelic replacement mutagenesis, a cumbersome, low-

efficiency procedure. Genetic systems available in *T. denticola* are limited owing to a paucity of selectable markers and vectors (Li *et al.*, 1996; Fenno *et al.*, 1998; Chi *et al.*, 1999; Slivienski-Gebhardt *et al.*, 2004). Genomic data suggest that *T. denticola* possesses restriction systems that are very likely active and detrimental to the introduction of exogenous DNA. Taken together, these factors are a major obstacle to molecular analysis of cultivable treponemes. In many organisms, some of these limitations are overcome by use of counterselectable markers that are recovered by a secondary selection step following initial mutagenesis. These systems not only permit construction of multiple mutations in organisms with limited selectable markers but also permit construction of defined unmarked mutations that are often important for functional analysis of mutated genes.

Orotate phosphoribosyltransferase (PyrE) can incorporate the pyrimidine analog 5-fluoro-orotic acid (5-FOA) to form 5-fluoro-orotylidate (5-F-OMP) instead of the normal OMP. This product is then used as a substrate by the OMP decarboxylase (PyrF), producing 5F-UMP instead of UMP. While 5-FOA is not directly toxic, accumulation of 5F-UMP-containing cellular components leads to cell death due to severe toxicity. This feature is the basis for the URA3 (*pyrF*) selection system used widely in *Saccharomyces cerevisiae* for both positive and negative selection in URA3-deficient strains (Boeke *et al.*, 1984). This system has been used rarely in other microorganisms, especially where other selectable markers are available. However, in recent years, *pyrF*-based counterselection methodology has been developed for a few bacteria in which other methods are impracticable (Knipfer *et al.*, 1997; Galvao & de Lorenzo, 2005) as well as for several genera of archaea (Peck *et al.*, 2000; Lucas *et al.*, 2002; Bitan-Banin *et al.*, 2003; Sato *et al.*, 2003).

To identify potential counterselectable markers for *T. denticola*, we focused on TDE2110, (*pyrF*) present in only a single copy in the genome. To determine whether TDE2110 encodes a functional OMP decarboxylase, we constructed and characterized 5-FOA resistance in a *pyrF*-deleted strain and showed that *T. denticola pyrF* complemented an *E. coli pyrF* mutant. Our results demonstrate the presence of both *de novo* and salvage pyrimidine synthesis pathways in *T. denticola* and provide proof of concept for use of a *T. denticola pyrF* mutant as a host strain for *pyrF*-based counterselection mutagenesis.

Materials and methods

Bacterial strains and growth conditions

Treponema denticola ATCC 35405 and isogenic mutants were grown in NOS broth medium as described previously (Haapasalo *et al.*, 1991), with erythromycin (40 $\mu g\,mL^{-1}$), uracil (10 or 20 $\mu g\,mL^{-1}$) or 5-fluoroorotic acid (5-FOA, Zymo Research, Orange, CA; 1–10 mM) added as appropriate. 5-FOA toxicity was assayed by its ability to inhibit *T. denticola* growth in NOS medium. A stock solution of 5-FOA (50 $mg\,mL^{-1}$ in DMSO) was diluted serially in DMSO and equal volumes of resulting 5-FOA solutions (including DMSO alone as negative control) were added to NOS medium. For growth studies, actively growing cultures were diluted 1 : 100 in NOS with or without supplement of interest and growth was monitored daily by OD at 600 nm for up to 2 weeks, by which point cultures were in stationary phase. Growth studies were conducted at least twice, with triplicate samples. Cultures were examined by phase contrast microscopy for purity and typical strain morphology. *Escherichia coli* NovaBlue (Novagen) and JM110 (Yanisch-Perron *et al.*, 1985), used as hosts for cloning and plasmid preparations, were grown in Luria–Bertani (LB) broth or agar medium supplemented with kanamycin (50 $\mu g\,mL^{-1}$) or erythromycin (200 $\mu g\,mL^{-1}$) as appropriate. For growth studies, *E. coli* YA149 carrying plasmids of interest was grown in M9 minimal medium containing 20 μM $CaCl_2$, 0.2% glucose, 0.1% glycerol, 0.1% casamino acids and kanamycin (50 $\mu g\,mL^{-1}$), with uracil (10 $\mu g\,mL^{-1}$) or 5-FOA (5 mM) added as appropriate. Overnight cultures grown in LB medium were diluted 1 : 500 in M9 medium and incubated at 37 °C with shaking. Growth was monitored by OD at 600 nm over a 10 h time course, by which point cultures were in stationary phase.

Allelic replacement mutagenesis

For allelic replacement of *T. denticola pyrF*, a genomic DNA fragment containing TDE2109-TDE2110-TDE2111 and part of TDE2112 was amplified with primers CX421 (5′-TTGCAGCATTTTTAGGCATAGTA-3′) and CX422 (5′-TCATACTTCTTCCTCTTTCCGTTT-3′) and cloned in plasmid vector pSTBlue-1 (Novagen). TDE2109 is annotated as *deaD*, encoding an ATP-dependent RNA helicase. TDE2111 encodes an uncharacterized *Treponema*-specific protein. Both genes are transcribed away from *pyrF*. To facilitate subsequent steps, this plasmid was then digested with ScaI and then self-ligated to remove a small portion of the vector, the 5′ end of TDE2111 and the 3′ end of TDE2112. The resulting plasmid, digested with HindIII and PpuMI to remove a 550 bp internal fragment of *pyrF*, was treated with Klenow fragment of DNA polymerase to create blunt ends and ligated to the *ermF-ermB* cassette from pSY118 (Lee *et al.*, 2002a), yielding pCF382. Plasmid pCF382, with the *ermF-ermB* cassette in the same transcriptional orientation as *pyrF*, was linearized with BsaBI and EcoRI to remove vector DNA and then electroporated into *T. denticola* as described previously (Li *et al.*, 1996; Fenno *et al.*, 1998). Mutants were selected for resistance to

erythromycin (Em^R) in NOS/GN agar (Chan *et al*., 1997). Mutations were verified by PCR analysis of the target region in genomic DNA.

Transcription analysis

Total RNA was isolated from *T. denticola* cultures harvested during logarithmic growth (3 days) using the RNeasy Mini Kit (Qiagen). DNAse-treated RNA samples were reversed transcribed with random hexamer primers using the SuperScript™ First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR; Invitrogen). One microlitre of the resulting first-strand cDNA was amplified for 30 cycles using *Taq* DNA polymerase (Invitrogen), with 16S rRNA gene serving as an internal control for normalization between samples. The GeneFisher algorithm (Giegerich *et al*., 1996) was used to design gene-specific T_m-matched primer sets yielding 80–120 bp amplicons. RT-PCR products, including negative controls (no RT enzyme), were analyzed by agarose gel electrophoresis.

Results and discussion

Overview of *T. denticola* pyrimidine synthesis pathway

Genes encoding most enzymes required for *de novo* pyrimidine synthesis have been provisionally identified in the *T. denticola* genome (Seshadri *et al*., 2004) or in updated annotations (http://www.oralgen.lanl.gov/). These include *pyrB, C, D, E* and F (Table 1). Of these, only *pyrB*, encoding aspartate carbamoyltransferase, has been functionally confirmed (Ishihara *et al*., 1992). Unlike many bacteria, *T. denticola* does not appear to possess a glutamine-dependent carbamoyl-phosphate synthase (PyrA). However, consistent with its peptide-dependent metabolism (Mäkinen & Mäkinen, 1996), *T. denticola* likely synthesizes carbamoyl phosphate directly from NH_3, CO_2 and ATP by means of carbamate kinase activity encoded by TDE2476 (*arcC*). It is noteworthy that the *T. denticola* genes in the *de novo* pyrimidine pathway do not appear to be linked at the level of transcription. The two most closely located genes, *pyrC* and *pyrF*, are transcribed in opposite directions and are separated by two apparently unrelated genes. TDE1612, recently annotated as *upp,* encodes uracil phosphoribosyltransferase activity required for one-step interconversion of uracil and UMP in the salvage pathway. The alternative two-step pathway (5′-ribonucleotide phosphohydrolase followed by uridine hydrolase) has not been identified in *T. denticola* as of this writing. TDE2085 (*pyrH*) encodes a uridylate kinase that catalyzes the same step in both the *de novo* and salvage pathways. To date, no *T. denticola* gene encoding nucleoside diphosphate kinase has been identified. In contrast, *T. pallidum* lacks all the genes for *de novo* pyrimidine biosynthesis, and is thus reliant on its mammalian host for uracil (Fraser *et al*., 1998).

Allelic replacement mutagenesis of *T. denticola* pyrF

The *T. denticola* genome annotation identifies TDE2110 as *pyrF*. The deduced polypeptide exhibits high homology (BLAST score > 200) with PyrF in a wide range of prokaryotic organisms. Interestingly, this list does not include PyrF of *E. coli* and other enterics, and *E. coli* PyrF does not have a homolog in *T. denticola* (data not shown). To demonstrate that TDE2110 encodes OMP decarboxlyase, the key final step in *de novo* synthesis of UMP, we constructed an isogenic mutant by allelic replacement of TDE2110 with a gene cassette encoding Em^R (Fletcher *et al*., 1995). The strategy for mutagenesis of *pyrF* is shown in Fig. 1. Positive Em^R clones resulting from double crossover homologous recombination following electroporation with linearized pCF382 were verified by PCR using CX421 and CX422, as well as primers specific for the *ermF-ermB* cassette and the deleted portion of *pyrF* (data not shown). A validated *pyrF*-deleted isolate designated *T. denticola* D2110E (for *D*eleted TDE*2110* with *E*rythromycin marker) was preserved for further analysis.

Table 1. *Treponema denticola* pyrimidine biosynthesis genes

Gene ID	Gene*	E.C. number	Enzyme	Product
TDE2476	*arcC*	2.7.2.2	Carbamate kinase	Carbamoyl-phosphate
TDE0129	*pyrB*	2.1.3.2	Aspartate carbamoyltransferase	N-carbamoyl-L-aspartate
TDE2107	*pyrC*	3.5.2.3	Dihydroorotase	Dihydroorotate
TDE0982	*pyrD*	1.3.3.1	Dihydroorotate oxidase	Orotate
TDE1001	*pyrE*	2.4.2.10	Orotate phosphoribosyltransferase	Orotidine-5-phosphate
TDE2110	*pyrF*	4.1.1.23	Orotidine-5′-P decarboxylase	Uridine-5-phosphate
TDE2085	*pyrH*	2.7.4.22	Uridylate kinase	Uridine diphosphate
TDE1612	*Upp*	2.4.2.9	Uracil phosphoribosyltransferase	Uracil

**arcC* and *pyrB-F* encode enzymes in the *de novo* pathway. The *upp* product functions in the salvage pathway. The *pyrH* product functions in both pathways.

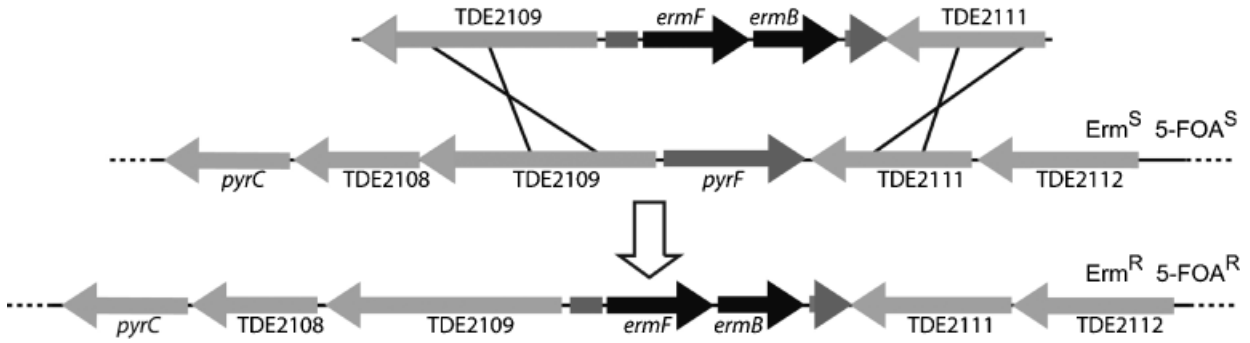


Fig. 1. Strategy for pyrF mutagenesis in *Treponema denticola* 35405. An isogenic *T. denticola pyrF* mutant was constructed as described in the text. *Treponema denticola* was electroporated with a linear DNA fragment consisting of the nonvector portion of pCF382, in which *ermF-ermB* replaced a 550 bp internal fragment of *pyrF*. Em^R strain *T. denticola* D2110E resulting from double crossover homologous recombination at *pyrF*-flanking regions was validated by PCR and by resistance to 5-FOA. Black arrows: *ermF-ermB*. Dark grey arrows: *pyrF* and *pyrF* fragments. Light grey arrows: genes flanking *pyrF*.

(a)

T. denticola strain	[5-FOA]	O.D.*
35405	0	0.484
	1 mM	0.023
	5 mM	0.019
	10 mM	0.012
D2110E	0	0.305
	1 mM	0.371
	5 mM	0.250
	10 mM	0.032

* Optical density at 600 nm, stationary phase.

(b)

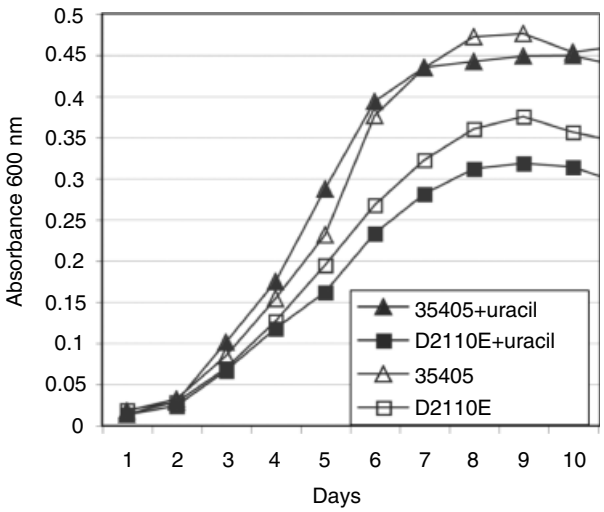


Fig. 2. Effects of 5-FOA and excess uracil on *Treponema denticola* growth. OD of *T. denticola* cultures was measured by absorbance at 600 nm. Results of representative growth studies are shown. Sample variability (not shown) was not significant. (a) Growth of *T. denticola* 35405 was inhibited by 1 mM 5-FOA, while *pyrF* mutant D2110E grew in the presence of up to 5 mM 5-FOA. (b) *Treponema denticola* 35405 and D2110E growth in NOS medium or NOS medium supplemented with 20 μg mL^{-1} uracil.

Effects of 5-FOA and uracil on *T. denticola* growth

Because some *T. denticola* Em^R mutant strains have been shown to contain subpopulations of wildtype, spontaneous point mutants to Em^R (Lee *et al.*, 2002b), we further validated D2110E by comparing its growth on NOS-GN agar containing both erythromycin and 5 mM 5-FOA to that of 35405 grown on NOS-GN agar alone or with 5 mM 5-FOA. Strain D2110E grew in the presence of 5-FOA while 35405 did not (data not shown). To further characterize the contribution of PyrF activity both to *T. denticola* growth and to its sensitivity to the toxic metabolites of 5-FOA, two growth studies were performed. *Treponema denticola* 35405 and D2110E were grown in the presence of a range of 5-FOA concentrations (0, 1, 5 and 10 mM) and growth was monitored for up to 2 weeks (Fig. 2a). 35405 did not grow in NOS medium containing 1, 5 or 10 mM 5-FOA. D2110E

grew equally well in NOS alone or NOS supplemented with 1 or 5 mM 5-FOA, but did not grow in 10 mM 5-FOA. Next, both strains were tested for growth in NOS with or without additional uracil supplementation. Addition of up to 20 μg mL^{-1} uracil had no significant effect on the growth of either strain. As illustrated in Fig. 2b, *T. denticola* D2110E grew at a slower rate and attained a lower final OD than 35405, independent of additional uracil. This indicates that NOS medium contains sufficient uracil to sustain growth in the absence of *de novo* pyrimidine synthesis. The difference in growth rate is consistent with the considerably slower appearance of D2110E colonies in semisolid medium (*c.* 3 weeks vs. 8–10 days for 35405, data not shown). Interestingly, supplementation with excess uracil (20 μg mL^{-1}) resulted in decreased sensitivity of *T. denticola* 35405 to 5-FOA (data not shown). Since both PyrE and PyrF activity are required for 5-FOA toxicity, the lack of toxicity under conditions of excess uracil likely reflects the activity of the salvage pathway, and may be due in part to downregulation of *de novo* pyrimidine synthesis when exogenous uracil is available.

Transcription of *T. denticola pyr* genes in response to uracil

In many bacterial systems, at least some of the *pyr* genes are organized in an operon (Theisen *et al.*, 1987; Ghim & Neuhard, 1994). As shown in Table 1, this is not the case in *T. denticola*. In both bacteria and yeast, pyrimidine synthesis is regulated at the transcriptional level, by availability of exogenous uracil or pyrimidine triphosphates (Bach *et al.*, 1979; West, 2005). Because PyrE and PyrF catalyze the two final steps in UMP synthesis and are required for 5-FOA toxicity, we focused on analysis of transcription of these genes. We used RT-PCR to assay expression of *pyrE, pyrF* and *pyrH* in *T. denticola* 35405 grown under conditions of limited or excess uracil. As shown in Fig. 3, transcription of *pyrF* was greatly reduced when grown in excess uracil. We did not detect changes in *pyrE* transcription in response to excess uracil. As expected, transcription of *pyrH* was not affected by uracil because PyrH functions in both the *de novo* and salvage pathways. It should be noted that NOS broth is a complex medium containing yeast extract and serum, and thus likely contains available uracil adequate for *T. denticola* growth. Therefore, *de novo* pyrimidine synthesis may be at less than maximum levels in this complex medium. To date, there have been no reports of growth of *T. denticola* in a chemically defined serum-free medium. [Correction added 22 January 2007: A seminal paper by Wyss (1992) reported growth of several *T. denticola* strains (not including the type strain ATCC 35405) in chemically defined serum-free OMIZ-W1 medium. A related study is currently in press in this journal (Wyss, 2007).]

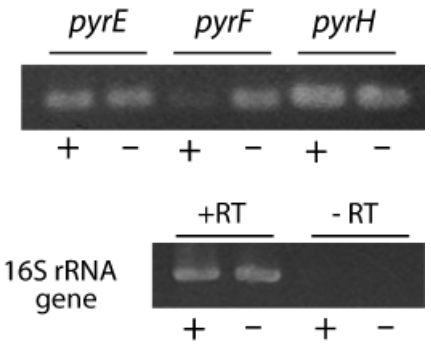


Fig. 3. Expression of pyrF mRNA is reduced in the presence of excess uracil. *Treponema denticola* 35405 was grown in NOS medium (−) or NOS medium supplemented with 40 μg mL^{-1} uracil (+). RT-PCR results from cells in logarithmic growth phase are shown. Primer sets are shown in Table 2. 16S rRNA gene controls were conducted on templates prepared with or without reverse transcriptase (RT). Results representative of three independent experiments are shown.

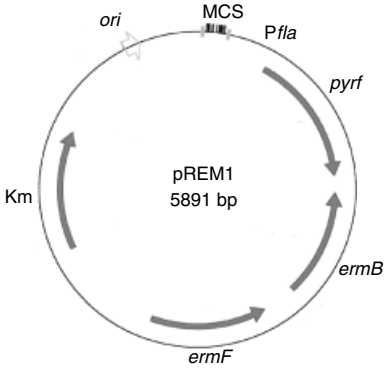
Table 2. Oligonucleotide primers for RT-PCR studies

Primer	Target	Sequence
16S-UF	Universal 16S rRNA gene (F)	TCCTACGGGAGGCAGCAGT
16S-UR	Universal 16S rRNA gene (R)	GGACTACCAGGGTATCTAATCCTGTT
CX608	*pyrE* (F)	AACCCTTTACTTGGGCATC
CX609	*pyrE* (R)	ATTCGGGGTCAAAGTCAAC
CX610	*pyrF* (F)	TTTTCGGGTTCCGAAAG
CX611	*pyrF* (R)	GTCGAAGGCTTCGATAG
CX612	*pyrH* (F)	GGCAAATACTGGTTGCTG
CX613	*pyrH* (R)	TTAGGATCGTCGGTATAGAC

Complementation of *E. coli pyrF* mutant by *T. denticola pyrF*

To confirm the identity and activity of PyrF, we introduced a plasmid carrying *T. denticola pyrF* into *E. coli pyrF* mutant YA149 (Beckwith *et al.*, 1962). A derivative of pSTBlue-1 was constructed in which the *ermF-ermB* cassette from pSY118 (Lee *et al.*, 2002a) replaced the 3′ end of the multiple cloning site (MCS), the f1 phage origin and most of the *bla* gene of pSTBlue-1. The constitutive *fla* promoter and ribosome binding site (P*fla*) from pBFC (Slivienski-Gebhardt *et al.*, 2004) was cloned in the MCS of the resulting plasmid. *T. denticola pyrF* was amplified using primers complementary to the 5′ and 3′ ends of the ORF and cloned directly downstream of P*fla*. The resulting plasmid (pREM1; Fig. 4a) contains *T. denticola pyrF* under transcriptional control of P*fla*. Plasmids pREM1 and pSTBlue-1 were maintained in *E. coli* by selection with Km, as described in Materials and methods. As shown in Fig. 4b, YA149/pSTBlue-1 growth in modified M9 minimal medium was dependent on supplementation with uracil (2 μg mL^{-1}). YA149/pREM1 grew in

(a)



(b)

E. coli strain	Ura^{10*}	O.D.**
YA149/pSTBlue-1	-	0.046
YA149/pREM1	-	1.07
YA149/PSTBlue-1	+	1.09
YA149/pREM1	+	1.07

* Medium supplemented with uracil (10 μg ml^{-1})

** Optical density at 600 nm, stationary phase.

Fig. 4. Complementation of pyrF mutation in *Escherichia coli*. (a): Plasmid pREM1 contains *Treponema denticola pyrF* under transcriptional control of a constitutive promoter (P*fla*). pREM1 also carries the MCS, origin of replication (ori) and kanamycin resistance gene (Km) from pSTBlue-1 and the *ermF-ermB* from pSY118. (b): ODs of *E. coli* YA149/pSTBlue-1 and *E. coli* YA149/pREM1 at stationary growth phase in minimal medium with kanamycin (50 μg mL^{-1}), with or without uracil (10 μg mL^{-1}). OD was measured by absorbance at 600 nm. Results of a representative experiment are shown.

minimal medium (with our without uracil) at a similar rate and final OD as YA149/pSTBlue-1 grown with uracil. When grown in LB broth, YA149/pSTBlue-1 was resistant to 5 mM 5-FOA, while YA149/pREM1 was unable to grow in the presence of 5 mM 5-FOA when the plasmid was maintained with kanamycin (data not shown).

Conclusions

Our findings demonstrate that *T. denticola* TDE2110 (*pyrF*) encodes an active orotidine 5′ phosphate decarboxylase activity and that *pyrF* transcription is downregulated in response to uracil availability. Mutagenesis of *pyrF* conferred resistance to 5-FOA, and *T. denticola pyrF* complemented an *E. coli pyrF* mutant. Our functional confirmation of *T. denticola pyrF* annotation provides strong evidence of both the *de novo* and salvage pathways for pyrimidine biosynthesis. Viability of the *T. denticola pyrF* mutant demonstrates that *T. denticola* possesses the salvage pathway for pyrimidine biosynthesis, while its resistance to 5-FOA confirms existence of the key enzymes PyrE and PyrF in the *de novo* pathway. While *pyrF* is not an essential gene in *T. denticola*, it appears to be necessary for optimal growth, suggesting that the salvage pathway is relatively inefficient. In contrast, *T. pallidum* appears to possess only the salvage pathway and is thus reliant on the uptake of uracil from its mammalian host. While the complex NOS medium used in the present study was sufficient to detect the effects of large excesses of uracil on *pyrF* transcription, we anticipate that growth in the complete absence of uracil would result in more dramatic changes in the transcription of pyrimidine pathway genes. A partially defined medium for oral spirochetes [OMIZ-P4 (Riviere *et al.*, 1999; Coico *et al.*, 2006); ATCC Medium 2131] that is used by several laboratories contains uracil, yeast extract and serum, and would likely yield similar results. More complete characterization of these pathways will require development of a chemically defined serum-free minimal medium for *T. denticola*.

Construction and properties of *T. denticola* D2110E provide proof of concept that a *pyrF*-based counter-selection mutagenesis system, such as is widely used in *S. cerevisiae*, is feasible in *T. denticola*. Having determined that it is not an essential gene, we are currently exploring use of *pyrF* as a selectable marker for both positive and negative selection in *T. denticola*. Because 5-FOA is toxic in the presence of active PyrF, it should be possible to construct an unmarked *pyrF* deletion mutant by allelic replacement and direct selection with 5-FOA without antibiotic selection. Plasmid pREM1 could then be used as a vector for two-step counterselection mutagenesis, resulting in defined, unmarked chromosomal mutations.

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