

# Molecular and biochemical characterization of the tetralin degradation pathway in *Rhodococcus* sp. strain TFB

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## Summary

**The tetralin biodegradation pathway in *Rhodococcus* sp. strain TFB, a Gram-positive bacterium resistant to genetic manipulation, was characterized using a proteomic approach. Relative protein expression in cell free extracts from tetralin- and glucose-grown cells was compared using the 2D-DIGE technique. Identification of proteins specifically expressed in tetralin-grown cells was used to characterize a complete set of genes involved in tetralin degradation by reverse genetics. We propose a tetralin degradation pathway analogous to that described for *Sphingomonas macrogolita* strain TFA. TFB *thn* genes are organized into three operons; two contain all of the structural genes and are transcribed in the same direction, while the third operon, *thnST*, is transcribed in the opposite direction and encodes a two-component regulatory system, whose transcription is higher in tetralin-grown cells. In addition to tetralin induction, TFB *thn* structural genes are subject to glucose repression. Primer extension assays and translational *thnA1::gfp* and *thnS::gfp* fusions were used to characterize putative promoter regions. A mutational analysis of the *thnA1* promoter region allowed us to define nucleotides within the *cis* regulatory elements that are important for the control of *thn* gene expression.**

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## Introduction

Members of the Gram-positive genus *Rhodococcus* are known to have important roles in biodegradation because of their broad metabolic diversity and ubiquity in contaminated environments (Bell *et al.*, 1998). However, rhodococcal catabolic pathways have been less well characterized at the molecular level than those of other Gram-negative bacteria involved in biodegradation, such as *Pseudomonas*. This is mainly due to the limited availability of genetic tools for *Rhodococcus* and the fact that some strains are resistant to genetic manipulation. The redundancy of metabolic pathways and genes in this genus, confirmed by analysis of the complete *Rhodococcus* sp. RHA1 genome sequence (van der Geize and Dijkhuizen, 2004; McLeod *et al.*, 2006), complicates the elucidation of whole biodegradation pathways. As an alternative to genetic analysis, proteomics has been shown to be a powerful tool for high-throughput analysis of rhodococcal metabolism (Navarro-Llorens *et al.*, 2005; Patrauchan *et al.*, 2005; Tomás-Gallardo *et al.*, 2006).

Analysis of *Rhodococcus* genome sequences has identified a number of transcriptional regulators, the most abundant belonging to the LysR-type and IclR-like families. Several two-component regulatory systems have also been found (see <http://www.rhodococcus.ca>). Some transcriptional regulators involved in regulating the degradation of aromatic compounds by rhodococcal strains have been characterized. In *Rhodococcus erythropolis* CCM2595, the IclR-like transcriptional regulator CatR represses the expression of the catechol degradation operon (Vesely *et al.*, 2007). In *Rhodococcus* sp. strains M5 (Labbé *et al.*, 1997) and RHA1 (Takeda *et al.*, 2004), a two-component regulatory system (BphST) has been characterized as the transcriptional regulatory system for biphenyl degradation. The response regulator BphT is a member of the LuxR family of transcriptional regulators. Two-component regulatory genes are also known to be involved in *o*-xylene degradation by *Rhodococcus* sp. strain DK17 (Kim *et al.*, 2005). However, very little is known about the molecular mechanism by which these regulatory systems control the activity of catabolic pathways in response to environmental signals.

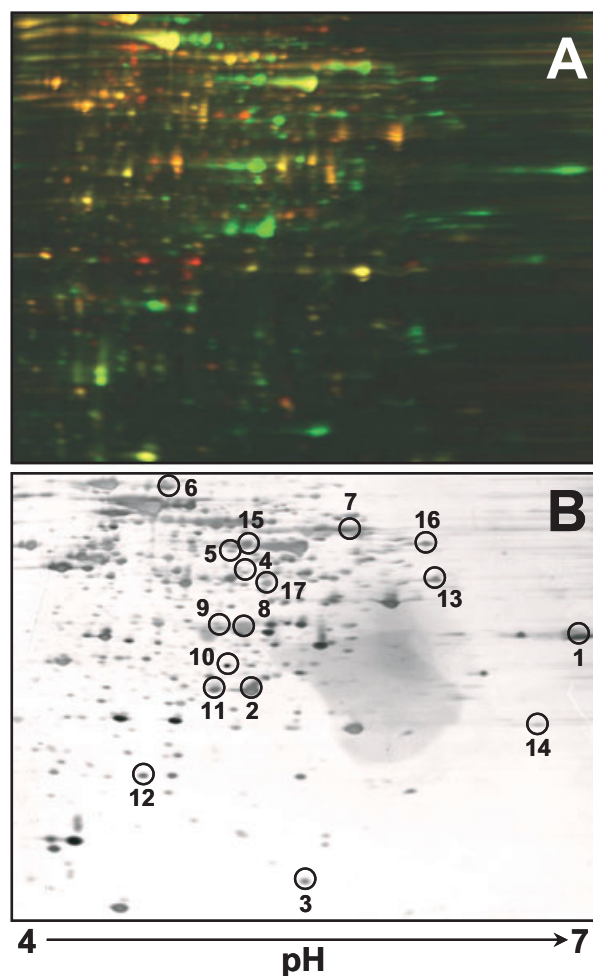
Tetralin (1,2,3,4-dihydronaphthalene) is a toxic organic solvent, which is composed of an aromatic and an alicyclic ring that share two carbon atoms. A number of bacterial strains able to grow aerobically on tetralin as the sole carbon and energy source have been described previously (Sikkema and de Bont, 1991). The tetralin catabolic pathway of *Sphingomonas macrogolitabida* strain TFA has been the best characterized at molecular and biochemical levels (Hernández *et al.*, 1999). In this pathway, the aromatic tetralin ring is initially modified by a ring-hydroxylating dioxygenase and a dehydrogenase (Moreno-Ruiz *et al.*, 2003). The resulting catechol derivative is then further metabolized through reactions sequentially catalysed by an extradiol dioxygenase, a hydrolase, a hydratase and an aldolase (Andújar *et al.*, 2000; Hernández *et al.*, 2000; Hernández *et al.*, 2002). This set of enzymes is able to cleave both the aromatic and the alicyclic rings of tetralin to render pyruvate and pimelic semialdehyde (Hernández *et al.*, 2002). Structural and regulatory TFA *thn* genes are clustered into two closely linked operons, which are transcribed in opposite directions (Hernández *et al.*, 1999; Moreno-Ruiz *et al.*, 2003). The regulatory system responsible for the induction of TFA *thn* genes in response to tetralin includes a LysR-type transcriptional activator, ThnR and a ferredoxin reductase-like protein, ThnY (Martínez-Pérez *et al.*, 2004). In addition, TFA *thn* genes are subjected to catabolite repression in the presence of preferential carbon sources (Martínez-Pérez *et al.*, 2004). An integrated response to several inducers through communication between the catabolic pathway and the regulatory system has also been recently reported in strain TFA. This additional level of regulation prevents efficient TFA *thn* gene induction by molecules that are not substrates of the catabolic pathway, thus preventing gratuitous induction (Martínez-Pérez *et al.*, 2007).

Previously, we have reported that growth on phthalate, tetralin or naphthalene induces the expression of specific proteins in *Rhodococcus* sp. strain TFB (Tomás-Gallardo *et al.*, 2006). In this paper, we extend our initial proteomic approach in order to characterize the tetralin catabolic pathway in *Rhodococcus* sp. strain TFB at the molecular level.

## Results

### *Proteome analysis of tetralin- versus glucose-grown TFB cells and identification of differentially expressed proteins*

Qualitative and quantitative analysis of tetralin-induced proteins was carried out using the Ettan DIGE fluorescent 2D gel electrophoresis system (GE Healthcare). Equal amounts of soluble protein extract (50 µg) from *Rhodococcus* sp. strain TFB grown on either glucose or tetralin were labelled with Cy5 or Cy3 dyes respectively. Pro-



**Fig. 1.** 2D-DIGE gel analysis of glucose- (red spots) versus tetralin- (green spots) grown cell proteomes (A) and silver staining of the same gel (B). Fifty micrograms of proteins from each condition was labelled with Cy3 or Cy5. As internal standard, 25 µg of each sample was mixed and labelled with Cy2. Numbered spots were digested in-gel with trypsin and analysed by MALDI-MS(/MS) or ESI-IT for protein identification.

teins specifically expressed in tetralin- (green spots) or glucose-grown cells (red spots) were detected after scanning (Fig. 1A). A total of 151 (or 13.5%) out of 1115 distinct spots showed quantitative differences in their expression between glucose- and tetralin-grown cells. Of the 151 spots, 47 could be individually selected from the 2D-DIGE gel for analysis by MALDI-MS(/MS) (maldi-assisted laser desorption/ionization tandem mass spectrometry) or ESI-IT MS(/MS) (electrospray ionization-ion trap tandem mass spectrometry), which identified 16 different proteins (Table 1). Of these proteins, we identified eight with probable roles in the conversion of tetralin to a linear compound: the  $\alpha$  and  $\beta$  subunits of a dioxygenase most similar to an ethylbenzene dioxygenase (ThnA1 and ThnA2; spots 7 and 12), a ferredoxin reductase (ThnA4, spot 5), a *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (ThnB,

**Table 1.** Identification of tetralin induced *Rhodococcus* sp. strain TFB proteins.

Spot	MASCOT score	Accession code	Predicted protein	Organism	Mw <sup>a</sup>	pI	Peptide sequence <sup>b</sup>	thn gene
1	53	gil110567587	4-(2-Oxocyclohexyl)-2-hydroxy-buta-2,4-dienoic acid hydrolase	<i>Sphingomonas macrogolliabidus</i> TFA	32	6.11	<u>CGHWAQLER</u>	<i>thnD</i>
2, 11	110	gil29647412	Cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase	<i>Rhodococcus</i> sp. RHA1	28	4.95	<u>LDTFVGNAAIWFDFSTK</u> <u>TATGAIINCDDGGMGVFR</u>	<i>thnB</i>
3	112	gil110825065	Possible sterol transfer protein	<i>Rhodococcus</i> sp. RHA1	14	5.17	TPDFVLATK LDVWRQFADGKLR HLPYERPPLSK	<i>thnU</i>
5	66	gil2072113	Ferredoxin reductase	<i>Escherichia coli</i> K12	43	5.6	<u>VFANSCPHR</u>	<i>thnA4</i>
7	99	gil111026201	Ethylbenzene dioxygenase alpha subunit	<i>Rhodococcus</i> sp. RHA1	51	5.28	<u>VCFADAGNR</u> MMPVAQVASYK MAYYNDLDMIFTR DEDRLVGSREDTWR	<i>thnA1</i>
12	181	gil110825065	Ethylbenzene dioxygenase beta subunit	<i>Rhodococcus</i> sp. RHA1	21	5.06	VYSNFFAFR	<i>thnA2</i>
14	137	gil42475486	Hypothetical protein	<i>Rhodococcus rhodochrous</i> K37	18	5.47	DMIVVPAGVPR YVSGGATGNHENDNR HEDWDTLGFQAK	
15	103	gil52783628	Isocitrate lyase	<i>Rhodococcus equi</i> 103P <sup>+</sup>	47	4.8	NGIEPCIAR VLIPTQQHIR EGMTAFVQLQER LAADVADVPTVIAR TDAEAATLLTSDVDER AMIAAGVAGSHWEDQASEK EVGAGYFDSIATTVDPNTSTAALK EAVFEPR EFHFPEPR	
16	122	gil706845	N,N'-dimethyl-4-nitrosoaniline oxidoreductase	<i>Rhodococcus erythropolis</i> N186/21	46	5.4	LLIMTTGLR VWEYNLPSSR DVGIPDNFGQVR NINEFEFQAK NLLTVQAADAAVEAAIR YAQLAGALGVDTFR YFAPTCVR	
17	54	gil10176610	Transposase	<i>Bacillus halodurans</i> C-125	50	8.9		

**a.** Mw and pI were calculated from the predicted protein sequences.  
**b.** Peptides subjected to MS/MS analysis are underlined.

Spot	Accession code	Predicted proteins	Organism	Mw <sup>a</sup>	pI	Tentative sequence	thn gene
4	gil119718512	Acyl-CoA dehydrogenase	<i>Nocardiaoides</i> sp. JS614	41	5.06	VMTLYEGTSQIQK	<i>thnV</i>
6	gil118470801	ATPases of the AAA+ class	<i>Mycobacterium smegmatis</i> MC2-155	65	4.85	SVLDTGAPGLR AIDTESNTGGYL IKIERPDAESAQDIFSK DFNSGAMIQINVDNR LLGLEGAVEYK	
8, 9	gil63148158	Catechol 2,3-dioxygenase	<i>Rhodococcus</i> sp. YU6	34	4.99	DIFGHDNEVEGYGLDPIPLK GAVGTPVFMHCNDR QEIVHVCQAIAR APEVFPFK	<i>thnC</i>
10	gil116332030	Undecaprenyl pyrophosphate synthetase	<i>Synechocystis</i> sp. PCC 6803	28	6.6		
13	gil91787128	Beta-ketoadipyl CoA thiolase	<i>Polaromonas</i> sp. JS666	40	5.38		<i>thnI</i>

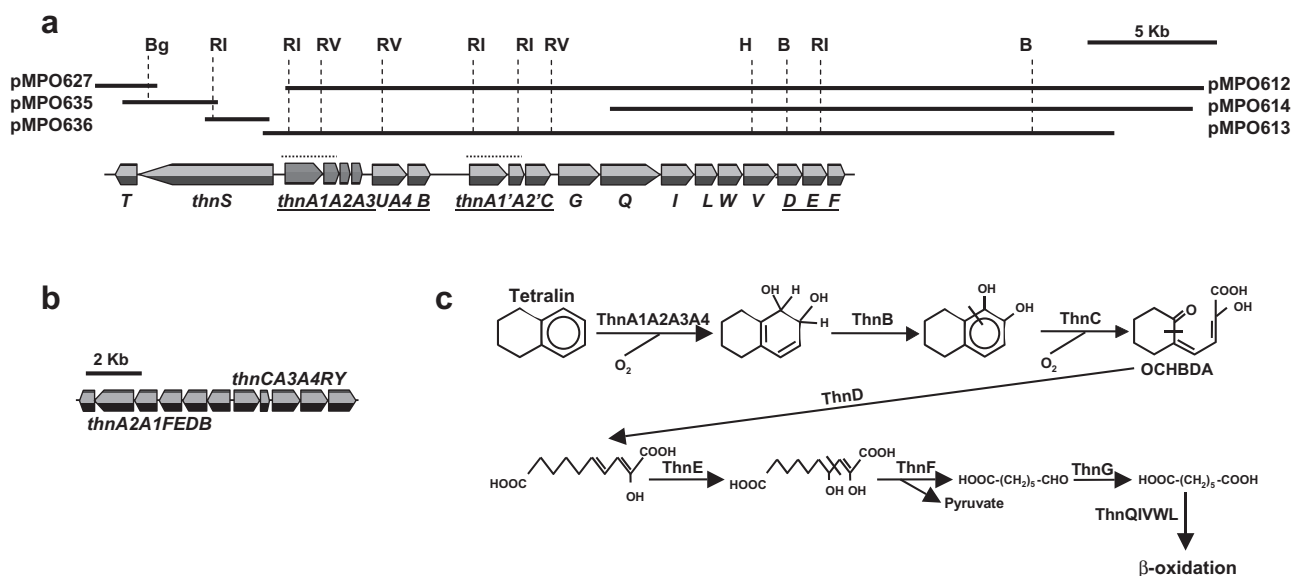
**a.** Mw and pI were calculated from the predicted protein sequences.

spots 2 and 11), a catechol 2,3-dioxygenase (ThnC; spots 8 and 9) and a 4-(2-oxocyclohexyl)-2-hydroxy-buta-2,4-dienoic acid hydrolase (ThnD, spot 1). We also identified a protein (ThnU, spot 3) that may be involved in sterol transfer and is encoded by a gene previously linked to others involved in biodegradation (Kulakov *et al.*, 2005; Maruyama *et al.*, 2005). Two additional proteins, a beta-ketoadipyl CoA thiolase (ThnI, spot 13) and an acyl-CoA dehydrogenase (ThnV, spot 4), were also identified, which could be involved in further processing of the linear tetralin derivative via  $\beta$ -oxidation.

The other tetralin-induced proteins that we have identified (Table 1) do not appear to be directly involved in tetralin catabolism. Spot 6 is similar to a group of proteins with chaperone-like functions that assist in the assembly or disassembly of protein complexes (Neuwald *et al.*, 1999). Spot 10 was identified as a protein similar to an enzyme involved in cell wall synthesis. We found that spot 14 corresponded to a hypothetical protein encoded by a gene located in a biphenyl degradation operon of *Rhodococcus rhodochrous* (Taguchi *et al.*, 2004). Spot 15 (Table 1A) was identified as an isocitrate lyase of *Rhodococcus equi* (Kelly *et al.*, 2002), an enzyme that catalyses the reversible cleavage of isocitrate to glyoxylate and succinate (glyoxylate cycle). Spot 16 was found to match ThcE of *R. erythropolis* (Nagy *et al.*, 1995), an enzyme induced by atrazine and thiocarbamate herbicides. Finally, spot 17 was found to correspond to a protein similar to a *Bacillus halodurans* transposase.

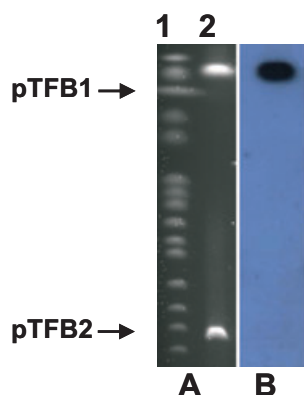
#### Cloning and identification of the *thn* genes of *Rhodococcus* sp. strain TFB

Based on the peptide sequences obtained from the proteomic analysis, degenerate primers (HIDFw and HIDRv; Table S1) were designed to amplify a region of the putative *thnD* gene. A 477 bp PCR product, obtained using total DNA from *Rhodococcus* sp. strain TFB as template, was cloned and sequenced. Its nucleotide sequence shows 70% identity to the corresponding region of the *Rhodococcus* sp. RHA1 *bphD* gene (GenBank CP000433) that encodes a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase. We used the putative *thnD* PCR product to screen a TFB library, identifying three positive cosmids (Fig. 2A). Cosmid pMPO613 was selected for further subcloning and sequencing. A 23 Kb fragment from this cosmid contained 18 ORFs oriented in the same direction. The deduced amino acid sequences of these ORFs were screened against the peptide sequences obtained from the proteome analysis to determine whether these ORFs encoded any of the proteins induced in tetralin-grown cells. Remarkably, peptides from spots 1, 2, 3, 4, 5, 6, 7, 8, 11, 12 and 13 were perfect matches to sequences of several of these ORFs (Table 1). As this suggests that the 23 Kb cosmid fragment contains one or more operons specifically induced by growth on tetralin medium, we designated each of these sequences as *thn* genes. Database comparison revealed high similarity between the TFB *thn* genes and the *bph* genes of *Rhodococcus* sp. RHA1 (Table S2). Based



**Fig. 2.** Genetic organization of *thn* genes.

A. Cosmids and plasmids used for TFB *thn* genes sequencing and genetic organization. Genes functionally homologous to TFA *thn* genes are underlined. A perfect duplication containing *thnA1A2* genes is denoted by a dotted line. B, BamHI; Bg, BglII; H, HindIII; Ri, EcoRI; Rv, EcoRV. C. Proposed tetralin degradation pathway in TFB.



**Fig. 3.** Plasmidic location of *thn* genes in TFB. A. Agarose pulse-field gel stained with ethidium bromide. Lane 1, molecular mass marker (New England). Lane 2, TFB linear plasmids pTFB1 and pTFB2. B. Southern blot hybridization using *thnD* as probe.

on information from previously characterized Thn proteins (GenBank AF157565), we propose a putative tetralin degradation pathway for strain TFB (Fig. 2C), analogous to the pathway that seems to be operating in *S. macroglotibida* strain TFA (Fig. 2B). Nevertheless, differences between the genetic organization of the *thn* genes in both bacteria are evident. In strain TFB, the structural genes are transcribed in the same direction, although seem to be arranged into two operons: one made up of *thnA1A2A3UB* and another comprising of a perfect duplication of *thnA1A2* (*thnA1' A2'*) plus the *thnCGQLWVDEF* genes (Fig. 2A). In addition, the *thnU* gene, which encodes a putative sterol transfer protein, is missing in strain TFA. We also found that a set of five TFB strain genes (*thnQILWV*) encodes proteins similar to those involved in  $\beta$ -oxidation pathways. DNA sequences resembling the remnants of transposons were found between both operons.

Analysis of the sequence upstream of *thnA1*, at the end of the cosmid insert, revealed a partial sequence similar to the two component regulatory system of *Rhodococcus* sp. RHA1 *bphST* (Fig. 2A). Inverse PCR was used to obtain a 5.83 kb fragment containing the complete sequence of *thnST*, as this fragment was not present in the TFB library. Spots corresponding to the protein products of these genes were not found during our proteomic analysis.

Given that the *bph* genes in *Rhodococcus* sp. RHA1 are located on the linear plasmids pRHL1 and pRHL2 (Masai *et al.*, 1997; McLeod *et al.*, 2006), we decided to isolate large plasmids from TFB bacteria. Pulse field electrophoresis analysis revealed two large linear plasmids, denoted pTFB1 and pTFB2, with estimated sizes of 1100 and 280 kb respectively (Fig. 3A). Using a *thnD* DNA fragment as the probe for southern blotting, we found that the *thn* genes are located on the pTFB1 plasmid (Fig. 3B).

### Characterization of the *ThnD* hydrolase strain from TFB

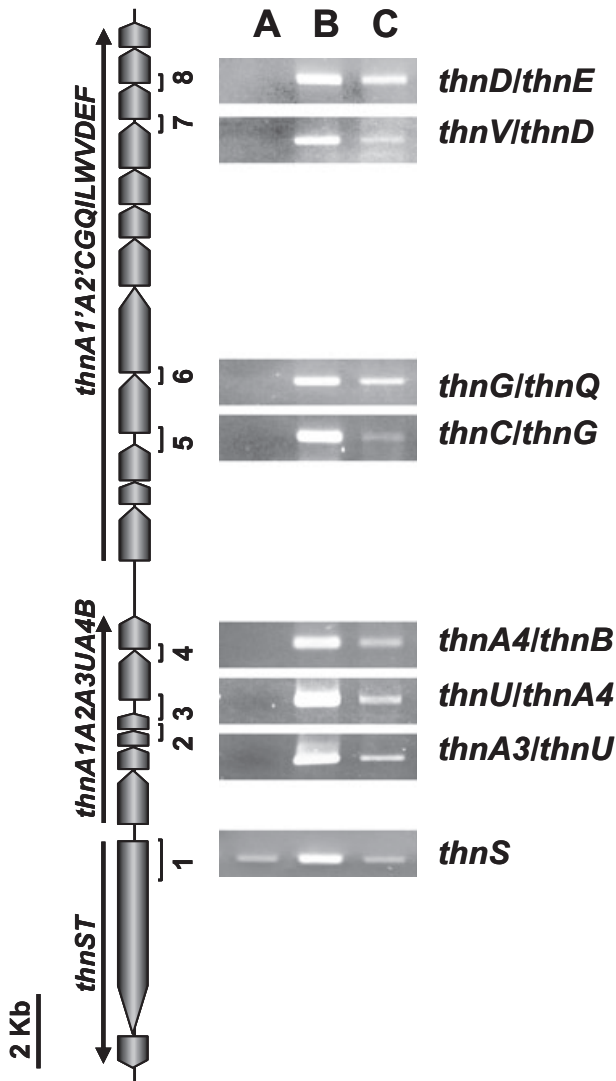
One of the most important enzymes in tetralin degradation is *ThnD*, a hydrolase able to open the tetralin alicyclic ring, rendering a linear compound, 2-hydroxydeca-2,4-dienedioic acid. In order to characterize its activity, the *thnD* gene of strain TFB was cloned into *Escherichia coli* under  $P_{T7}$  control (plasmid pMPO611, Table S1). Crude *E. coli* extracts showed hydrolytic activity when 2-hydroxy-4-(2-oxocyclohexyl)-2,4-butadienoic acid (OCHBDA) the aromatic ring fission product in the tetralin degradation pathway, see Fig. 2C) or 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (6-phenyl-HODA, the aromatic ring fission product in the biphenyl degradation pathway) were used as substrates. However, *ThnD* hydrolase activity using OCHBDA as a substrate was 40% of that obtained using 6-phenyl-HODA (data not shown). To estimate the optimal reaction temperature, enzyme activity assays were performed at different temperatures using 6-phenyl-HODA as substrate. The rate of substrate consumption increased with temperature until reaching an optimal temperature of 70°C.

In order to demonstrate the specific role of TFB-*ThnD* as a hydrolase in the tetralin biodegradation pathway, we introduced a plasmid (pMPO620) expressing TFB-*thnD* under a  $P_{tac}$  promoter into a *Thn*<sup>-</sup> TFA mutant that bears a K1XX insertion in *thnD* (K6 mutant; Hernáez *et al.*, 1999). We found that mutant K6 bacteria containing TFB-*thnD* were able to grow on a tetralin substrate (data not shown), thus confirming the functional role of TFB-*ThnD* in tetralin metabolism.

### Transcriptional analysis of *thn* genes

Total RNA isolated from TFB cells grown to mid-log phase with glucose or tetralin was used to perform semiquantitative RT-PCR with different combinations of oligonucleotide pairs to amplify intergenic or intragenic *thn* regions. The PCR products of the sizes expected for the structural genes were detected only in cells grown in the presence of tetralin (Fig. 4), indicating that expression of the identified *thn* genes is induced by this compound. From these results we could define three different operons (Fig. 4): one codes for the regulatory genes *thnST*, a second encompasses the genes from *thnA1* to *thnB* and a third one extends from *thnA1'* to *thnF*.

In order to determine whether or not the *thn* genes are subject to catabolite repression, TFB cells were grown to mid-log phase on tetralin, then incubated in a 2% glucose solution in the presence of tetralin for 2 h. Total RNA was isolated and converted to cDNA for semiquantitative RT-PCR analysis. Glucose treatment resulted in reduced PCR amplification of all the structural genes tested (Fig. 4), indicating a partial glucose repression of these genes.



**Fig. 4.** Expression of *thn* genes in strain TFB analysed by RT-PCR. RNA was isolated from cells grown on glucose (A), tetralin (B) or glucose plus tetralin (C) as carbon sources. The expected fragments of each PCR reaction are numbered. The three defined operons are denoted by arrows.

The *thnST* regulatory genes showed higher expression in tetralin-grown cells compared with glucose or glucose plus tetralin-grown cells (Fig. 4). However, in order to detect the *thnST* transcript, the number of PCR cycles had to be increased to 30, indicating a very low expression of these genes even in the presence of tetralin.

#### Determination of the 5' ends of *thn* transcripts and analysis of the promoter regions

Primer extension assays were carried out to characterize the 5' end of the *thnA1* and *thnS* transcripts. Total RNA extracts from glucose- or tetralin-grown cells were subjected to reverse transcription with primers P-Ext-*thnSA1*

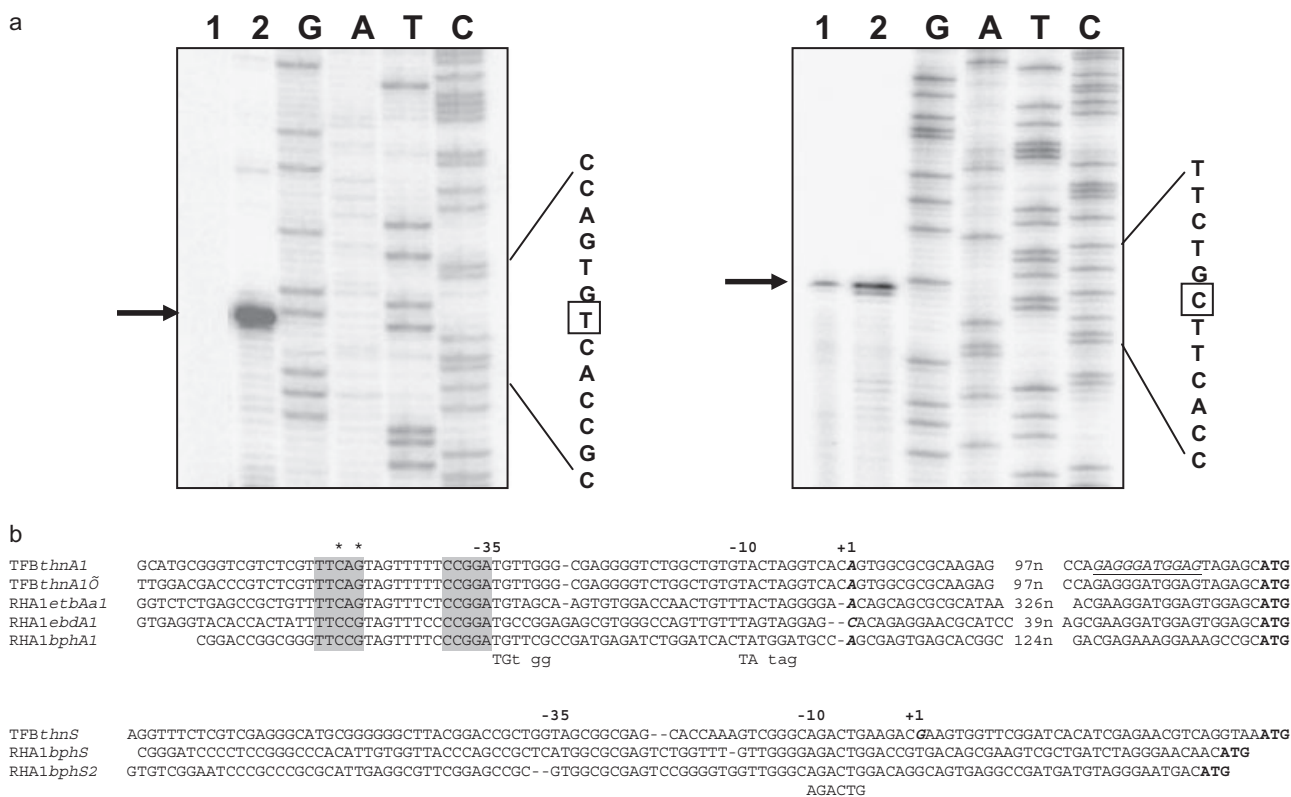
and P-Ext-*thnS* (Table S1). As shown in Fig. 5A, an intense band for *thnA1* was obtained only with RNA extracts from tetralin-grown cells. Comparison with parallel sequencing reactions using the same primer, indicates that the *thnA1* mRNA transcript starts at an A (a T in the DNA, Fig. 5A) and has a 132 bp non-translated leader. As expected, the primer extension assay detected the same start point for the two main *thn* gene transcripts because of the perfect duplication of the *thnA1A2* genes and upstream sequences. For *thnS*, the transcription start point was detected 35 nucleotides upstream of the first ATG codon (Fig. 5A). Basal transcription of *thnS* from the same promoter was also detected in glucose-grown cells (Fig. 5A).

*In silico* analysis of the promoter sequences revealed the presence of two nucleotide regions conserved in other *Rhodococcus* genes (Takeda *et al.*, 2004). These regions, 6 and 33 nucleotides upstream of the *thnA1* transcription start point, could be acting as  $-10$  and  $-35$  promoter sequences (Fig. 5B). No equivalent sequences were found within the *thnS* promoter, but a region at  $-10$  seems to be conserved with the putative RHA1 *bphS* gene promoters. Additionally, an 18 bp sequence, which matches sequences present in biphenyl-inducible promoters of *Rhodococcus* sp. RHA1 (Takeda *et al.*, 2004), was identified 44 bp upstream of *thnA1* and *thnA1'* (Fig. 5B). This region has been proposed to be specifically involved in the transcriptional regulation of aromatic degradation enzymes in *Rhodococcus* (Takeda *et al.*, 2004).

#### Expression of *gfp* translational fusions in strain TFB and mutational analysis of the *thnA1* promoter

In order to study the transcriptional control of *thn* genes, we developed a translational GFP fusion system for TFB cells. Translational *thnA1::gfp* (pMPO633) and *thnS::gfp* (pMPO639) fusions were expressed in TFB cells grown on glucose, glucose plus tetralin or tetralin. Subsequent fluorescence measurements corroborated the expression pattern previously determined by RT-PCR (Table 2).

Recently, Antunes and colleagues (2008) described the critical nucleotides for LuxR binding sites in *Vibrio fischeri*. As ThnT is similar to members of the LuxR family of regulators, we postulated that ThnT might act in an analogous way to regulate *thnA1* expression. We tested this hypothesis by performing a mutational analysis of the *thnA1* promoter region. To this end, we assayed two different point mutations, C(-51)A and G(-49)T, using the *gfp* expression system (see Fig. 5B). The C(-51)A mutation reduced expression to 30% of that of the wild-type promoter, but maintained the same regulatory pattern: low tetralin induction in the presence of glucose and higher induction in its absence. In contrast, the G(-49)T mutation resulted in a constitutive basal expression in all the con-



**Fig. 5.** Characterization of *thnA1* and *thnS* promoter regions.

**A.** Primer extension assays for *thnA1* (left) and *thnS* (right) transcriptional start point mapping. Total RNA from glucose- (lanes 1) or from tetralin- (lanes 2) grown cells were used as templates for primer extension reactions. The transcriptional start point is marked with a square. **B.** Alignment of the *thnA1* and *thnS* promoter sequences (coding strand) with other RHA1 promoter sequences. In bold and italic, transcriptional start points experimentally determined. Underlined, putative *thnA1* ribosome binding site. Proposed -10 and -35 consensus sequences are indicated under the alignment. Putative *thn* boxes, important for tetralin induction, are shadowed and asterisks denote the nucleotides mutated in plasmids pMPO645 (C-51A) and pMPO646 (G-49T).

ditions tested. These results indicate that the upstream region affected by these mutations is likely to be recognized by the transcriptional activator of the *thn* genes and play a key role in controlling the response of TFB cells to aromatic compounds.

#### Overexpression of *thnST* genes

As it is not currently possible to generate mutants in strain TFB we tested the effect of overexpressing *thnST* in TFB

cells on *thnA1* expression to test the role of ThnST as transcriptional regulators of the *thn* genes. To achieve this, we introduced plasmid pMPO647, harbouring the *thnST* genes expressed from their own promoter, together with the translational *thnA1::gfp* fusion into TFB cells. Fluorescence was monitored in glucose-, tetralin- and glucose plus tetralin-grown TFB cells. In all cases, fluorescence was higher in TFB cells carrying extra copies of *thnST* than in cells that contained *thnA1::gfp* alone (Table 2). Basal expression of *thnA1::gfp* in glucose-grown TFB cells was

**Table 2.** Expression of *gfp* translational fusions in *Rhodococcus* sp. strain TFB.

	Arbitrary fluorescence units ( $\times 10^4$ )		
	Glucose	Glucose plus tetralin	Tetralin
pMPO634 (promoterless <i>gfp</i> )	n.d.	0.3 $\pm$ 0.2	n.d.
pMPO633 (WT <i>thnA1::gfp</i> )	1.4 $\pm$ 0.4	79 $\pm$ 6.4	399 $\pm$ 24
pMPO645 (C-51A <i>thnA1::gfp</i> )	1.3 $\pm$ 0.1	18 $\pm$ 1.2	127 $\pm$ 16
pMPO646 (G-49T <i>thnA1::gfp</i> )	0.98 $\pm$ 0.08	0.93 $\pm$ 0.06	1.07 $\pm$ 0.08
pMPO639 (WT <i>thnS::gfp</i> )	1.49 $\pm$ 0.35	1.63 $\pm$ 0.62	2.95 $\pm$ 0.39
pMPO647 ( <i>thnST</i> plus <i>thnA1::gfp</i> )	5.4 $\pm$ 0.3	137 $\pm$ 1.4	449 $\pm$ 43

n.d., non detected.

increased by the presence of *thnST* in the plasmid, and increased almost twofold in the presence of glucose plus tetralin. Taken together, our results suggest that *thnST* genes encode positive regulators involved in the induction of TFB *thn* genes in response to tetralin.

## Discussion

*Rhodococcus* sp. strain TFB is able to grow on different aromatic compounds as its sole carbon and energy source (Tomás-Gallardo *et al.*, 2006). Previous studies with this strain have shown that growth on such compounds is associated with an induction of specific proteins. Growth on tetralin involves the induction of a significant number of proteins different from those induced by growth on other aromatic carbon sources (e.g. phthalate; Tomás-Gallardo *et al.*, 2006). Of the 18 *thn* gene products, MALDI-MS(/MS) analysis or *de novo* peptide sequencing of tetralin-induced spots identified nine of them (ThnA1, ThnA2, ThnU, ThnA4, ThnB, ThnC, ThnI, ThnV and ThnD). The proteomic analysis of tetralin-grown cells and sequencing of the genes encoding the induced proteins allow us to propose a tetralin degradation pathway operating in strain TFB, which is identical to that described for *S. macrogolita* strain TFA. The aromatic ring of tetralin is cleaved by an extradiol dioxygenase (ThnC) after its dioxygenation and dehydrogenation (catalysed by ThnA1A2A3A4 and ThnB respectively). The alicyclic ring is subsequently cleaved by the serine hydrolase ThnD, thus rendering a linear molecule that is then broken down into central metabolites (Fig. 2C).

One of the most important enzymes in the tetralin degradation pathway is ThnD. This protein hydrolyses a C–C bond of the alicyclic ring of tetralin to produce a dicarboxylate product (Hernández *et al.*, 2000). Like *S. macrogolita* strain TFA-ThnD protein, TFB-ThnD is a thermostable protein with activity at high temperatures, an interesting feature in organic synthesis that might reflect a common origin. However, of the two hydrolases found in *Rhodococcus* sp. RHA1, ThnD from strain TFA is more closely related to EtbD while TFB-ThnD is most similar to BphD1. In addition, unlike the hydrolase from strain TFA (Hernández *et al.*, 2000), TFB-ThnD is more active towards 6-phenyl-HODA (an intermediate in the biphenyl pathway) than towards OCHBDA (the tetralin ring fission product), which supports the notion that this enzyme has been recruited from a biphenyl biodegradation pathway. However, other TFB-Thn enzymes, like the ring hydroxylating dioxygenase system (ThnA1A2A3A4) or extradiol dioxygenase (ThnC), are more similar to the ethylbenzene dioxygenase (EBDO) and EtbC, from strain RHA1. The combination of enzymes from the ethylbenzene pathway in the TFB tetralin degradation pathway could be explained by the fact that EBDO transforms naphthalene (a molecule with two aromatic

rings sharing two carbons) more efficiently than biphenyl (Iwasaki *et al.*, 2007) and that biphenyl dioxygenases transform mono-aromatic compounds more efficiently than EBDO (Patrauchan *et al.*, 2008).

In addition to proteins directly involved in tetralin metabolism, other proteins were also induced following growth on tetralin. These could reflect a change in the metabolic status of TFB cells during growth on tetralin. Tetralin-induced isocitrate lyase, a key enzyme of the glyoxylate cycle that cleaves isocitrate into succinate and glyoxylate, indicates that TFB cells adapt their central metabolism depending on the contaminant. In this case, the putative tetralin degradation products (pyruvate and acetyl-CoA) would be used as carbon sources via the glyoxylate cycle, whose end-product is succinate. Induction of central metabolism enzymes in response to organic contaminants has been previously described. Growth of strain TFB on phthalate induces a succinate dehydrogenase, a component of the citric acid cycle (Tomás-Gallardo *et al.*, 2006). Phthalate metabolism renders acetyl-CoA and succinate, which suggests that the induction of succinate dehydrogenase may be required to deal with the succinate excess, although in this case a glyoxylate bypass, to use phthalate as a carbon source, would not be required. Similar results have been obtained in *Rhodococcus* sp. strain RHA1 (Navarro-Llorens *et al.*, 2005; Patrauchan *et al.*, 2005) in which growth on benzoate, phthalate or phenylacetate upregulates citric acid cycle enzymes, while growth on pyruvate upregulates the glyoxylate cycle (Patrauchan *et al.*, 2005). Tetralin is toxic to bacteria at concentrations above 15  $\mu$ M (Schreiber and Winkler, 1983) and, due to its lipophilic character, may interact with biological membranes causing changes in their structure and function, impairing growth and cell activity (Sikkema *et al.*, 1992; 1994). The induction of proteins involved in cell wall synthesis (spot 10) or with chaperone-like functions (spot 6) might be a response to tetralin toxicity.

The *thn* genes from strain TFB are most similar to *bph* and *etb* genes from other *Rhodococcus* strains (see Table S2). Despite their homology, the genetic organization is different. While in *Rhodococcus* sp. strain RHA1 several copies of *bph* and *etb* genes are located on the linear plasmids pRHL1 and pRHL2 (McLeod *et al.*, 2006), TFB *thn* genes are clustered together on the plasmid pTFB1 (a linear plasmid similar to pRHL1 in size) and no additional copies have been found elsewhere (data not shown). The DNA sequences most closely related to TFB *thn* genes are the *etb* genes present on pRHL2 and pDK2 from *Rhodococcus* sp. strain DK17. In plasmid pRHL2, a duplication of the *etbAa1Ab1* genes (homologous to *thnA1A2*) is also present. In this case, there is only a single nucleotide difference between *etbAa1Aba* and *etbAa2Ab2*. This is



another example of the genetic rearrangements that can take place between different replicons of members of the *Rhodococcus* genus.

Our *in silico* analysis of the promoter regions of TFB-*thn* genes and RHA1-*etb-ebd-bph*-genes allowed us to define putative -10 and -35 consensus sequences that do not follow the *E. coli* consensus. However, the -10 sequence of *thnS* does not match the consensus found in *thnA1*. This fact could be related to transcriptional differences between regulatory (with a basal expression in glucose) and structural genes (only expressed in the presence of the inducer).

A stretch of conserved nucleotides, centered at -44, which has been described in other *etb* and *bph* gene promoters as possibly being involved in a specific transcriptional regulatory mechanism (Takeda *et al.*, 2004), is also present at *thnA1* and *thnA1'* promoters. This sequence shows a palindromic structure that resembles the DNA binding sites of LuxR-type transcriptional regulators (White and Winans, 2007; Antunes *et al.*, 2008). As ThnT is similar to this kind of transcriptional regulators, we have functionally analysed the putative *thn* box within the *thnA1* promoter. Our finding that single-base substitution at nucleotide 3 or 5 of this region reduce or eliminates induction, respectively, indicates the importance of the *thn* box in ensuring correct *thnA1* transcription. Given the conservation of the -44 *thnA1* box sequence with promoters of other *Rhodococcus* genes involved in aromatic compound degradation, which appear to be controlled by homologous two-component LuxR-type regulatory systems, we postulate a common regulatory mechanism with conserved binding specificity that operates to modulate gene expression in response to the presence of aromatic compounds. However, in *S. macrogolita* strain TFA, the response to tetralin involves a LysR-type transcriptional activator, ThnR and a co-activator, ThnY (Martínez-Pérez *et al.*, 2004). This difference in the regulatory systems of the same biodegradation pathway is another example of how the evolution of metabolic pathways and their regulatory systems are independent, the latter conferring selective advantage by improving the ability of some bacteria to adapt their catabolic capability and general metabolism to the available resources (Cases and de Lorenzo, 2001; Tropel *et al.*, 2004).

## Experimental procedures

### *Chemicals, bacterial strains, culture conditions, plasmids and primers*

Dihydroxytetralin was chemically synthesized (Andújar *et al.*, 2000). Tetralin and 2,3-dihydroxybiphenyl were purchased from Sigma. The OCHBDA and 6-phenyl-HODA were biologically synthesized (Andújar *et al.*, 2000). Bentone MA was a gift of Zeus Química (Barcelona, Spain).

*Rhodococcus* sp. strain TFB (Tomás-Gallardo *et al.*, 2006), *S. macrogolita* strain TFA and the *thnD* mutant derivative K6 lacking the hydrolase activity (Hernández *et al.*, 1999; Hernández *et al.*, 2000) were grown on MM medium (Dorn *et al.*, 1974) at 30°C with tetralin concentration in vapour phase, 11.11 mM glucose (for strain TFB) or 40 mM  $\beta$ -hydroxybutyrate (for strain TFA) as carbon and energy sources. Luria-Bertani broth (LB; Sambrook *et al.*, 1989) or MML (Andújar *et al.*, 2000) were used as rich media for TFB and TFA respectively. *Escherichia coli* strains were grown in LB medium containing the appropriate antibiotics depending on the plasmid carried (see Table S1). *Escherichia coli* DH5 $\alpha$  (Hanahan, 1983) was used for DNA manipulation. *Escherichia coli* NCM631/pIZ227 (Govantes *et al.*, 1996) was used to overproduce the tetralin hydrolase ThnD.

Bacterial strains, plasmids and primers used in this work are listed in Table S1.

Conjugation of plasmids to *S. macrogolita* strain TFA was carried out by triparental mating as described previously (de Lorenzo and Timmis, 1994).

### *ThnD overexpression, crude extract preparation and hydrolase activity assay*

For TFB tetralin hydrolase overexpression, the *thnD* gene was amplified with NdeI-hid and 608Hid-Ter primers (Table S1) and cloned in pT7-7 (Tabor and Richardson, 1985) with NdeI and PstI to make pMPO611. *Escherichia coli* NCM631/pIZ227 was transformed with pMPO611 and cell free extracts were obtained as described in Hernández and colleagues (2000). Protein concentration was determined using the Bradford method (Bradford, 1976).

Hydrolase activity towards different ring fission products was assayed as described in Hernández and colleagues (2000). One unit of enzyme activity was defined as the amount of enzyme that converts 1  $\mu$ mol of substrate per min.

### *Protein sample preparation, fluorophore labelling and 2D electrophoresis analysis*

TFB cell free extracts, fluorophore labelling, 2D electrophoresis and protein identification were carried out as described by Tomás-Gallardo and colleagues (2006). For protein quantification, fluorescent gel images were obtained by scanning using a Typhoon TM 9400 Scanner and analysed with the DeCyder Differential Analysis software 5.01 version (GE Healthcare) with default parameters. Theoretical molecular masses and isoelectric points of the proteins of interest were calculated using EXPASY tools.

### *Screening for genes encoding hydrolases*

A total TFB DNA library (Tomás-Gallardo *et al.*, 2006) was screened by colony hybridization, using a probe targeting serine hydrolase genes. The probe was obtained by PCR using degenerate primers HIDFw and HIDRv (Table S1), whose design was based on the peptide sequence obtained from the proteomic analysis. PCR was carried out using 50 ng of total DNA (isolated as described in Tomás-Gallardo *et al.*, 2006) as template and the puRe-Taq Ready-To-Go

PCR Beads (GE Healthcare) under the following amplification conditions: 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 50 s. The 477 bp fragment was cloned into EcoRV-cleaved pBluescript II SK+ (Stratagene) to make pMPO602. This fragment was used to probe a TFB library by Southern analysis (Sambrook *et al.*, 1989). A 23 kb fragment from cosmid pMPO613 was sequenced commercially on both strands.

Plasmids pMPO627, pMPO635 and pMPO636 were obtained by inverse PCR as follows: total DNA from strain TFB was cut with EcoRI, BglII or BamHI and religated. Each religation product was used as template for PCR reactions with primers PCRInvEcoRI619 and PCRInvthnSFw, PCRInvBglII626 and PCRInvBglII627, and PCRInvBamHI624 and PCRInvBamHI635 respectively. The amplification products were cloned in pBluescript SK+.

The obtained DNA sequence was compared with those in the databases using BLASTN 2.2.6 (Altschul *et al.*, 1997) and submitted to GenBank under Accession Number FJ183471.

#### *Plasmid isolation, pulsed field gel electrophoresis, linearity determination and Southern blot hybridization*

For detection of extrachromosomal DNA, plasmid isolation and pulse field gel electrophoresis assays were developed as described by König and colleagues (2004). Yeast Chromosome PFG marker (New England Biolabs) was used as reference. After electrophoresis, DNA was transferred to Hybond-N<sup>+</sup> nylon membranes (GE Healthcare) and subjected to Southern hybridization with the pMPO602 *thnD* probe.

#### *Total RNA sample preparation, RT-PCR and primer extension analysis*

Total RNA of *Rhodococcus* sp. strain TFB was isolated following the method described by Tomás-Gallardo and colleagues (2006). PCR amplification of 16S rDNA and some *thn* genes with RNA samples as template was conducted to test for DNA contamination.

DNA-free RNA samples (2 µg) were converted to cDNA using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. Equal amounts of cDNA were used as templates in PCR reactions with primers listed in Table S1. To detect intergenic and intragenic mRNA, combinations of primers were: 608-5SG with 8836G for *thnV-thnD*, 8837G with 9273L for *thnD-thnE*, 2-608ESf570 with β-ox1rev for *thnG-thnQ*; RT-Exdio with RT-Aldh for *thnC-thnG*; RT-FerRed with RT-DihyDehy for *thnA4-thnB*, RTfer-fw with RTorf7-rev for *thnA3-orf7*, RTorf7-fw with 3-624rev for *thnU-thnA4*, ThnS-RTPCR-Fw with ThnS-RTPCR-Rev for *thnS*. PCR reactions were performed with 30 ng of cDNA for structural genes and 5 ng of cDNA for *thnS* using the puReTaq Ready-To-Go PCR beads (GE Healthcare) and consisted of 20 cycles of 30 s at 94°C, 30 s at 59°C and 45 s at 72°C (30 cycles for *thnS* RT-PCR). As loading control, primers f27 and r519 (Hugenholtz *et al.*, 1998) were used with 20 pmol of cDNA.

Primer extension reactions were performed as described previously (Govantes *et al.*, 2000), with 40 µg of RNA as a template, <sup>32</sup>P-end-labelled oligonucleotides P-Ext-thnSA1

and P-Ext-thnS, and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Sequencing reactions with the same primers and pMPO647 as template were performed with the Thermo Sequenase cycle sequencing kit (USB, Cleveland, OH), according to the manufacturer's instructions. Samples were run on polyacrylamide sequencing cells. Dried gels were exposed to radiosensitive screens, which were subsequently scanned with a Typhoon 9410 scanner (GE Healthcare).

#### *Construction of a gfp-based promoter probe vector and gene fusions*

A promoter probe vector was constructed using the *Rhodococcus-E. coli* shuttle vector, pNC9503 (H. Saeki, Japan Energy Corporation, Japan) and a variant of the GFP (Cormack *et al.*, 1996) as the reporter gene. The *gfp* gene was cloned as a PCR product with Scal-XbaI ends (pMPO634) and the tested promoters were cloned as Sall-ScaI PCR fragments. The used primers and the obtained plasmids are described in Table S1 and electroporation of the plasmids into TFB cells was carried out as described by Tomás-Gallardo and colleagues (2006). *thnA1* promoter region mutagenesis was carried out by overlapping PCR using plasmid pMPO633 as template. Primers 633C3AFw and GFPXbaIPCRsolap and primers 633C3Arev and thnSA1SallIPCRsolap were used to amplify 210 and 850 bp fragments respectively. These fragments were used as templates with primers thnSA1SallIPCRsolap and GFPXbaIPCRsolap to obtain a 1060 bp fragment that includes the point mutation C-51A and that was cloned in pMPO634 to obtain pMPO645. The same strategy was applied to obtain pMPO646 (which included G-49T point mutation) with the overlapping primers 633G5TFw and 633G5Trev.

Fluorescence measurements were carried out with a Typhoon 9410 scanner (GE Healthcare). Cells were grown in mineral medium with glucose, tetralin or glucose plus tetralin as carbon sources to an OD<sub>600</sub> of 1.5–2. The excitation and emission wavelength used were 488 and 532 nm respectively. The intensity of fluorescence is expressed as its relative value per unit of optical density at 600 nm (arbitrary units). Basal fluorescence of the plate was subtracted in each experiment. Data were the result of triplicate measurements of independent cultures of strain TFB carrying each plasmid.

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### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Bacterial strains, plasmids and primers.

**Table S2.** Database comparison of TFB-*thn* genes.

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