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Identification and characterization of an acyl-CoA dehydrogenase from *Pseudomonas putida* KT2440 that shows preference towards medium to long chain length fatty acids

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Diverse and elaborate pathways for nutrient utilization, as well as mechanisms to combat unfavourable nutrient conditions make *Pseudomonas putida* KT2440 a versatile micro-organism able to occupy a range of ecological niches. The fatty acid degradation pathway of *P. putida* is complex and correlated with biopolymer medium chain length polyhydroxyalkanoate (mcl-PHA) biosynthesis. Little is known about the second step of fatty acid degradation (β-oxidation) in this strain. *In silico* analysis of its genome sequence revealed 21 putative acyl-CoA dehydrogenases (ACADs), four of which were functionally characterized through mutagenesis studies. Four mutants with insertionally inactivated ACADs (PP_1893, PP_2039, PP_2048 and PP_2437) grew and accumulated mcl-PHA on a range of fatty acids as the sole source of carbon and energy. Their ability to grow and accumulate biopolymer was differentially negatively affected on various fatty acids, in comparison to the wild-type strain. Inactive PP_2437 exhibited a pattern of reduced growth and PHA accumulation when fatty acids with lengths of 10 to 14 carbon chains were used as substrates. Recombinant expression and biochemical characterization of the purified protein allowed functional annotation in *P. putida* KT2440 as an ACAD showing clear preference for dodecanoyl-CoA ester as a substrate and optimum activity at 30 °C and pH 6.5–7.

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INTRODUCTION

Pseudomonas putida KT2440, an important and versatile model micro-organism, is currently one of the most promising strains for numerous biotechnological applications, e.g. biopolymer production [medium chain length polyhydroxyalkanoate (mcl-PHA)], and a source of numerous industrially relevant enzymes (Dos Santos *et al.*, 2004; Puchałka *et al.*, 2008).

Abbreviations: ACAD, acyl-CoA dehydrogenase; CDW, cell dry weight; mcl-PHA, medium chain length PHA; PHA, polyhydroxyalkanoate.

PHA is a biological polyester used for a range of applications on account of its biodegradability and biocompatibility (Chen, 2009; Zinn *et al.*, 2001). The utilization of fatty acids for microbial production of biopolymers, such as mcl-PHA, has gained importance, due to the finding that polymer production is more efficient when these carbon sources are used, compared to PHA-unrelated substrates (Elbahloul & Steinbüchel, 2009; Steinbüchel, 2001; Sun *et al.*, 2007). In addition, tailoring of biopolymer properties can be achieved using different fatty acids as substrates (Hartmann *et al.*, 2004, 2006; Hazer & Steinbüchel, 2007; Sun *et al.*, 2009) and through metabolic engineering of the β-oxidation pathway (Escapa

et al., 2011; Liu *et al.*, 2011). The length of 3-hydroxyalkanoic acid incorporated into PHA plays a key role in determining the physical properties of the biopolymer. In general, all alkanoic acids can be divided into three groups: short chain length (scl, 1–5 carbons), medium chain length (mcl, 6–12 carbons) and long chain length (lcl, $\geqslant$13 carbons).

Mcl-PHA biosynthesis in *P. putida* involves a network of metabolic pathways that converge onto a specific pathway consisting of proteins required for PHA synthesis, hydrolysis and intracellular granule formation encoded by the *pha* cluster. Metabolic pathways, such as β-oxidation and fatty acid *de novo* synthesis, process fatty acids and carbohydrate substrates, respectively, to generate (*R*)-3-hydroxyacyl-CoAs, the substrates of PHA synthases (Prieto *et al.*, 2007; Rehm, 2007). These metabolic pathways underpin the ability of *P. putida* KT2440 to utilize a multitude of carbon sources for growth and mcl-PHA accumulation (Nelson *et al.*, 2002; Prieto *et al.*, 2007). Mcl-PHAs contain a variety of (*R*)-3-hydroxyalkanoic acid monomers varying from 6 to 12 carbons in length. Identification of proteins in the β-oxidation pathway that show a preference for specific chain lengths would allow future metabolic engineering to promote the accumulation of PHA with a tailor-made monomer composition.

Availability of the whole genome sequence of *P. putida* KT2440 has unlocked its considerable genetic potential, revealing ubiquity, metabolic versatility and adaptability of this particular strain (Nelson *et al.*, 2002). However, its genotype–phenotype relationship is complex and cannot be predicted simply by *in silico* annotation and assignment of gene functions (Puchałka *et al.*, 2008; van Duuren *et al.*, 2013). For instance, multiple copies of genes that encode β-oxidation enzymes exist, but studies on identification and biochemical characterization of the translated proteins involved in *P. putida* β-oxidation are lacking (Fig. 1).

Previously, we showed that FadD, the first enzyme of the β-oxidation pathway, is a long fatty acyl-CoA synthase that allows incorporation of longer PHA monomers when overexpressed in the wild-type *P. putida* CA-3 strain (Hume *et al.*, 2009). The second step of the β-oxidation pathway is catalysed by acyl-CoA dehydrogenases (ACAD; Fig. 1). ACADs belong to a large family of flavoproteins and show activity towards a broad range of substrates (Ghisla & Thorpe, 2004). The reaction of α,β-dehydrogenation presents the rate-limiting step in β-oxidation, since ACADs have the lowest activity among all β-oxidation enzymes (Lu *et al.*, 2003; Qi *et al.*, 1998). Moreover, these enzymes are usually present as different isozymes within bacterial organisms. In the genome of *P. putida* KT2440, 21 ORFs are predicted to code for ACADs (Nelson *et al.*, 2002). A number of ACADs from *P. putida* KT2440 have been characterized *in vitro*. The protein encoded by the PP_2216 gene has been shown to be specific for short chain aliphatic fatty acyl-CoAs (McMahon *et al.*, 2005) and the PP_0368 gene identified as an inducible phenylacyl-CoA dehydrogenase (McMahon & Mayhew, 2007). However, no studies on *P. putida* KT2440 to date have examined the relationship between individual ACADs and mcl-PHA accumulation or identified the ACADs responsible for medium to long chain fatty acid metabolism. To elucidate these issues, we generated several insertional and knockout

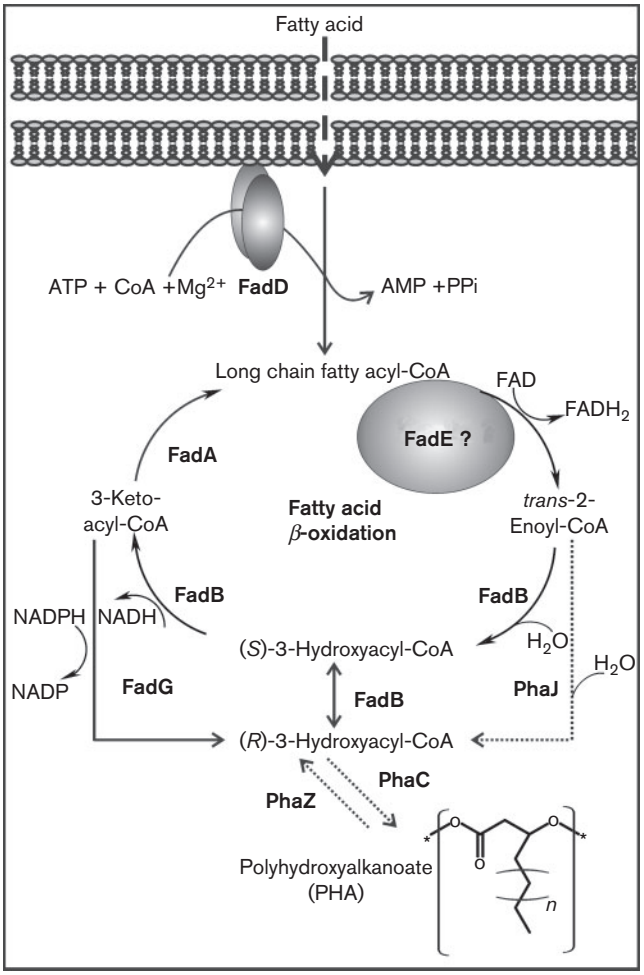

Fig. 1. Proposed fatty acid degradation (β-oxidation) pathway in *Pseudomonas putida* (Fiedler *et al.*, 2002; Hume *et al.*, 2009; Liu *et al.*, 2011). FadD (acyl-CoA synthase; PP_4549;) activation of fatty acids to their acyl-CoA esters proceeds through ATP-dependent covalent binding of AMP to fatty acid with release of inorganic pyrophosphate, and subsequently, AMP. FadE is an ACAD and more than a dozen potential homologues exist in the *P. putida* genome. The action of other β-oxidation enzymes (NAD-dependent (*S*)-3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase, FadB; ketoacyl-CoA thiolase, FadA) results in shortening of an acyl moiety by two carbons. In parallel, intermediates of the β-oxidation pathway can be polymerized into the PHA molecule by PhaC (PHA polymerase) using three potential routes: hydration of *trans*-enoyl-CoA by PhaJ [(*R*)-specific enoyl-CoA hydratase], epimerase activity of FadB or reduction of 3-ketoacyl-CoA by FabG (NADPH-dependent 3-ketoacyl-CoA reductase). PHA depolymerase (PhaZ) is able to hydrolyse PHA.

mutants of putative ACADs and assessed their growth and PHA accumulation characteristics. Based on characterization data, the enzyme encoded by the PP_2437 gene was recombinantly expressed, purified, and its activity and physiological role examined.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. For culture propagation, Luria–Bertani (LB) (Sambrook & Russell, 2001) or minimal salts medium (MSM; Schlegel *et al.*, 1961) was used. Where required, the medium was supplemented with carbenicillin, kanamycin or gentamicin (50 μg ml^{-1}). Stock cultures of all strains were maintained at −80 °C in LB broth with glycerol (15 %, v/v).

For mcl-PHA accumulation assays, bacteria were grown in 50 ml of nitrogen-limited MSM (0.25 g NH_4Cl l^{-1}). Except where stated otherwise, carbon substrates were included in the medium at a concentration of 1.95 g C l^{-1}. Bacterial cultures were grown for 48 h at 30 °C with shaking (250 r.p.m.).

Generation of *P. putida* KT2440 gene insertional and deletion mutants. Gene sequence analysis and assignment was assisted using predictions in the *Pseudomonas* Genome Database (http://www.pseudomonas.com/index.jsp; Lewenza *et al.*, 2005; Winsor *et al.*, 2011). The Conserved Domain Database (CDD) for the functional annotation of proteins was also used (Marchler-Bauer & Bryant, 2004; Marchler-Bauer *et al.*, 2009, 2011). All general molecular biology techniques were performed following the procedures described by Sambrook & Russell (2001). Gene inactivation was performed using pKNOCK-Gm vectors designed for insertional mutagenesis (Table 1; Alexeyev, 1999). A construct for specific PP_2437 gene deletion via a double cross-over recombination event was generated following the procedure described by Hume *et al.* (2009). Electrocompetent cells of *P. putida* were prepared and transformed using the protocol of Choi *et al.* (2006). Recombinant strains of *P. putida* KT2440 with inactivated putative ACAD were generated by introduction of the corresponding plasmids from the pKN : ins series (Table 1). The deletion mutant, KT2440 ΔPP_2437, was created by the introduction of pGEM-ΔPP_2437 plasmid into *P. putida* KT2440. All generated vectors and insertional mutants were confirmed by restriction digestion and sequencing (GATC Biotech) using the appropriate primers. The ΔPP_2437 deletion mutant was confirmed using Southern blot analysis.

Complementation of ACAD deletion. Complementation of the *P. putida* deletion mutant, ΔPP_2437, was carried out by introducing the pJB861 : PP_2437 plasmid via electroporation to generate *P. putida* KT2440 ΔPP_2437 + PP_2437 (Table 1). This strain was examined for PHA accumulation under the same conditions as WT in the presence of 50 μg kanamycin ml^{-1} and induced with 1 mM *m*-toluic acid. Appropriate controls, including strains carrying the pJB861 vector containing no insert (*P. putida* KT2440 wild-type and KT2440 ΔPP_2437), were additionally included in the study.

Recombinant expression and purification of PP_2437 protein in *Escherichia coli*. For recombinant expression of PP_2437 protein in *E. coli* BL21(DE3), the 1245 bp coding sequence was ligated into pRSET-B expression vector to yield pRSETB : PP_2437exp (Table 1). The recombinant strain, *E. coli* PP_2437 (Table 1), was grown in LB medium supplemented with carbenicillin (50 μg ml^{-1}). Cultures were grown (25 °C with shaking at 200 r.p.m.) until optical density reached OD_{600} of 0.4 (Helios delta; Unicam), at which point cells were induced with 0.1 mM IPTG at 25 °C. After 12 h of culture, cells were harvested and the ACAD purified using a His-Trap column procedure described previously (Hume *et al.*, 2009). Fractions (2 ml) were collected and analysed with 10 % SDS-PAGE under denaturing conditions. The enzyme concentration was determined spectrophotometrically at 448 nm (Helios delta; Unicam), using the molar absorption coefficient ε=14890 M^{-1} cm^{-1} (McMahon *et al.*, 2005).

Analysis of the ACAD activity of purified PP_2437 protein. A modified ACAD activity assay (O'Brien & Frerman, 1977) was used to characterize the activity of PP_2437 protein. Specifically, 50 mM potassium phosphate buffer, pH 7, 0.9 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1.7 mM phenazine methosulfate (PMS), 40 μM acyl-CoA (C_3–C_{16} aliphatic fatty acid derivatives) and enzyme were combined in a total volume of 200 μl at 30 °C. The course of the reaction was followed spectrophotometrically at 580 nm for 20 min (Spectra MAX 340; Molecular Devices), and activity calculated from the initial rates using the molar absorption coefficient of formazan (ε=22800 M^{-1} cm^{-1}). All acyl-CoA substrates were synthesized from the mixed anhydride of fatty acids, as described previously (Hume *et al.*, 2009; Stadtman, 1957). Kinetic parameters were obtained according to the protocols of Martin *et al.* (2008) and Molloy *et al.* (2013). Values for k_{cat} are presented as mol of substrate oxidized per mol of enzyme-FAD per min.

Using dodecanoyl-CoA as substrate, pH and temperature optima were determined for PP_2437 protein at a range of temperatures between 20 and 60 °C. Optimum pH was determined by varying the standard buffer pH from 3 to 10. To test enzyme stability under different pH conditions, PP_2437 protein was pre-incubated for 30 min in 50 mM sodium phosphate buffer adjusted to a range of pH 3 to 10. After adjusting the buffer pH to 7, the standard assay was carried out. Similarly, stability of the enzyme was tested over a range of temperatures (4–70 °C).

PHA content and monomer determination. The polymer content of lyophilized whole bacterial cells was determined by subjecting cells to acidic methanolysis and subsequent gas chromatography analysis (Brandl *et al.*, 1988; Hume *et al.*, 2009; Lageveen *et al.*, 1988). Commercially available (*R*)-3-hydroxyalkanoic acids (Bioplastech) were used for determination and quantification of PHA monomers.

Statistical analysis. Growth and PHA data are presented as mean values of at least three independent experiments with SD, with the exception of Table 2, where SDs have been removed to improve readability. Data were analysed using one-way ANOVA and ad hoc Dunnett's test in IBM SPSS Statistics 20, a statistical program, to compare growth and PHA results of all mutant and overexpression strains against those obtained for wild-type *P. putida* KT2440 grown on fatty acids. Results were accepted as statistically significant at P value <0.05.

RESULTS AND DISCUSSION

In silico analysis and insertional mutagenesis of putative ACADs of *P. putida* KT2440

In order to identify FadE homologues in the genome of *P. putida* KT2440, the amino acid sequence of ACAD from *Pseudomonas aeruginosa* PAO1 (PA_2815) was used for a BLAST search against *P. putida* KT2440 ORFs (Altschul *et al.*, 1997). Among the 21 putative sequences predicted to code for ACADs in the genome of *P. putida* KT2440, 14 showed 22–75 % identity to PA_2815 (data not shown). Amino acid sequences of the FadE homologues identified were further

Table 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Relevant characteristics*	Source or reference
Plasmids		
pJB861	Broad-host-range vector, expression under P_m promoter, Kan^R	Blatny *et al.* (1997)
pGEM-T Easy	For cloning of PCR products, f1 *ori*, Amp^R pGEM-5zf(+) derivative	Promega
pKNOCK-Gm	Broad host range for insertional mutagenesis, RP4 *oriT*, R6K *ori*, Gm^R	Alexeyev (1999)
pPS856	Source of gentamicin cassette, Gm^R Amp^R	Hoang *et al.* (1998)
pRSET-B	T7 promoter, N-terminal 6 × His tag; N-terminal Xpress epitope; enterokinase cleavage site	Invitrogen
pGEM : ins 1893	pGEM-T Easy derivative containing 680 bp of internal region of *P. putida* KT2440 gene PP_1893	This study
pGEM : ins 2039	pGEM-T Easy derivative containing 480 bp of internal region of *P. putida* KT2440 gene PP_2039	This study
pGEM : ins 2048	pGEM-T Easy derivative containing 660 bp of internal region of *P. putida* KT2440 gene PP_2048	This study
pGEM : ins 2437	pGEM-T Easy derivative containing 600 bp of internal region of *P. putida* KT2440 gene PP_2437	This study
pKN : ins 1893	pGEM-T Easy derivative containing 680 bp of internal region of *P. putida* KT2440 gene PP_1893	This study
pKN : ins 2039	pGEM-T Easy derivative containing 480 bp of internal region of *P. putida* KT2440 gene PP_2039	This study
pKN : ins 2048	pGEM-T Easy derivative containing 660 bp of internal region of *P. putida* KT2440 gene PP_2048	This study
pKN : ins 2437	pGEM-T Easy derivative containing 600 bp of internal region of *P. putida* KT2440 gene PP_2437	This study
pGEM : PP_2437_ext	pGEM-T Easy derivative containing 3.2 kb fragment including 1.2 kb sequence of *P. putida* KT2440 gene PP_2437 and 1.0 kb flanking regions	This study
pGEM : PP_2437_SmaI_1	Plasmid derived from pGEM-PP_2437_ext, with introduced *Sma*I enzyme restriction site at 48th bp of PP_2437 gene	This study
pGEM : PP_2437_SmaI_fin	Plasmid derived from pGEM-PP_2437_*Sma*I_1, with introduced *Sma*I enzyme restriction site at 1197th bp of PP_2437 gene	This study
pGEM : ΔPP_2437	Plasmid derived from pGEM-PP_2437_*Sma*I_fin, containing gentamicin cassette flanked by 48 bp from beginning of PP_2437 gene and 47 bp from end of PP_2437 gene from *P. putida* KT2440	This study
pJB861 : PP_2437	Expression plasmid containing 1.2 kb fragment of PP_2437 gene and an artificial RBS	This study
pRSETB : PP_2437exp	pRSET-B derivative containing 1245 bp (PP_2437 gene)	This study
Bacterial strains		
E. coli XL1-Blue MRF′	F^- *recA1 endA1 relA1 lac*, cloning host	Stratagene
E. coli BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λ[DE3 (*lacI lacUV5-T7* gene *ind1 sam7 nin5*)], recombinant protein expression host	Invitrogen
E. coli PP_2437	*E. coli* BL21(DE3) strain carrying pRSETb : PP_2437 expression plasmid	This study
P. putida KT2440	Wild-type strain	ATTC 47054
P. putida KT2440 insPP_1893	*P. putida* KT2440 PP_1893 insertional mutant	This study
P. putida KT2440 insPP_2437	*P. putida* KT2440 PP_2437 insertional mutant	This study
P. putida KT2440 insPP_2039	*P. putida* KT2440 PP_2039 insertional mutant	This study
P. putida KT2440 insPP_2048	*P. putida* KT2440 PP_2048 insertional mutant	This study
P. putida KT2440 ΔPP_2437	*P. putida* KT2440 PP_2437 deletion mutant, Gm^R	This study
P. putida KT2440 ΔPP_2437 + PP_2437	*P. putida* KT2440 PP_2437 deletion mutant expressing PP_2437 copy from pJB861 : PP_2437 plasmid, Kan^R Gm^R	This study

*Kan, kanamycin (50 μg ml^{-1}); Amp, ampicillin (50 μg ml^{-1}); Gm, gentamicin (50 μg ml^{-1}).

analysed for the presence of ACAD conserved domains using the CDD. Proteins predicted to be involved in the metabolism of amino acids (glutaryl-CoA dehydrogenase encoded by PP_0158, and isovaleryl-CoA dehydrogenase encoded by PP_4064 involved in leucine catabolism) or organic sulfur metabolism (PP_3226 and PP_3259) were omitted from further investigation. The functionally characterized ACADs from *P. putida* KT2440, PP_2216 and PP_0368 (McMahon *et al.*, 2005; McMahon & Mayhew, 2007) were not considered for this study. The phylogenetic relationship

Table 2. Growth and PHA accumulation of *P. putida* KT2440 and four mutants bearing an insertionally inactivated putative ACAD gene (PP_1893, PP_2039, PP_2048 or PP_2437) on a range of alkanoic acids [propionic (C_3) to palmitic (C_{16})]

Substrate	*P. putida* KT2440		*P. putida* KT2440 insPP_1893		*P. putida* KT2440 insPP_2039		*P. putida* KT2440 insPP_2048		*P. putida* KT2440 insPP_2437	
	CDW (g l^{-1})	PHA (g l^{-1})	CDW (g l^{-1})	PHA (g l^{-1})	CDW (g l^{-1})	PHA (g l^{-1})	CDW (g l^{-1})	PHA (g l^{-1})	CDW (g l^{-1})	PHA (g l^{-1})
C_3	0.60	0.21	0.56	**0.15**	0.55	**0.16**	0.56	**0.15**	0.56	**0.17**
C_4	0.61	0.22	0.61	0.18	0.57	0.18	0.57	0.19	0.62	0.22
C_5	0.59	0.24	0.63	**0.04**	0.53	**0.04**	0.55	**0.08**	0.65	**0.12**
C_6	0.91	0.47	0.84	**0.29**	**0.72**	**0.23**	**0.65**	**0.18**	0.86	**0.33**
C_7	0.85	0.48	0.96	0.47	**1.07**	**0.64**	**1.12**	**0.65**	**1.03**	**0.60**
C_8	0.92	0.55	0.89	0.57	0.90	**0.64**	0.88	**0.60**	0.94	**0.63**
C_9	0.76	0.47	0.78	0.52	0.76	0.51	0.75	0.51	0.75	0.47
C_{10}	0.96	0.57	0.92	**0.40**	**0.88**	0.59	0.90	0.58	**0.78**	**0.50**
C_{11}	0.77	0.31	0.73	**0.23**	0.69	**0.22**	**0.68**	**0.24**	**0.70**	**0.19**
C_{12}	0.81	0.49	0.92	**0.55**	1.12	**0.74**	1.04	**0.64**	**0.60**	**0.43**
C_{13}	0.96	0.56	0.96	0.60	0.90	0.53	0.81	0.49	**0.78**	0.49
C_{14}	0.96	0.61	1.04	0.57	0.94	0.53	0.97	0.53	**0.76**	**0.52**
C_{15}	0.82	0.43	0.85	0.39	**0.97**	0.43	**0.99**	0.41	0.87	0.43
C_{16}	1.01	0.55	0.94	0.49	0.92	0.47	0.90	0.47	0.99	0.52

C_3–C_{16}, carbon chain length of the substrate.

Data represent the average of at least three independent experiments. For clarity, standard deviations have been removed and significant decrease or increase ($P<0.05$) relative to wild-type *P. putida* KT2440 marked in bold.

between the remaining eight sequences and their annotated counterparts in *E. coli* K-12 and *P. aeruginosa* PAO1 were examined using a neighbour-joining clustering method (Fig. 2a). The amino acid sequence of the putative protein, PP_1893, displayed the highest similarity with that of *E. coli* FadE involved in the oxidation of acyl-CoA to 2-enoyl-CoA (Fig. 2a). These two proteins shared the same FadE multi-domains, an all-alpha helix N-terminal domain, as well as a domain of unknown function predominantly found in prokaryotic ACADs (data not shown). Although PP_2048 displayed the least similarity to FadE proteins from *P. aeruginosa* and *E. coli* (12 % query coverage and 26 % identity), the protein was selected for further study due to its genomic context, as it is positioned directly upstream of *fadB*, a gene coding for 3-hydroxyacyl-CoA dehydrogenase, along with PP_2039 positioned directly upstream of *fadD* (Hume *et al.*, 2009) and PP_2437, which contains a *fadE2* domain. Analysis of conserved amino acid residues in the ACAD active site domains from the four selected putative fatty acyl-CoA dehydrogenases revealed a high degree of conservation (Fig. 2b).

Four mutants, each bearing insertionally inactivated putative ACAD (PP_1893, PP_2039, PP_2048 and PP_2437), were generated and their growth and PHA accumulation on a range of aliphatic fatty acids as the sole source of carbon and energy assessed (Table 2). Long chain fatty acids are poorly soluble in aqueous solution and form a second phase when added to growth medium. To overcome this, alkanoic acids were supplemented as sodium salts to facilitate solubility in the liquid medium and avoid second phase formation. When the mutants were grown on butyric (C_4), nonanoic (C_9) and palmitic (C_{16}) acid, neither biomass nor mcl-PHA accumulation levels were affected (Table 2). In general, when grown on short chain length aliphatic fatty acids (C_3–C_5), all four insertional mutants exhibited biomass accumulation comparable to that of wild-type *P. putida* KT2440 (Table 2). However, mcl-PHA accumulation levels in the mutants were lower than wild-type when supplied with propionic acid (C_3) or valeric acid (C_5). The negative effect on PHA accumulation for mutants grown on C_3 and C_5 (uneven short chain fatty acids), compared to butyric acid (even short chain), may be due to easier condensation of even carbon units, compared to uneven units (Cerrone *et al.*, 2014; Olivera *et al.*, 2001). With C_6–C_8 alkanoic acids as substrates, differential behaviour of mutants was observed. Hexanoic (C_6) acid substrate negatively affected PHA accumulation more than growth. *P. putida* KT2440 insPP_2039 and *P. putida* KT2440 insPP_2048 accumulated up to 1.4-fold lower biomass and up to 2.6-fold lower PHA, respectively, in comparison to wild-type. *P. putida* KT2440 insPP_1893 and *P. putida* KT2440 insPP_2437 biomass levels were not affected, while PHA accumulation was lower, compared to that of wild-type (Table 2). Heptanoic (C_7) acid as a substrate caused up to 1.4-fold increase in cell dry weight (CDW) in all mutants, except *P. putida* KT2440 insPP_1893, which showed a 1.3-fold increase in PHA accumulation. The same insertion

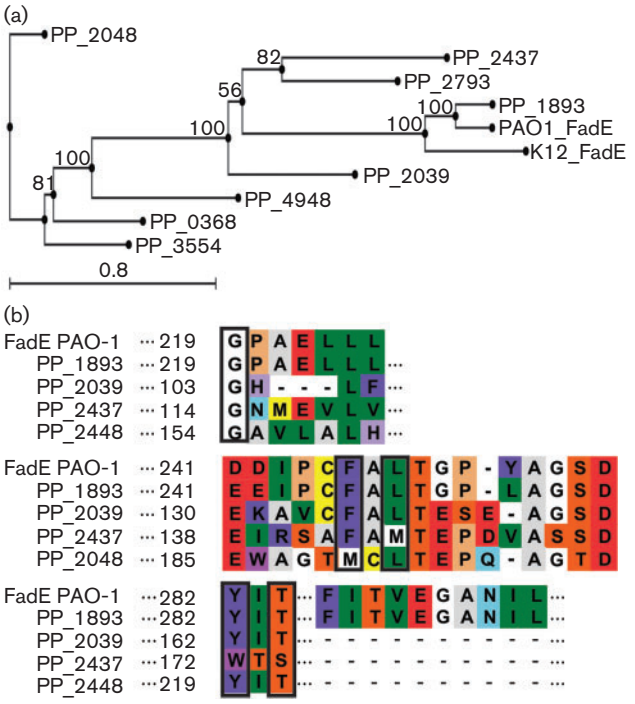

Fig. 2. *In silico* identification of FadE-homologous genes in *P. putida* KT2440. (a) Neighbour-joining phylogenetic tree based on FadE amino acid sequences showing the relationship between putative ACADs encoded by homologous genes of *P. putida* KT2440, FadE from *P. aeruginosa* PAO1 and FadE from *E. coli* K-12. (b) Alignment of selected ACAD-homologous motifs of *P. putida* KT2440 with *P. aeruginosa* PAO1 conserved amino acid residues present in the ACAD active site domain. Amino acids with similar properties are assigned the same colour based on the CLC sequence viewer 6 software (www.clcbio.com). Conserved residues important for the ACAD activity are marked with black rectangles. The scale bar represents 0.8 substitutions per nucleotide position.

mutants accumulated more PHA when supplied with octanoic acid (C_8), although biomass levels remained unaffected (Table 2). When medium to long chain length fatty acids (C_{10}–C_{14}) were used as substrate, the insertional mutants of *P. putida* KT2440 generally exhibited a decrease in both CDW and PHA accumulation (Table 2). Biomass accumulation by insPP_2039 and 2048 was higher than that by wild-type supplied with pentadecanoic (C_{15}) acid, while biomass and mcl-PHA levels of the other two mutants were not affected (Table 2).

The PHA monomer composition for all four mutants differed from the corresponding monomer composition of PHA in wild-type *P. putida* KT2440 by less than 5 % (Fig. 3). The monomer composition of PHA was as predicted from fatty acid substrates (Hume *et al.*, 2009; Ward & O'Connor, 2005), with the predominant monomer being either equal in chain length or an even number of carbons less than the substrate supplied, e.g. (*R*)-3-hydroxyoctanoic acid was

the predominant monomer from octanoic acid while decanoic acid was the predominant monomer of PHA that accumulated in cells supplied with dodecanoic acid (Fig. 3).

Overall, although noted and assigned as *fadE* with high confidence (http://www.pseudomonas.com/getAnnotation.do?locusID=PP_1893), growth of the *P. putida* KT2440 insPP_1893 mutant was not affected using fatty acids of different chain lengths as the sole source of carbon and energy. However, PHA accumulation was decreased for a number of fatty acid substrates (Table 2).

Mutants *P. putida* KT2440 insPP_2039 and *P. putida* KT2440 insPP_2048, located close to *fadD* and *fadB* on the

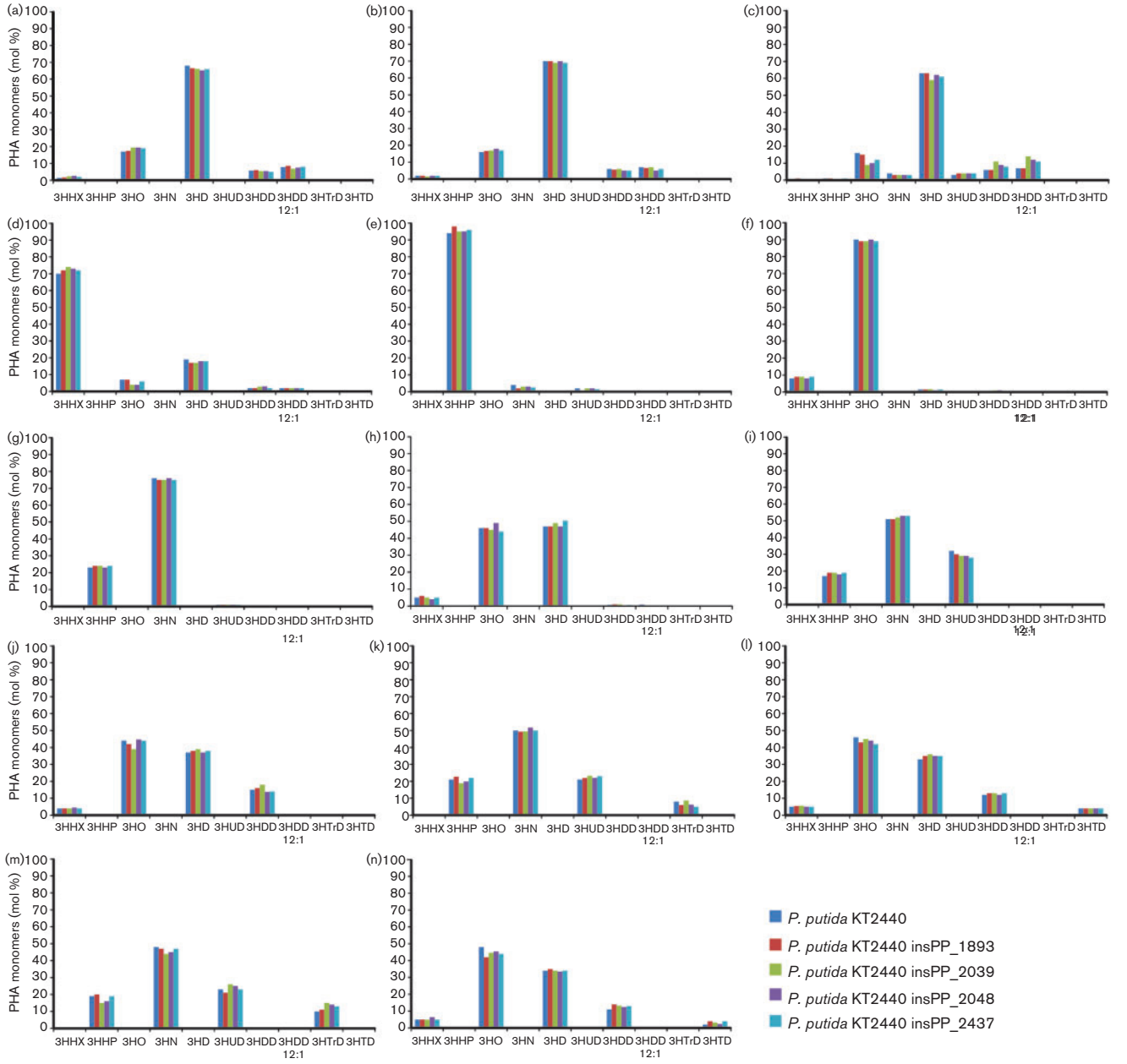

Fig. 3. Monomer composition of mcl-PHA accumulated by wild-type *P. putida* KT2440 and insertional mutants using (a) propionic, (b) butyric, (c) valeric, (d) hexanoic, (e) heptanoic, (f) octanoic, (g) nonanoic, (h) decanoic, (i) undecanoic, (j) dodecanoic, (k) tridecanoic, (l) tetradecanoic, (m) pentadecanoic and (n) hexadecanoic acids as substrate. 3HHX, (*R*)-3-hydroxyhexanoic acid; 3HHP, (*R*)-3-hydroxyheptanoic acid; 3HO, (*R*)-3-hydroxyoctanoic acid; 3HN, (*R*)-3-hydroxynonanoic acid; 3HD, (*R*)-3-hydroxydecanoic acid; 3HUD, (*R*)-3-hydroxyundecanoic acid; 3HDD, (*R*)-3-hydroxydodecanoic acid; 3HDD 12 : 1, (*R*)-3-hydroxydodecenoic acid; 3HTrD, (*R*)-3-hydroxytridecanoic acid; 3HTD, (*R*)-3-hydroxytetradecanoic acid.

genome, exhibited similar patterns in terms of ability to grow and accumulate PHA using fatty acids as a carbon source, with growth levels affected on C_6, C_7, C_{15} and PHA levels on C_3, C_5, C_6, C_7, C_8, C_{11} and C_{12} substrates (Table 2).

Medium chain length fatty acids are of interest as sources of mcl-PHAs with desirable polymer properties, i.e. polymers predominantly composed of (*R*)-3-hydroxyoctanoic acid and (*R*)-3-hydroxydecanoic acid have properties combining both elasticity and strength. The only mutant with a clearly reduced ability to grow and accumulate mcl-PHA on C_{10} to C_{14} acids was *P. putida* KT2440 insPP_2437. This finding, in combination with gene and protein sequence analyses (Fig. 2), was the basis for selection of PP_2437 as a possible long to medium chain length-specific fatty acyl-CoA dehydrogenase for further functional analysis.

Specific deletion of PP_2437 in *P. putida* KT2440

To eliminate polar effects of insertion mutants, the PP_2437 gene was specifically deleted and a complementation study carried out. The constructed knockout vector, pGEM-ΔPP_2437, was introduced into electrocompetent *P. putida* KT2440 wild-type strain to generate a *P. putida* ΔPP_2437 recombinant strain (Table 1). The growth of the mutant with specific deletion of the PP_2437 gene (*P. putida* ΔPP_2437) resulted in up to 1.5-fold decrease in biomass on all 14 fatty acids (Fig. 4a). Biomass accumulation was negatively affected to the greatest extent when C_3 and C_9 acids were used as substrates, while no significant change in growth was observed when a strain bearing the insertion mutant for the same gene (*P. putida* KT2440 insPP_2437) was cultured on these substrates (Table 2), indicating possible polar effects of the mutant bearing the insertionally inactivated gene. However, the level of CDW decrease when insertion and deletion mutants were grown on medium to long chain length fatty acids (C_{10}–C_{14}) was well correlated. CDW decrease with C_{10}–C_{14} as substrates was more pronounced when fatty acids contained odd numbers of carbons in the chain (C_{10} vs C_{11}, etc.; Fig. 4a).

PHA accumulation in *P. putida* ΔPP_2437 decreased 1.7- to 3.8-fold with fatty acids (Fig. 4b), which was higher than the change observed for the corresponding insertional mutant *P. putida* KT2440 insPP_2437 (up to 1.9-fold). The PHA monomer composition was unaffected, compared to that of WT KT2440 grown on the corresponding acids (data not shown).

The correct in-frame deletion was confirmed by Southern blot (data not shown) and complementation analyses (Fig. 5). Gene deletion was complemented in *P. putida* ΔPP_2437 by introducing the pJB861 : PP_2437 plasmid (Table 1). Recovery of the wild-type phenotype was achieved (from 87 to 100 %) with all acid substrates, confirming that no polar effects are caused by the specific gene deletion (Fig. 5).

Limited information is available on the effect of ACAD from the β-oxidation pathway on PHA accumulation.

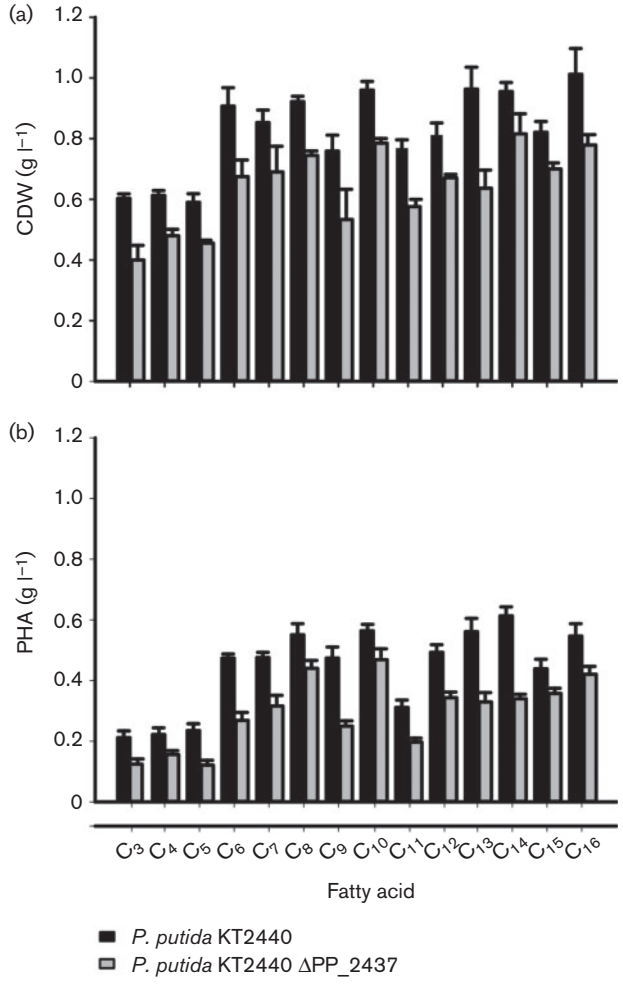

Fig. 4. Growth (a) and PHA accumulation (b) of the wild-type strain, *P. putida* KT2440, and the deletion mutant, *P. putida* KT2440 ΔPP_2437.

Co-expression of *fadE* along with *(R)*-specific enoyl-CoA hydratase (*phaJ* gene) and polymerase (*phaC* gene) from *Aeromonas caviae* resulted in a fourfold increase in the amount of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), compared to that obtained upon expression of *phaJ* and *phaC* genes only (Lu *et al.*, 2003). This finding clearly demonstrates that overriding the β-oxidation pathway rate-limiting step is beneficial for the efficient supply of PHA precursors. In another study reporting the construction of a poly-knockout mutant of *P. putida* KT2442 (Δ*fadB*, Δ*fadA*, Δ*fadB2x*, Δ*fadAx*, Δ*phaG*) harbouring additional deletion of a putative ACAD (PP_2048), the engineered strain produced a homopolymer of 3-hydroxydecanoate when grown on decanoic acid, suggesting an effect of the genes involved on the monomer composition (Liu *et al.*, 2011). To date, no studies have clearly demonstrated an influence of acyl-CoA dehydrogenases involved in the β-oxidation pathway on PHA accumulation in *P. putida* KT2440. In this strain, two genes responsible for dehydrogenation of the C-C bond between β- and γ-

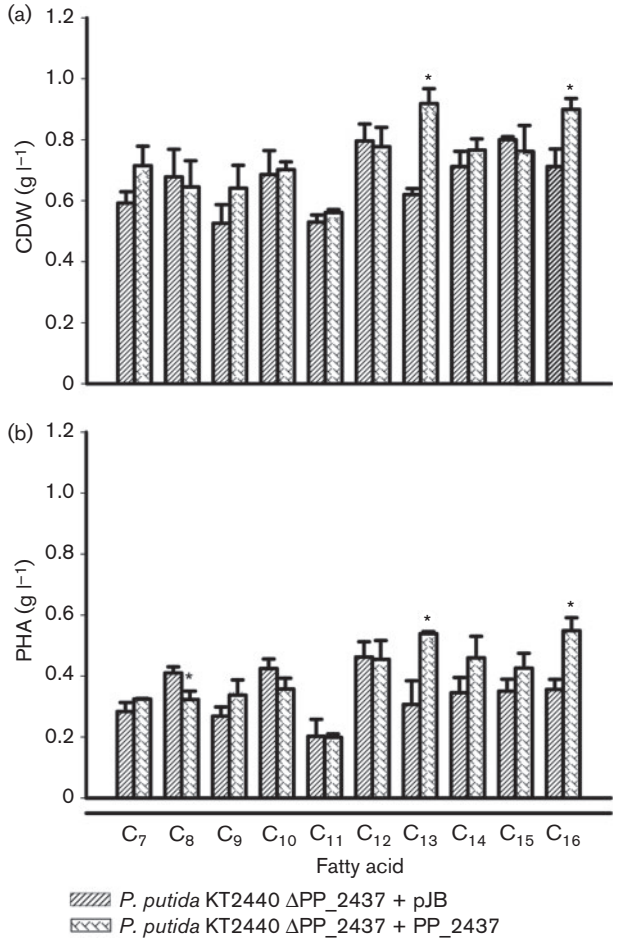

Fig. 5. Growth (a) and PHA accumulation (b) of the deletion mutant, *P. putida* KT2440 ΔPP_2437, and its complement variant, *P. putida* KT2440 ΔPP_2437+PP_2437; *significantly different ($P<0.05$).

carbon atoms of the acyl group in the acyl-CoA moiety are proposed to directly participate in fatty acid degradation, specifically, PP_1893 (ACAD or *fadE*) and PP_2437 (ACAD domain-containing protein, *fadE2*), while the 19 remaining putative ACADs have a probable role in this process (http://www.genome.jp/kegg/). These two ACADs share 90–99 % identity with analogues from different *P. putida*, *P. aeruginosa* and *P. syringae* strains, indicating wide distribution and conservation among pseudomonads. The first gene, *fadE* (PP_1893), shares 52 % identity with the *E. coli* K-12 substrain MG1655 *fadE* gene.

Among the *P. putida* KT2440 genes, PP_1893, PP_2039, PP_2048 and PP_2437 are not essential, but contribute to the metabolism of aliphatic fatty acids. This observation [especially with regard to gene inactivation/deletion data obtained with PP_1893 and PP_2437, particularly annotated as *fadE*s based on homology to other strains (http://www.genome.jp/kegg/)], is inconsistent with results obtained for the *fadE* deletion mutant of *E. coli*, which failed to grow on aliphatic fatty acids (Campbell & Cronan, 2002).

Recombinant expression, purification and biochemical characterization of PP_2437 ACAD

Complete solubilization of the recombinant protein, PP_2437, in *E. coli* BL21(DE3) cells was achieved by lowering the growth and induction temperature from 37 °C to 25 °C. N-terminally histidine tagged PP_2437 protein was purified to homogeneity with affinity chromatography using a nickel-chelating column and increasing concentrations of imidazole. The yellow coloured eluted fractions contained a protein of ~46 kDa (Fig. 6a) showing a typical absorption spectrum of a flavoprotein (Fig. 6b; McMahon *et al.*, 2005; McMahon & Mayhew, 2007).

The substrate range of the purified PP_2437 enzyme was investigated using 14 different CoA esters of aliphatic fatty acids (C_3–C_{16}). PP_2437 showed activity towards all tested substrates (Fig. 7). However, activity was distinctly low

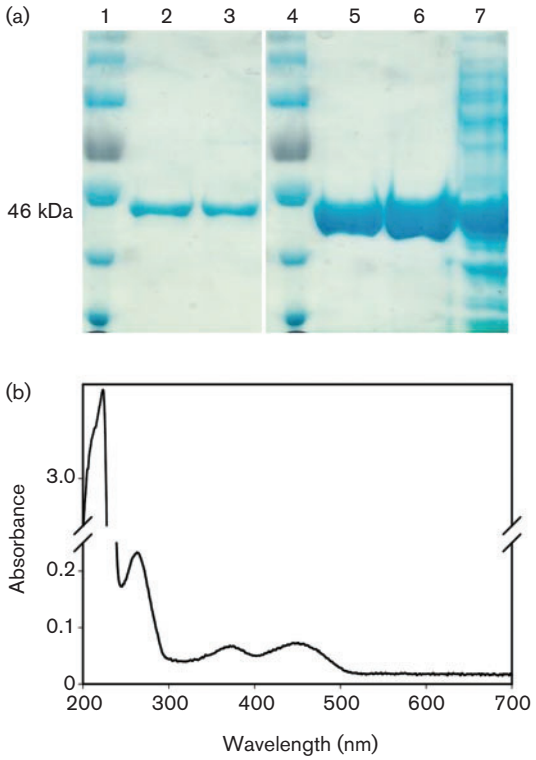

Fig. 6. SDS-PAGE analysis (a) of protein fractions and cell-free lysates of recombinantly expressed and purified acyl-CoA dehydroganse encoded by the PP_2437 gene of *P. putida* KT2440. Lanes 1 and 4 correspond to protein ladder (EZ-Run Pre-Stained *Rec* Protein Ladder, Fisher BioReagents), 2, 3, 5 and 6 represent fractions containing pure protein after column purification, and 7 represents cleared cell lysate. UV-vis spectrum (b) of recombinant PP_2437 ACAD.

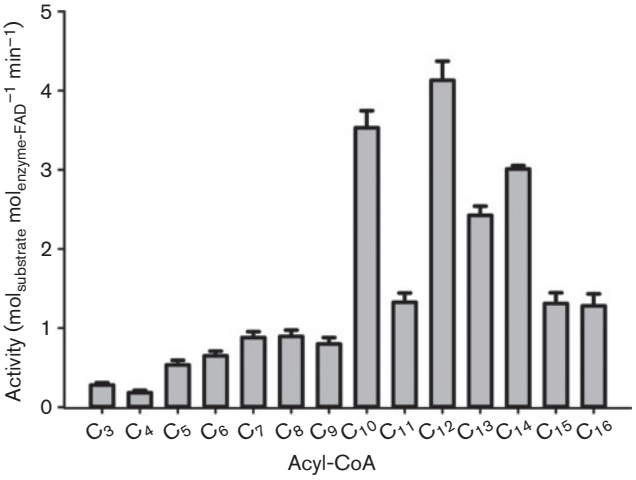

Fig. 7. Activity of recombinant PP_2437 ACAD towards a range of acyl-CoA esters with carbon chain lengths of C_3–C_{16}.

($\leqslant$1 mol substrate per mol enzyme-FAD complex min^{-1}) with C_3–C_9 acyl-CoA esters as substrate. A 22.5-fold difference in activity was observed between the poorest butyryl-CoA (C_4) and best dodecanoyl-CoA (C_{12}) ester substrates. On average, highest activity was observed with C_{10}–C_{14} CoA esters. Activity was almost ninefold higher, compared to that with C_3–C_5 CoA esters, 3.5-fold higher than that with C_6–C_9 CoA esters and 2.3-fold higher than that with C_{15}–C_{16} CoA esters (Fig. 7). In the C_{10}–C_{15} fatty acyl-CoA ester range, a preference towards esters of even chain length (C_{10} vs C_{11} etc.) was observed, with 2.7- and 1.7-fold higher activities with decanoyl- and dodecanoyl-CoA, respectively, in comparison to undecanoyl- and tridecanoyl-CoAs (Fig. 7).

The medium chain acyl-CoA dehydrogenase was further characterized in terms of temperature and pH range using the substrate with which highest activity was obtained in the above experiments, i.e. dodecanoyl-CoA. The optimal temperature for recombinant PP_2437 activity was 30 °C. The enzyme remained stable after storage at 4 °C for 24 h, and at −20 °C for seven days. The activity of PP_2437 was decreased by 20 % following incubation at temperatures from 20 °C to 30 °C for 30 min, and further decreased by 60 % after incubation at 40 °C. The optimal pH for enzyme activity was determined as between pH 6.5 and 7.0. Pre-incubation of the enzyme at different pH revealed that PP_2437 is stable within the pH range 6.0 to 7.5, with a marked decrease in stability at pH below 5.5 or above 8.0.

The catalytic properties of purified PP_2437 were further investigated using five CoA esters of even medium to long chain length fatty acids (C_8, C_{10}, C_{12}, C_{14} and C_{16}) (Table 3). The k_{cat} with dodecanoyl-CoA (C_{12}) ester was the highest in comparison to other substrates, while the K_m for this substrate was lower relative to K_m values with octanoyl-CoA and hexadecanoyl-CoA (Table 3). The overall catalytic efficiency of PP_2247 for dodecanoyl-CoA ester was from 1.5-fold to 13.5-fold higher in comparison to C_{10} and C_8 CoA esters respectively (Table 3). The catalytic properties of the enzyme encoded by PP_2437 gene in *P. putida* KT2440 were those of a flavoprotein with ACAD characteristics. The substrate specificity of any ACAD classifies the enzyme into one of the following groups: short, medium, long or very long chain acyl-CoA dehydrogenases, although the boundary of classification is not tight, as substrate specificities of particular enzymes can overlap (Ghisla & Thorpe, 2004; Kim & Miura, 2004). Only two *P. putida* KT2440 ACADs have been characterized in detail with regard to utilization of aliphatic substrates: a short chain length ACAD (McMahon *et al.*, 2005) and a long/very long chain phenylacyl-CoA dehydrogenase (McMahon & Mayhew, 2007). In this case, the enzyme encoded by the PP_2437 gene falls into the medium to long chain length acyl-CoA family. To our knowledge, this is the first verified ACAD in the *P. putida* KT2440 genome with such a substrate preference.

CONCLUSIONS

Based on the effects of deleting genes encoding putative ACADs, we conclude that many of these enzymes are redundant in *P. putida* KT2440 or overlap in terms of function when the organism is grown on aliphatic fatty acids. However, mutagenesis and biochemical characterization analyses showed that gene PP_2437 of *P. putida* KT2440 encodes a medium to long chain length acyl-CoA

Table 3. Kinetic constants (V_{max}, k_{cat} and K_m) of purified PP_2437 ACAD with fatty acyl-CoA esters as substrates

Substrate	k_{cat} (mol $_{substrate}$ $mol_{enzyme\text{-}FAD^{-1}}$ min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
Octanoyl-CoA	6.95 ± 0.16	119.10 ± 9.81	0.06
Decanoyl-CoA	6.11 ± 0.39	19.43 ± 1.25	0.31
Dodecanoyl-CoA	26.22 ± 3.51	32.41 ± 4.34	0.81
Tetradecanoyl-CoA	15.58 ± 3.11	28.65 ± 5.72	0.54
Hexadecanoyl-CoA	5.56 ± 0.06	45.36 ± 0.51	0.12

Data represent mean ± SD of three independent determinations.

dehydrogenase that plays a major role in the metabolism of medium to long chain fatty acids.

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