Brief Report

Unravelling the thioesterases responsible for propionate formation in engineered *Pseudomonas putida* KT2440

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Summary

***Pseudomonas putida* KT2440 is becoming a new robust metabolic chassis for biotechnological applications, due to its metabolic versatility, low nutritional requirements and biosafety status. We have previously engineered *P. putida* KT2440 to be an efficient propionate producer from L-threonine, although the internal enzymes converting propionyl-CoA to propionate are not clear. In this study, we thoroughly investigated 13 genes annotated as potential thioesterases in the KT2440 mutant. One thioesterase encoded by locus tag *PP_4975* was verified to be the major contributor to propionate production *in vivo*. Deletion of *PP_4975* significantly decreased propionate production, whereas the performance was fully restored by gene complement. Compared with thioesterase HiYciA from *Haemophilus influenza*, thioesterase PP_4975 showed a faster substrate conversion rate *in vitro*. Thus, this study expands our**

knowledge on acyl-CoA thioesterases in *P. putida* KT2440 and may also reveal a new target for further engineering the strain to improve propionate production performance.

Introduction

Propionic acid (PA) is recognized as the safest, most economical and effective food preservative in the world which is used for preventing grain caking, grain preservation and forage preservation. PA is also widely used in cellulose esters, plastics dispersible, herbicides, pharmaceuticals, spices and perfumes. Chemical and biological synthesis has been developed for PA production, while biotechnological production from renewable resources is attracting more attentions (Dishisha *et al.*, 2013; Zhuge *et al.*, 2015). To date, *Propionibacterium* is the main host for PA biosynthesis, in which propionyl-CoA produced from Wood–Werkman cycle is further converted to PA with succinate as co-substrate by CoA transferase, as shown in Fig. S1 (Falentin *et al.*, 2010). Overexpression of CoA transferase has been shown to promote PA production (Wang *et al.*, 2015), while succinate and acetate become inevitable impurities in this fermentation process.

In contrast to *de novo* fermentation from glucose by Wood–Werkman cycle, the L-threonine degradation pathway in *E. coli* has also been proposed to be a feasible and energetically efficient pathway for PA production (Gonzalez-Garcia *et al.*, 2017). In this way, L-threonine was deaminated and cleaved to propionyl-CoA and then to PA. While the *E. coli* pathway yields formate, in which 2-ketobutyrate is cleaved by pyruvate formate-lyase to propionyl-CoA (Hesslinger *et al.*, 1998), *Pseudomonas putida* uses the enzyme of branched-chain alpha-keto acid dehydrogenase complex to produce propionyl-CoA from 2-ketobutyrate with only CO_2 as the by-product. Thus, *P. putida* has the obvious advantage of producing high-purity PA, since no other organic acids remained in the broth. We have previously developed *P. putida* KT2440 as a PA hyper-producer by engineering the internal L-threonine degradation route (Ma *et al.*, 2020).

Additionally, one major facilitator superfamily transporter (PP_1271-1273) was also verified to be involved in PA tolerance in *P. putida* KT2440 (Ma *et al.*, 2021). In the process of engineering the final strain of PS10, the intermediate strain PS08, which the final step from propionyl-CoA to PA was not engineered, could also accumulate certain amount of propionate from L-threonine (Ma *et al.*, 2020). Thus, internal transformation pathways should exist in the native strain to accomplish the reaction. However, the catalysing enzymes responsible for this step in *P. putida* KT2440 remained unknown.

The aim of this study is to identify the native enzyme(s) responsible for the hydrolysis of propionyl-CoA to PA in the engineered *P. putida* KT2440 strain. To date, three different pathways from propionyl-CoA to PA in organisms are known, as, respectively, catalysed by phosphate acetyltransferase/acetate kinase system (route a), acyl-CoA thioesterase (route b) and acetyl-CoA synthetase (route c) (Fig. 1). Route a has been widely reported in *E. coli*, which is achieved by the Pta-AckA system (Sumantran *et al.*, 1990; Hesslinger *et al.*, 1998). However, no *ack*A gene is found in the genome of *P. putida* KT2440, and interestingly, propionate production even decreased slightly when the *E. coli* Pta-AckA was co-expressed in the *P. putida* strain (Ma *et al.*, 2020). Thus, route a was excluded, and the activities of thioesterase and acetyl-CoA synthetase were investigated in this study.

Results and discussion

Acetyl-CoA synthetases were not involved in PA production in P. putida KT2440

The genetic background of strain PS08, which is derived from *P. putida* KT2440, is shown in Fig. S2. Besides blocking the branch pathways and strengthening the
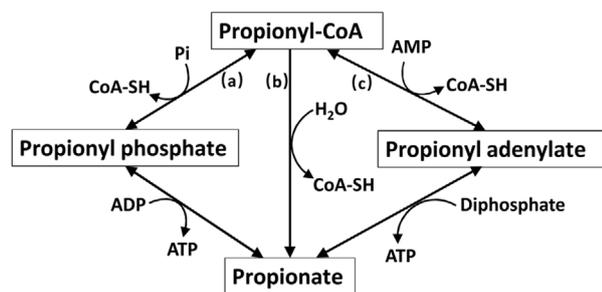
steps from L-threonine to propionyl-CoA, we also deleted the gene *prpE* (PP_2351) encoding a propionyl-CoA synthetase, as the isoenzyme of acetyl-CoA synthetase. *PrpE* is part of the *prpBCDE* operon, generally recognized to catalyse the activation of propionate during propionate breakdown (Horswill and Escalante-Semerena, 1999). Strain PS08 with the deletion of *prpE* showed only a slight increase of propionate production. Thus, we dismissed the possibility of the involvement of PrpE.

Four possible acetyl-CoA synthetase genes (*PP_2213*, *PP_3458*, *PP_4487* and *PP_4702*) were annotated from the genome of KT2440 (Belda *et al.*, 2016). Although the literature indicated that acetyl-CoA synthetase might catalyse an irreversible reaction *in vivo* from acetate to acetyl-CoA (Kumari et al., 2000), we still conducted the gene deletion experiments to exclude such a possibility. Additionally, the acetyl-CoA synthetase gene null strain could provide an ideal platform to further investigate the function of any potential thioesterase *in vivo*, since the propionate degradation pathway was completely blocked. Thus, based on strain PS08, we knocked out the four acetyl-CoA synthetase genes one by one to explore the possible contributors. The strains and recombinant plasmids used in this study are listed in Table S1. Even when the four acetyl-CoA synthetase genes were knocked out simultaneously (the strain was designated as TV04 in the following experiments), no obvious effects on the propionate production performance were observed (Fig. 2). The results indicated that the acetyl-CoA synthetase catalysed reaction *in vivo* is not the way for propionate production in *P. putida* KT2440. Besides acetyl-CoA synthetase genes, propionyl-CoA transferase (EC 2.8.3.1) could reversibly transform propionyl-CoA to propionate with succinate or other organic acids as co-substrate (Volodina *et al.*, 2014). One gene was annotated as propionyl-

Fig. 1. The schematic diagram of three pathways from propionyl-CoA to propionate.
A. The phosphate acetyltransferase (EC 2.3.1.8; encoded by *pta*) converted propionyl-CoA to propionyl phosphate, which is further catabolized to propionate by acetate kinase (EC 2.7.2.1; encoded by *ackA*).
B. Propionyl-CoA is directly transformed into propionate by acyl-CoA thioesterase (EC 3.1.2.20).
C. Propionyl-CoA is catalysed by two consecutive steps to propionate by acetyl-CoA synthetase (EC 6.2.1.1).

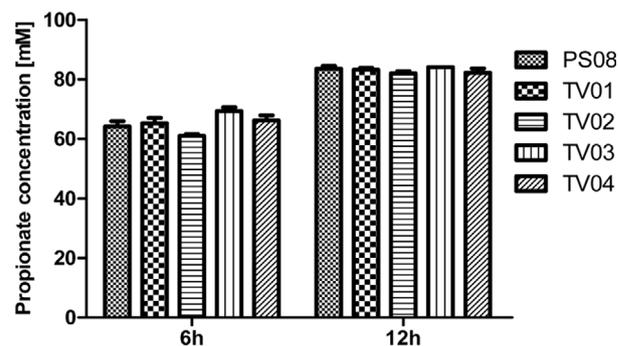**Fig. 2.** Bioconversion assays of 2-ketobutyrate to propionate by acetyl-CoA synthetase gene deletion strains. The 100 mM 2-ketobutyrate was added at the beginning of the assay at $OD_{600} = 30$. The concentrations of propionate produced in the conversion were sampled and measured at 6 and 12 h respectively. The figure shows the mean values of at least three independent assays. The standard deviation is represented by *error bars*.

CoA:succinate CoA transferase (*scp*C, PP_0154) in the *P. putida* KT2440 genome. Although propionyl-CoA transferases are unlikely to be involved since there were not enough organic acids in the resting cells to support such an efficient process, we still conducted gene deletion to exclude such a possibility. As expected, knocking out the *scp*C gene had no effects on propionate production (Fig. S3). Given the results above, the acyl-CoA thioesterase catalysed reaction (route b) should be the only way in which propionate is formed from propionyl-CoA in *P. putida*, since the other two pathways have been excluded by the experimental verification.

Deletion of Acyl-CoA thioesterases significantly decreased PA production

We screened the acyl-CoA thioesterase genes in the genome of *P. putida* KT2440 (Belda *et al.*, 2016) and found 13 possible annotated thioesterase genes (Table 1). Based on strain TV04, we have further sequentially knocked out the thioesterase genes to identify the contributors. To simplify the reaction, 2-ketobutyrate was added as substrate in the bioconversion assays to compare the catalytic capacities of different acyl-CoA thioesterase gene deletion strains. The bioconversion assays showed that only deletion of *PP_4975* (strain TV06) and *tesB* (strain TV10) obviously decreased propionate production, although the final strain TV17 still retained about 24% capacity to produce propionate (Fig. 3). The results clearly indicate that these two acyl-CoA thioesterases are involved in propionate production, especially PP_4975, since the propionate production titre sharply decreased by 51% in strain TV06. Further deletion of *tesB* gave a total decrease rate of more than 75% for propionate
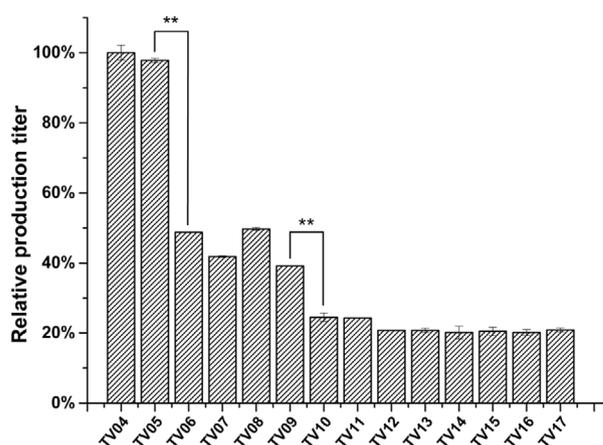

Fig. 3. Bioconversion assays of 2-ketobutyrate to propionate by a series of thioesterase gene deletion strains. The 100 mM 2-ketobutyrate was added at the beginning of the assay with the biomass set at $OD_{600} = 30$. The final concentrations of propionate produced in the conversion were measured by HPLC. The figure shows the mean values of at least three independent assays, and the standard deviation is represented by *error bars*. The production titre obtained by strain TV04 was set at 100%, in which no thioesterase gene was disrupted. The *t*-tests compared the propionate production of strain TV06 against TV05, as well as strain TV10 against TV09 (**$p < 0.01$).

production, as compared to the starting strain TV04. To confirm the functionality of TesB, the single gene deletion of *tes*B was also conducted based on strain TV05 to be consistent with *PP_4975* gene deletion. As shown in Fig. S4, without deletion of *PP_4975*, single knockout of *tes*B only slightly decreased the propionate production. These data clearly indicate the main contributory role of PP_4975 for propionate production in *P. putida* KT2440.

PA production was restored by Acyl-CoA thioesterase complementary strains

To further verify the roles of these two thioesterase genes in the pathway of propionate production, we constructed the overexpression plasmids, pUCP18-*4975* and pUCP18-*tesB*, by inserting the responsible thioesterase genes into pUCP18, whose gene expression was driven by *lac* promoter. The *PP_4975* and *tes*B genes were PCR-amplified from the genome of *P. putida* KT2440 with the NCBI accession number of NC_002947.4. Then, the plasmids were transformed into *P. putida* TV17, in which all possible thioesterase genes had been deleted. As shown in Fig. 4, both gene complementary strains all restored the propionate production performance, even more efficient than strain TV04, which solidly confirmed the contributory role of genes *PP_4975* and *tes*B for propionate production *in vivo*. Although it is a little surprising that the *tes*B gene complementary strain could restore an equivalent performance as that of the *PP_4975*, it is still reasonable

Table 1. Genes annotated as thioesterases in *P. putida* KT2440.

| Gene	Description	Locus tag	null strains
PP_5331	acyl-CoA thioesterase	PP_5331	TV05
PP_4975	acyl-CoA thioesterase	PP_4975	TV06
vdlD	thioesterase	PP_5356	TV07
PP_3807	thioesterase	PP_3807	TV08
PP_1466	acyl-CoA thioesterase	PP_1466	TV09
tesB	acyl-CoA thioesterase II	PP_4762	TV10
tesA	acyl-CoA thioesterase I/protease I/ lysophospholipase L1	PP_2318	TV11
ybgC	tol-pal system-associated acyl-CoA thioesterase	PP_1218	TV12
PP_2308	thioesterase family protein	PP_2308	TV13
paaY	phenylacetate degradation detoxifying thioesterase	PP_3285	TV14
paaI	hydroxyphenylacetyl-CoA thioesterase	PP_3281	TV15
PP_1980	acyl-CoA thioesterase	PP_1980	TV16
PP_2050	acyl-CoA thioesterase	PP_2050	TV17
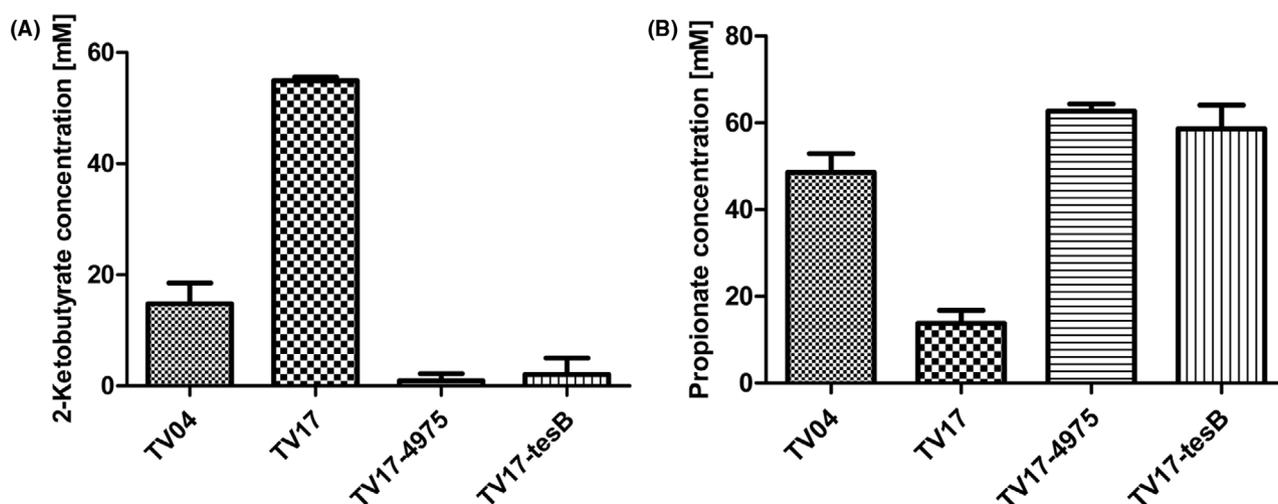
Fig. 4. The performance of propionate production from 2-ketobutyrate by thioesterase gene complimentary strains.
A. The remained 2-ketobutyrate and (B) the produced propionate in the reaction. The 100 mM 2-ketobutyrate was added at the beginning of the assay with the biomass set at OD_{600} = 30. The figure shows the mean values of at least three independent assays, and the standard deviation is represented by *error bars*.

since the *tes*B gene was overexpressed in the plasmid with a relatively high copy number, as compared to only one copy in the genome.

Enzymatic characterization of the identified thioesterase PP_4975

As TesB has been reported to hydrolyse β-hydroxyacyl-CoA thioesters (Naggert *et al.*, 1991), we focused on the investigation of the newly identified PP_4975. PP_4975 has a predicted molecular weight of 14.9 kDa. Analysis of the amino acid sequence similarity with other thioesterase proteins reveals that PP_4975 belongs to the acyl-CoA hydrolase family (Zhuang *et al.*, 2008). On the peptide chain of 1-109, there is a predicted hotdog ACOT-type domain. In our previous work, upon inserting the thioesterase HiYciA from *Haemophilus influenza* into the *P. putida* KT2440 mutant, the resultant final strain PS10 could efficiently transform 400 mM L-threonine into propionate with a molar yield of > 99% (Ma *et al.*, 2020). HiYciA is also a hexameric hotdog thioesterase homologous to the YciA from *E. coli* (Willis *et al.*, 2008). It was used as an experimental control in this study for its high catalytic activity for a series of acyl-CoA substrates, including propionyl-CoA. The thioesterase activity was determined using steady-state kinetic methods. The initial reaction rates were measured with different concentrations of propionyl-CoA as substrate. The saturation curves of PP_4975 and HiYciA for propionyl-CoA were fitted according to the Michaelis–Menten equation in the Origin software (version 8.0), and they both reached near saturation values. The kinetic parameters are recorded in Table 2. HiYciA showed higher affinity to

Table 2. Kinetic parameters of PP_4975 and HiYciA with substrate propionyl-CoA.

Thioesterase	$K_{cat\ (s)}^{-1}$	$K_{m\ (\mu M)}$	$K_{cat}/K_{m\ (\mu M}^{-1}\ s^{-1})$
PP_4975	1.3×10^{3}	144.13	9.02
HiYciA	4.9×10^{2}	26.84	18.26

propionyl-CoA than PP_4975, but the K_{cat} values indicated PP_4975 had a faster substrate conversion rate under this experimental condition. Although the activity of PP_4975 was lower than that of HiYciA with substrate propionyl-CoA, this result confirmed the functionality of PP_4975 under pure enzyme condition. Notably, since PP_4975 is not as efficient as the HiYciA, this result also supports our previous study that introducing an exogenous thioesterase, such as HiYciA, is necessary to improve the production performance (Ma *et al.*, 2020).

Thioesters participate in metabolism, membrane synthesis, signal transduction and gene regulation. Thioesterases convert thioesters to the thiol and carboxylic acid components, which endorse thioesterases the important cellular roles (Swarbrick *et al.*, 2020). This study demonstrated the native enzymes responsible for the hydrolysis of propionyl-CoA to propionate in the engineered *P. putida* strain. Deletion and overexpression studies identified a clear role for thioesterase PP_4975 in the engineered propionate-producing *P. putida* strain. Moreover, thioesterase PP_4975 was not the sole contributing thioesterase. Deletion of *tes*B further decreased propionate production while the *tes*B complementary strain could fully restore the performance, even when no PP_4975 existed (Fig. 4). Compared to thioesterase I

(TesA) which favours medium-chain-length acyl-CoA (Magnuson *et al.*, 1993), thioesterase II (TesB) was previously reported to have broad specificity in catalysing acyl-CoA of C6-C18 carbon length to their correspondent free fatty acids (Naggert *et al.*, 1991). TesB from *E. coli* K12 was successfully employed to directly hydrolyse 3-hydroxybutyrate-CoA (Liu *et al.*, 2007). In this study, TesB in *P. putida* KT2440 was also shown to be involved in catalysing C3 acyl-CoA. Our study confirmed the versatile substrate specificities of TesB.

Compared to the research in *E. coli* and other microorganisms, thioesterases in *Pseudomonas* are rarely investigated. Thioesterases are a large superfamily of enzymes with a broad range of substrate preference. The newly found thioesterase PP_4975 was demonstrated to play a key contributory role in propionate production, which was verified by both *in vivo* gene deletion and *in vitro* pure enzyme activity test. It should be mentioned that propionyl-CoA might not be its favourite substrate, since *P. putida* KT2440 is not a natural propionate producer. Without deletion of the branch pathways in KT2440, no propionate could accumulate (Ma *et al.*, 2020). However, the positive impact of PP_4975 overexpression in Fig. 4 showed the potential of evaluating thioesterases within a specific pathway to improve the product titres. Two *P. aeruginosa* hotdog thioesterases were reported to have activity against 3-hydroxy-3-methylglutaryl-CoA (PA5202) and octanoyl-CoA (PA2801) respectively. PA5202 and PA2801 shared a similar structure but exhibited different substrate preferences and functions (Gonzalez *et al.*, 2012). Thus, although PP_4975 shared a high similarity with the hotdog thioesterases from other species, this study experimentally broadens our knowledge on acyl-CoA thioesterase in *P. putida* KT2440. Additionally, even with all potential thioesterase genes deleted in strain TV17, it still remained the low capacity to produce propionate. Some unknown enzymes or pathways must exist in KT2440 to catalyse the formation of propionate, which is expected to be revealed under further investigations.

Conclusions

Pseudomonas putida has recently gained much attention as a new workhorse for bio-industries. Re-examination of *P. putida* KT2440 genome enlightens its value as a robust metabolic chassis. Thus, experimental verification of the genes responsible for important cellular roles is beneficial to fully understand this platform microorganism. Previously, we engineered *P. putida* KT2440 as a propionate producer from L-threonine with high efficiency, while the internal transformation enzyme from propionyl-CoA to propionate was not clear. In this study, based on the annotated genome information of *P. putida*

KT2440, we conducted systematic gene deletion experiments and successfully found two thioesterases responsible for propionate production, especially the newly found thioesterase PP_4975. This study not only expands our knowledge of L-threonine metabolism in *P. putida* KT2440 but will also facilitate further increase of the metabolic flux from propionyl-CoA to propionate by improving the internal enzyme activities.

Conflict of interest

No declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. The detailed information of experiment procedures was provided, including cultivation conditions, genetic manipulation methods, strains & plasmids used in this study, enzyme purification and characterization, as well as the HPLC analysis method.

Fig. S1. Propionate production pathway in *Propionibacterium* spp. via the Wood–Werkman cycle.

Fig. S2. The genetic background of strain PS08 and the propionate production performance of the engineered strains.

Fig. S3. Propionate production by strain TV15 and the propionyl-CoA:succinate CoA transferase (*scp*C, PP_0154) gene deletion strain.

Fig. S4. The performance of propionate production from 2-ketobutyrate by *PP_4975* and *tes*B gene single deletion strains, respectively.

Table S1. Strains and plasmids used in this study.