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The TetR family transcriptional regulator PccD negatively controls propionyl coenzyme A assimilation in *Saccharopolyspora erythraea*

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Abstract

Propanol stimulates erythromycin biosynthesis by increasing the supply of propionyl-CoA, a starter unit of erythromycin production in *Saccharopolyspora erythraea*. Propionyl-CoA is assimilated via propionyl-CoA carboxylase to methylmalonyl-CoA, an extender unit of erythromycin. We found that the addition of n-propanol or propionate caused a 4–16 fold increase in the transcriptional levels of the SACE_3398-3400 locus encoding propionyl-CoA carboxylase, a key enzyme in propionate metabolism. The regulator PccD was proved to be directly involved in the transcription regulation of SACE_3398-3400 locus by EMSAs and DNase I footprint analysis. The transcriptional levels of SACE_3398-3400 were upregulated 15-37-fold in the *pccD* gene deletion strain (Δ*pccD*) and downregulated 3-fold in the *pccD* overexpression strain (WT/pIB-*pccD*), indicating that PccD was a negative transcriptional regulator of SACE_3398-3400. Δ*pccD* strain has a higher growth rate than that of wild-type strain (WT) on Evans medium with propionate as the sole carbon source, whereas the growth of WT/pIB-*pccD* strain was repressed. As a possible metabolite of propionate metabolism, methylmalonic acid was identified to be an effector molecule of PccD, and repressed its regulatory activity. The higher level of erythromycin in the Δ*pccD* strain was observed compared with the wild-type strain. Our study reveals a regulatory mechanism in propionate metabolism and suggests new possibilities for designing metabolic engineering to increase erythromycin yield.

Importance:

Our work has identified the novel regulator PccD that controls the gene expression of propionyl-CoA carboxylase, a key enzyme in propionyl-CoA assimilation in *S. erythraea*. PccD represses the generation of methylmalonyl-CoA through carboxylation of propionyl-CoA, and reveals an effect on biosynthesis of erythromycin. This finding provides novel insight for propionyl-CoA assimilation, and extends our understanding of the regulatory mechanisms underlying the biosynthesis of erythromycin.

Introduction

Saccharopolyspora erythraea, a Gram-positive filamentous soil bacterium, is used for production of the antibiotic erythromycin. Erythromycin and its derivatives play a vital role in medicine, with annual sales reaching several billions of dollars annually [1]. Historically, multiple rounds of random mutagenesis and selection have been used to obtain overproducing mutants for industrial production. Nowadays, genetic engineering of the pathways involved in biosynthesis of secondary metabolites also holds promises to enhance production [2-4].

Production of one unit of erythromycin requires one propionyl coenzyme A (propionyl-CoA) to provide a starter unit and six (2S)-methylmalonyl-CoA to provide extender units to form 6-deoxyerythronolide B (6-dEB) [5]. Reeves' experiments indicated that methylmalonyl-CoA was a limiting factor for erythromycin biosynthesis [6]. Engineering of the methylmalonyl-CoA metabolic flux of *S. erythraea* through knockout of the methylmalonyl-CoA mutase operon resulted in a 50% increase in erythromycin production in an oil-based fermentation medium [7]. Integrating genomics, transcriptomics, and proteomics comparison between the wild-type strain and the industrial high-producing strain of *S. erythraea* revealed that the industrial strain has a high flux of metabolites towards erythromycin biosynthesis and degradation pathway of branched-chain amino acids [8]. Because branched-chain amino acids are a source of methylmalonyl-CoA, the significantly higher expression levels of *ilvB* (acetolactate synthase large subunit, SACE_4565), *acd* (acyl-CoA dehydrogenase, SACE_4125 and SACE_5025), and *mmsA* (methylmalonate-semialdehyde dehydrogenase, SACE_4672) in the high-producing strain may lead to an increased supply of propionyl-CoA and methylmalonyl-CoA, key precursors for erythromycin synthesis. This is also supported by an increased erythromycin yield when different genes of the branched-chain amino acid metabolism were overexpressed [9]. The quantitative analysis of metabolic carbon flux indicated that high consumption rate of propanol increased propionyl-CoA and methylmalonyl-CoA concentrations. The addition of n-propanol at a concentration of

1% (v/v) increased the erythromycin production by 20% [10]. Analysis of [^{18}O] n-propanol metabolic carbon flux suggested that the oxygen atoms of 6-dEB are largely derived from n-propanol [11]. Propanol is converted into propionate, which is subsequently metabolized to propionyl-phosphate or propionyl-CoA by propionate kinase or acetyl/propionyl-CoA synthetase. However, propionyl-CoA is an inhibitor of CoA-dependent enzymes such as pyruvate dehydrogenase, succinyl-CoA synthetase, and ATP citrate lyase [12-16]. Therefore, the assimilation of propionyl-CoA needs to be precisely regulated to prevent its accumulation within the cell.

In most microorganisms, so far, two pathways were found for propionyl-CoA assimilation: the methylcitrate cycle (MCC) that converts propionyl-CoA to pyruvate, and the propionyl-CoA carboxylase (PCC) pathway, which is responsible for the metabolism of propionyl-CoA to methylmalonyl-CoA. No gene encoding methylcitrate synthase has been found in the genome of *S. erythraea*. There are at least six genetic locus encoding putative biotin-dependent acetyl-CoA/propionyl-CoA carboxylases, including SACE_0026–0028, SACE_3241–3242, SACE_3398–3400, SACE_7038–7039, SACE_4237, and SACE_3856/6509-6510 [17]. Here, we show that only the genetic locus SACE_3398–3400 (herein named *pccB*, *pccC*, and *pccA*) plays an important role in propionyl-CoA assimilation when n-propanol is added for high production of erythromycin. The TetR family transcriptional regulator SACE_3396 (herein named PccD) can bind directly to the promoter regions of *pccBC* and *pccA*, and negatively regulate their transcription.

Results

The *pccBC* and *pccA* operons are induced by propanol and propionate in *S. erythraea*

It was reported that propionyl-CoA carboxylases were upregulated when propanol or propionate was added to microorganisms [18,19]. Propanol is converted into propionate, which is subsequently metabolized to propionyl-phosphate or propionyl-CoA by propionate kinase or acetyl/propionyl-CoA synthetase (Fig. 1A). The quantitative analysis of metabolic carbon flux indicated that a high consumption rate of propanol increased intracellular concentration of propionyl-CoA and methylmalonyl-CoA [11] to enhance the biosynthesis of erythromycin. In the genome of *S. erythraea*, at least six possible acetyl-CoA/propionyl-CoA carboxylases are annotated, namely SACE_0026–0028, SACE_3241–3242, SACE_3398–3400, SACE_7038–7039, SACE_4237, and SACE_6509 (Fig. S1). The co-transcription of genes in each locus was investigated (Fig. S2). We found that gene *pccB* (SACE_3398) was cotranscribed with the *pccC* (SACE_3399) (form an operon: *pccBC*), and wasn't cotranscribed with the *pccA* (SACE_3400). So far, it is still unclear which of the possible propionyl-CoA carboxylases make a contribution to erythromycin biosynthesis. To investigate which genetic locus were responsive to the addition of n-propanol or propionate, transcript levels of all locus encoding acetyl-CoA/propionyl-CoA carboxylases were examined using qRT-PCR (Fig. 1B). The results showed that transcriptional levels of *pccBC* operon and *pccA* gene increased 4- or 14-fold and 5- or 16-fold, respectively, by addition of propanol or propionate. No changes were observed in transcriptional levels of the other locus coding for acetyl-CoA/propionyl-CoA carboxylases (Fig. 1B). *pccB* encodes a β-subunit (PccB) that has a carboxyl transferase (CT) activity, and *pccA* codes for a larger α-subunit (PccA) comprising the biotin carboxylase (BC) and biotin carboxy carrier protein (BCCP). *pccC* encodes the small ε-subunit [17]. These results indicate that *pccBC* and *pccA* operons perform a major function in propionate metabolism and propionyl-CoA assimilation.

PccD (SACE_3396) binds to the promoter regions of *pccBC* and *pccA* operons

To identify the possible transcriptional regulator controlling *pccBC* and *pccA* in *S. erythraea*, we analyzed their genomic organization. It was found that SACE_3396 gene encoding a TetR/AcrR family transcriptional regulator (named as PccD) was located upstream of *pccBC* (Fig. 2A). To examine the binding activity of PccD to *pccBC* and *pccA* operons, electrophoretic mobility shift assays (EMSAs) were performed. As shown in Figure 2B, obvious band shifts were observed, as the entire promoter regions of *pccBC* and *pccA* were incubated with purified recombinant His-tagged PccD (the fractions G, H and I in Fig. S4). The results indicate that PccD was able to directly interact with the promoter regions of these two operons.

PccD is a transcriptional repressor of *pccBC* and *pccA* operons

To further investigate the regulatory effect of PccD on the *pccBC* and *pccA* operons, we constructed the *pccD*-deleted mutant (Δ*pccD*) and *pccD*-overexpressed strain (WT/PIB-*pccD*), and performed transcriptional analysis by qRT-PCR of wild-type (WT), Δ*pccD* and WT/PIB-*pccD* strains. These strains were cultivated in TSB medium, and RNA was extracted at the late exponential growth/early stationary phase. Results show that deletion of *pccD* resulted in a 15-fold (for *pccA*) and 37-fold (for *pccBC*) upregulation of the *pccA* and *pccBC* operons, and overexpression (approximately 5 folds) of *pccD* inhibited the expression of two operons by 3-fold as compared to that in the WT strain (Fig. 3A). Next, we examined the growth of WT, Δ*pccD,* and WT/PIB-*pccD* strains cultivated in Evans media with 20 mM sodium propionate as the sole carbon source. As shown in Figure 3B, differences in growth behavior were observed among these strains. On one hand, the deletion of *pccD* significantly promoted propionate metabolism and propionyl-CoA assimilation by inducing the transcription of *pccBC* and *pccA* operons, and increased the growth rate of *S. erythraea* on propionate medium (Fig. S5). On the other hand, the WT/PIB-*pccD* strain overexpressing *pccD* revealed a decrease in growth rate on propionate compared to the wild-type strain due to the low levels of *pccBCA* transcripts (Fig. 3A). Meanwhile, the WT/PIB-*pccD* strain showed a decrease in growth yield probably

due to the accumulation of toxic propionyl-CoA [12-16]. Three strains revealed similar growth curves on Evans media supplied with 2.5% glucose (Fig. 3C). Taken together, these observations further demonstrate that PccD is a transcriptional repressor of *pccBC* and *pccA* operons, and inhibits propionyl-CoA assimilation.

Identification of the PccD-binding sites in upstream regions of *pccBC* and *pccA*

To define the binding sites of PccD, the upstream region (300 bp) of *pccBC* bound by recombinant His-tagged PccD was investigated by DNase I footprint mapping. As shown in Figure 4A, a protected region of 27 nucleotides was detected at the position -140 to -114 relative to the translational start site in the *pccBC* promoter. The -35 promoter sequence (predicted by softberry website, http://www.softberry.com/) was located in the protected region (Fig. 4B). A putative PccD-binding motif (t/aTGACGg/cTGt/cTGt/a) was obtained from the protected sequence and the promoter sequence of *pccA* using MEME (http://meme-suite.org/) (Fig. 4C). To further confirm the putative PccD-binding motif, two short biotin-labeled dsDNA, named M-*pccBC* and M-*pccA*, containing PccD-binding sequences in the upstream regions of *pccBC* and *pccA* operons, respectively, were synthesized (Table S1) and incubated with purified recombinant His-tagged PccD. The EMSAs results showed that the two synthetic fragments containing the PccD-binding motif were sufficient to interact with the His-tagged PccD (Fig. 4D), thus confirming the PccD-binding motif predicted by DNase I footprint mapping and MEME method. No PccD-binding motif was observed in the promoter region of *pccD* gene. Indeed, EMSA experimental result revealed that PccD did not bind to its own gene promoter (data not shown).

The metabolite methylmalonic acid is an effector of PccD

TetR/AcrR family transcriptional repressors are homodimeric DNA-binding proteins. Their DNA-binding activity is allosterically inactivated by the binding of small-molecule ligands (effectors). PccD protein from *S. erythraea* contains two TetR domains, may act as a monomer for DNA-binding. To identify the effector of PccD,

we investigated several intermediate metabolites of the propionate metabolism as effector molecules, including propionyl-CoA, methylmalonic acid, methylmalonyl-CoA, and succinyl-CoA, as well as propionate itself. The effect of these compounds on PccD binding to the *pccBC* promoters was examined using the EMSA experiment. We found that propionate, propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA had no effect on the DNA-binding activity of PccD, whereas methylmalonic acid specifically inhibited PccD binding to the *pccBC* promoter (Fig. 5A). In order to further verify the important effect of methylmalonic acid on *pccBC* operon transcription in vivo in *S. erythraea*, 10 mM methylmalonic acid was added to TSB medium and the transcriptional level of *pccBC* operon was investigated. The results showed that *pccBC* operon transcriptional level was increased about more than 26-fold by addition of methylmalonic acid (Fig. 5B). This indicated that methylmalonic acid may exert an important effect on the transcription of *pccBC* operon, although there is no direct experimental evidence that methylmalonic acid is an effector of PccD regulator in vivo in *S. erythraea*.

Δ*pccD* probably promotes erythromycin production by activating propionyl-CoA assimilation

From the results described above, we speculated that enhancement of the propionyl-CoA carboxylation might result in an increase of intracellular methylmalonyl-CoA and promote erythromycin production. To verify this speculation, we determined the intracellular concentration of propionyl-CoA and methylmalonyl-CoA in WT and Δ*pccD* strains grown on TSB medium with 1% (v/v) n-propanol. As shown in Figure 6A, the result demonstrated that deletion of *pccD* promoted propionyl-CoA carboxylation, resulting in a decrease in propionyl-CoA concentration and an increase in methylmalonyl-CoA concentration. It is known that the biosynthesis of a unit erythromycin needs one propionyl-CoA and six (2S)-methylmalonyl-CoA. High ratio of methylmalonyl-CoA/propionyl-CoA in Δ*pccD* strain will facilitate the biosynthesis of erythromycin. Indeed, the Δ*pccD* strain revealed a higher erythromycin yield than the WT strain (Fig. 6B and 6C).

222

Discussion

Common and distinctive features of propionyl-CoA assimilation in actinobacteria

Propionyl-CoA is generated from the β-oxidation of odd chain fatty acids, branched-chain fatty acids, as well as branched-chain amino acids and cholesterol. Propionyl-CoA is toxic if accumulated inside the cell, and thus propionyl-CoA metabolism needs to be tightly regulated to prevent its accumulation and alleviate toxicity [12-16]. In most microorganisms, propionyl-CoA levels are controlled via at least two known metabolic pathways: MCC and the PCC/methylmalonyl-CoA pathway. The propionyl-CoA assimilation and metabolism have been investigated only in a few actinobateria, such as *Mycobacterium tuberculosis* and *Corynebacterium glutamicum* [20-23]. Previous studies showed that *M. tuberculosis* contains both pathways of propionyl-CoA assimilation [24,25]. It was found that *S. erythraea* has no MCC for propionyl-CoA assimilation. Genome sequence analysis suggests that PCC pathway plays a role in propionyl-CoA assimilation in *S. erythraea*. Six genetic locus may encode biotin-dependent carboxylases catalyzing the carboxylation of propionyl-CoA to methylmalonyl-CoA for biosynthesis of erythromycin.

The transcriptional control of gene expression involved in propionyl-CoA assimilation has been investigated in *C. glutamicum* and *M. tuberculosis*. In *M. tuberculosis*, the transcriptional regulator, LrpG (also known as PrpR and Rv1129c), control the expression of genes encoding enzymes of MCC pathway [24,26,27]. In *C. glutamicum*, PrpR regulator activates the *prpDBC2* operon encoding the enzymes 2-methylcitrate dehydratase (PrpD2), 2-methylisocitrate lyase (PrpB2), and 2-methylcitrate synthase (PrpC2), and 2-methylcitrate probably acts as co-activator of PrpR [23,28]. The transcriptional regulation of the genes encoding enzymes of the PCC pathway in bacteria remains unknown. Recently, Alber group found that RSP_2186 controls the transcript of propionyl-CoA carboxylase in *Rhodobacter sphaeroides* (thus designated PccR) [19]. They defined a novel family of short-chain fatty acyl-CoA regulators (ScfRs)

that modulates pathways for short-chain acyl-CoA assimilation in microorganisms, including PccR (PCC pathway for propionyl-CoA assimilation), MccR (MCC for propionyl-CoA assimilation), RamB (glyoxylate bypass for acetyl-CoA assimilation), and IbcR (for isobutyryl-CoA assimilation) [19]. ScfRs typically belong to the xenobiotic response element (XRE) family with a helix-turn-helix N-terminal domain for DNA binding and a C-terminal domain of unknown function (DUF2083) as common features. In this study, we found that the TetR/AcrR family transcriptional regulator PccD negatively controlled propionyl-CoA assimilation. *S. erythraea* PccD is a novel ScfR, as it does not belong to the XRE family regulator described by Alber et al. [19].

BldD, a key developmental regulator in actinobacteria, was identified to directly activate the transcription of all genes involved in the biosynthesis of erythromycin [29]. More recently, it was found that two TetR family regulators (SACE_7301 and SACE_3986) were associated with biosynthesis of erythromycin in *S. erythraea* A226. SACE_7301 activated the transcription of erythromycin biosynthetic gene *eryAI* and the resistance gene *ermE* by interacting with their promoter regions [30]. SACE_3986 negatively controlled erythromycin biosynthesis by reducing the transcription of its adjacent gene SACE_3985 coding for a short-chain dehydrogenase/reductase [31]. Herein, PccD, a TetR family regulator, was found to negatively regulate erythromycin biosynthesis by repressing feeder pathways.

The important role of propionyl-CoA carboxylation for high production of erythromycin with n-propanol supplementation

In *S. erythraea*, branched-chain amino acids (BCAA) are a source of propionyl-CoA and methylmalonyl-CoA, key precursors in erythromycin biosynthesis. As shown in Figure 1A, two degradation pathways of valine maybe exist for the generation of methylmalonyl-CoA from valine: MPMS pathway and the predicted MMMS pathway [32,33]. Methylmalonyl-CoA is a limiting factor for erythromycin biosynthesis. In actinobacteria, at least four pathways have been found to generate methylmalonyl-CoA: the PCC pathway through carboxylation of propionyl-CoA by

280 propionyl-CoA carboxylase, the MCM pathway that catalyzes the reversible
281 isomerization of succinyl-CoA and methymalonyl-CoA by methylmalonyl-CoA mutase,
282 the CCR pathway through crotonyl-CoA reductase or isobutyryl-CoA mutase, and the
283 meaA pathway from acetoacetyl-CoA [8,17]. No CCR or meaA pathway is found in *S.*
284 *erythraea*, whereas PCC and MCM pathways play important roles in the precursor
285 supply under different conditions [6-8,17]. Reeves et al. have proposed a metabolic
286 model in which, in carbohydrate-based fermentations, MCM acts as a drain on the
287 methylmalonyl-CoA metabolite pool and, in oil-based fermentations, MCM acts in
288 the reverse direction to fill the methylmalonyl-CoA pool [7]. Engineering of the
289 methylmalonyl-CoA metabolic flux of *S. erythraea* through duplication of the
290 methylmalonyl-CoA mutase operon resulted in a 50% increase in erythromycin
291 production in an oil-based fermentation medium [7]. Under industrial medium
292 conditions, the increase in methylmalonyl-CoA pool is mainly attributed to the PCC
293 pathway from propionyl-CoA. Some genes involved in the branched-chain amino acid
294 degradation pathway, such as *ilvB* (SACE_4565), *acd* (SACE_4125 and SACE_5025),
295 and *mmsA* (SACE_4672), are significantly upregulated in industrial high-producing
296 strains. This result indicates that propionyl-CoA can be derived from valine, leucine,
297 and isoleucine degradation [9]. The addition of n-propanol in medium significantly
298 increases the propionyl-CoA pool as a result of propionate metabolism by
299 acetyl/propionyl-CoA synthetases. Therefore, PccD-regulated PCC-pathway for
300 propionyl-CoA carboxylation plays an important role in high erythromycin
301 production.

302 In conclusion, short-chain acyl-CoAs are intermediates and precursors for the
303 biosynthesis of many antibiotics. The regulation of their supplies is crucial for
304 increasing antibiotic yield. Our work has identified the novel regulator PccD that
305 controls the gene expression of propionyl-CoA carboxylase, which is responsible for
306 propionyl-CoA assimilation in *S. erythraea*. PccD represses the generation of
307 methylmalonyl-CoA through carboxylation of propionyl-CoA, and reveals an effect on
308 biosynthesis of erythromycin. The higher level of erythromycin in the Δ*pccD* strain

was observed compared with wild-type strain. Our study reveals a regulatory mechanism in propionate metabolism and suggests new possibilities for designing metabolic engineering to increase erythromycin yield.

Material and methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. erythraea* NRRL2338 was grown on R2YE agar plates for 5-6 days at 30°C for sporulation. An agar piece of about 1 cm^2 was inoculated into a 150-mL flask containing 30 mL of tryptone soya broth (TSB) medium and grown at 30°C and 200 rpm for 48 h for seed-stock preparation. Next, about 0.5-1mL of the seed culture (before inoculation, the seed was centrifuged at 2,880 × g for 10 min at 4°C to form a pellet and were resuspended and washed three times with the next corresponding medium) was added to a 500-mL flask containing 50 mL TSB (to make the initial OD_{600} was 0.05) or minimal Evans medium [34] (to make the initial OD_{600} was 0.1) supplemented with various carbon sources grown at 30°C and 200 rpm for genomic DNA extraction, phenotype, or transcription studies.

RNA preparation and qRT-PCR

S. erythraea wild-type (WT), *pccD* deficient strain (Δ*pccD*), and *pccD* overexpression (WT/PIB-*pccD*) strains were grown to late-exponential growth/early stationary phase at 30 °C in TSB liquid media, TSB supplemented with 1% (v/v) propanol, or 20 mM propionate, or 10 mM methylmalonic acid. The cells were harvested at 36 h by centrifugation at 4 °C, 1,500 × g for 10 min. Total RNA was prepared using the RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China). RNA integrity was analyzed by 1% agarose gel electrophoresis. RNA concentration were quantitated by SynergyMx multi-mode microplate reader (BioTek, Winooski, USA). DNase digestion was performed to remove genomic DNA before reverse transcription for 5 min at 42 °C. Total RNA (1 μg) was reverse transcribed using the PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan). qPCR was conducted using the SYBR Premix ExTaq GC kit (Takara, Japan), and about 50 ng cDNA was added to a final PCR reaction volume of 20 μL. PCR was performed using the primers (Table S1) with final concentration of 0.25 μM. PCR assays were carried out using a CFX96 Real-Time System (Bio-Rad, CA, USA), and the thermocycling conditions were as follow: 95 °C for 5 min, followed by

40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s, with a final extension cycle at 72 °C for 10 min. 16S_rRNA is used for reference gene. qRT-PCR validation information on amplification efficiency, calibration curves with slope and R^2, and specificity (melt) were shown in DATASET S1. Data analysis of qRT-PCR were shown in DATASET S2, DATASET S3, and DATASET S4. STDEV indicate standard deviation from three independent experiments.

Protein overexpression and purification

To produce the PccD protein, the *pccD* gene was amplified from *S. erythraea* NRRL2338 by PCR using *pccD*-F/-R primers (Table S1) and cloned into pET-19b, generating the recombinant plasmid pET-*pccD* (Table 1). The correct gene sequences were confirmed by sequencing analysis and the recombinant plasmids were introduced into *E. coli* Rosetta (DE3) competent cells. The recombinant cells selected by ampicillin were grown in 5 mL LB medium with 100 mg L^{-1} ampicillin in an orbital shaker (200 rpm, 37°C) for 12 hrs. Next, 2.5 mL of the seed culture was added to a 250-mL flask containing 50 mL TB medium (24 g L^{-1} yeast extract, 12 g $\cdot L^{-1}$ tryptone, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) followed by incubation at 20°C for 20–24 hrs. His_6-tagged PccD protein (His-PccD) was purified from Rosetta (DE3)-harboring pET-*pccD* as previously described [37]. Purity of the purified protein was checked by SDS-PAGE (Fig. S4). The fractions G, H, I were pooled and used in the assay. The protein concentration was determined using the Bradford assay.

Electrophoretic mobility shift assay (EMSA)

The putative promoter regions of the target genes were amplified by PCR using the primers listed in Table S1. PCR products were labeled with biotin using a universal biotinylated primer (5'biotin-AGCCAGTGGCGATAAG-3'). The biotin-labeled PCR products were purified using the PCR Purification kit (Shanghai Generay Biotech, China) as EMSA probes. EMSAs were carried out using a chemiluminescent EMSA kit (Beyotime Biotechnology, China), as previously described [37]. Biotin-labeled DNA probes were incubated with a gradient concentration of proteins at 25°C for 20 min. For control groups, unlabeled specific probe (200-fold) or non-specific competitor DNA (200-fold, sonicated salmon sperm DNA) was used. Samples were separated by

6% non-denaturing PAGE gels in ice-cold 0.5× Tris-borate-EDTA at 160 V, and bands were detected by BeyoECL Plus (Beyotime Biotechnology, China).

To verify the conserved PccD-binding motif, two short biotin-labeled dsDNA named M-*pccBC* and M-*pccA* containing PccD-binding site in the promoters of *pccBC* and *pccA* operons, respectively, were synthesized (Table S1) and incubated with purified recombinant His-tagged PccD. In EMSA), an excess of poly[d(I-C)] was added during incubation to avoid unspecific binding of the protein to the DNA.

Construction of the *pccD* in-frame deletion mutant and overexpression strain

For an insertional deletion of the *pccD* gene, a previously described homologous recombination strategy was used [35]. 1.5 kb DNA fragments upstream and downstream of *pccD* gene locus were amplified from *S. erythraea* NRRL2338 genomic DNA by PCR using the primer pair pUC3396upF/R, pUC3396dwF/R (Table S1). The PCR products were digested with HindIII/XbaI and KpnI/EcoRI, and subsequently inserted into the corresponding sites of pUC19-tsr, creating pUC-*pccD* knockout plasmids (Table 1). The thiostrepton-resistance cassette amplified from pUC-*pccD* knockout plasmids by PCR using the primer pair pUC3396upF/ pUC3396dwR was transferred into *S. erythraea* by polyethylene glycol (PEG)-mediated transformation. The mutants were selected by thiostrepton on R_3M agar plates. The selected mutants were verified by PCR and DNA sequencing (Fig. S6). Primers are listed in Table S1.

For overexpression of the mutants, the *pccD* gene was PCR-amplified with the primer pair 3396over-F/R (Table S1). The PCR products were then cloned into NdeI/NotI sites of pIB139, creating the pIB-*pccD* plasmid (Table 1). The overexpression plasmids were introduced into the *pccD* mutant strain (SeryΔ*pccD*) by PEG-mediated transformation. The desired overexpression strains were screened by apramycin resistance and confirmed by PCR.

Erythromycin assay

Turbidimetric method in microbiological assay of antibiotics was used to quantify the erythromycin levels as previously described [38], with modifications. Briefly, the supernatant (cultivated in TSB with 1% (v/v) n-propanol) were collected after culture for 84 hours of *S. erythraea* wild-type (WT), *pccD* deficient strain (Δ*pccD*) and diluted

401 by 40 times before used for test samples. *Staphylococcus aureus* were cultivated on
402 medium (peptone 6 g, pancreatic digest of casein 4 g, beef extract 1.5 g, yeast extract
403 3 g, glucose monohydrate 1 g, and agar 15 g per 1000 ml water) for 16-18 h at 37 °C
404 for use as the sensitive microorganism. 15 µL erythromycin standards (0.1 µg-0.8 µg)
405 or test samples were added to a 96-well plate. 135 µL medium (peptone 6 g, beef
406 extract 1.5 g, yeast extract 3 g, sodium chloride 3.5 g, glucose monohydrate 1 g,
407 dipotassium hydrogen phosphate 3.68 g, potassium dihydrogen phosphate 1.32 g,
408 per 1000 ml Water) containing a suspension of *Staphylococcus aureus* (to the final
409 OD for 0.05) was added to the wells. Formaldehyde was used as blank to set the
410 optical apparatus. The 96-well plate was placed in microplate plate incubator at 37 °C
411 for 3-4 h (OD_{600} was controlled between 0.1-0.5). The growth of the microorganisms
412 were stop by adding 7.5 µL of formaldehyde to each well. OD was measured by
413 SynergyMx multi-mode microplate reader (BioTek, Winooski, USA). Three
414 independent experiments was performed to calculate standard deviation. The
415 bioassay-based erythromycin analysis was also carried out as previously described
416 [30].

417 **Determination of intracellular propionyl-CoA and methylmalonyl-CoA**
418 **concentrations**

419 For determination of propionyl-CoA and methylmalonyl-CoA concentrations, CoA
420 compound extraction and chromatography were performed as previously described
421 [39]. Cells grown at 36 h in TSB medium with 1% (v/v) n-propanol were harvested by
422 centrifugation, washed twice with phosphate-buffered saline (PBS, pH 8.0), and lysed
423 in lysis buffer (10% trichloroacetic acid, 2 mM dithiothreitol). The cell lysates were
424 frozen and thawed out repeatedly with liquid nitrogen and ice water 2-3 times,
425 centrifuged at 4°C, 15000 × *g* for 10 min, and transferred to an equilibrated
426 solid-phase extraction column (Sep-Pak, 1 cc, 50 mg tC18; Waters, Milford, MA).
427 After samples were adsorbed, the columns were washed by 0.1% trifluoroacetic acid
428 (TFA) and eluted by 40% acetonitrile with 0.1% TFA. The eluate was dried by Speed
429 Vac (Thermo Fisher, Waltham, MA) and stored at -80 °C. For chromatography, the
430 two mobile-phase solvents used were buffer A (75 mM KH_2PO_4, pH 5.5) and buffer B

(800 mL of 75 mM KH_2PO_4, pH 5.0 mixed with 200 mL of 20% acetonitrile). The chromatographic separations were performed at room temperature at a flow rate of 0.8 mL /min^{-1} on an ODS-C18 column, and the samples was monitored at 254 nm. The mobile phase composition profile was divided into several linear gradient segments, with sequential segment end-points of 10 min (when buffer B reached 28% from 10%), 15 min (buffer B reached 30% from 28%), 25 min (buffer B 40% reached from 30%), 26 min (buffer B reached 42% from 40%), and 35 min (buffer B reached 54% from 42%), 36 min (buffer B was reduced from 54% to 10%), and after a 14-min constant buffer B level of 10%, data collection was stopped at 50 min. Three independent experiments was performed to calculate standard deviation.

DNase I footprinting assay

The promoter region of *pccB* (SACE_3398) was amplified by PCR with primers pMD18T-3398F/R (Table S1), and the amplicon was cloned into the T-vector pMD-18T (Takara). For preparation of fluorescent FAM-labeled probes, the promoter region of *pccB* was PCR-amplified from the obtained plasmids using primers of M13R-48 (FAM) and M13F-47. The FAM-labeled probes were purified using a QIAquick gel extraction kit (Qiagen) and quantified using a NanoDrop 2000C (Thermo Fisher). DNase I footprinting assays were performed as previously described [40]. For each assay, 500 ng of probes were incubated with different amounts of His-PccD in a total volume of 40 µL. After incubation for 30 min at 25°C, a 10 µL solution containing about 0.015-unit DNase I (Promega), and 100 nmol freshly prepared $CaCl_2$ was added and further incubated for 1 min at 25°C. The reaction was stopped by adding 140 µL DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). Samples were first extracted with phenol/chloroform and precipitated with ethanol, and the pellets were dissolved in 30 µL MiniQ water. The preparation of the DNA ladder, electrophoresis and data analysis were previously described [40], except that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Figure Legends

Figure 1. *pccBC* (SACE_3398–3399) and *pccA* (SACE_3400) operons are induced by propanol and propionate in *S. erythraea*. A. In *S. erythraea*, branched-chain amino acids (BCAA) are a source of propionyl-CoA and methylmalonyl-CoA, key precursors in erythromycin biosynthesis. Valine and isoleucine were metabolized to propionyl-CoA through the degradation pathways. Propanol and propionate are two important external sources of propionyl-CoA, especially in the production of erythromycin. The metabolism of propionyl-CoA to (2S)-methylmalonyl-CoA is via the PCC pathway in *S. erythraea*, which plays an important role in propionyl-CoA assimilation when n-propanol or valine is added for high production of erythromycin. **B.** Total RNAs of *S. erythraea* NRRL2338 were extracted after 36 h of growth (Fig. S3) in TSB medium (white bars), TSB supplied with 1 % (v/v) n-propanol (gray bars), and TSB supplied with 20 mM propionate (black bars). Relative transcript levels were normalized to the 16S rRNA. The transcription value of each gene in *S. erythraea* in TSB medium was arbitrarily normalized to 1. Error bars represent the standard deviations from three biological replicates. MmsA, methylmalonate-semialdehyde dehydrogenase (acylating); Mut, methylmalonyl-CoA mutase; Mce, methylmalonyl-CoA epimerase. Enzymes in the dotted box are based on KEGG database and the literature [32,33], but their functions in *S. erythraea* have not yet been investigated.

Figure 2. PccD (SACE_3396) binds to the promoter regions of *pccBC* and *pccA*. A. Genetic organization of *pccD* gene in the *S. erythraea* genome. *pccB* (SACE_3398), *pccC* (SACE_3399), and *pccA* (SACE_3400) encode a propionyl-CoA carboxylase β-chain, acetyl-CoA carboxylase probable ε-subunit and acetyl/propionyl-CoA carboxylase α–subunit, respectively. The numbers between dashed lines show the length of promoter regions of *pccBC* and *pccA* used gel shift experiment shown in panel B respectively. **B.** EMSAs of His-PccD protein with promoter regions (separated by dashed lines shown in panel A) of *pccBC* or *pccA*. The biotin labeled DNA probe (about 15 ng, reaction system was 10 μL) was incubated with a protein concentration gradient (0, 1, 3, and 5 μM). Unlabeled specific probe (200-fold) or non-specific competitor DNA (200-fold, sonicated salmon sperm DNA) was used as controls. The free probes that did not bind with protein are shown by arrowheads.

Figure 3. PccD is a repressor of *pccBC* and *pccA* operons. **A.** qPCR analysis of the relative transcription levels of *pccD*, *pccBC*, *pccA* genes between the WT, Δ*pccD* and WT/PIB-*pccD* strains. Total RNAs of these strains were extracted after 36 h of growth in TSB medium (Fig. S5A). The expression levels of genes in the WT strain were arbitrarily set to 1.0. Error bars indicate SD from three independent experiments. **B** and **C.** Growth curves of *S. erythraea* strains WT, Δ*pccD,* and WT/PIB-*pccD* grown on the Evans medium supplemented with 20 mM sodium propionate (B) or 2.5% glucose (C) as the sole carbon source. Error bars indicate standard deviation from three biological replicates.

Figure 4. Identification of a PccD-binding site in *pccBC* and *pccA* operons. A. Electropherograms of a DNase I digest of *pccBC* promoter probe incubated without (top) or with 2.0 μg (bottom) of His-PccD. The nucleotide sequence protected by His-PccD is indicated below. **B.** Protected sequence stretches in the upstream regions of *pccBC*. Black lines indicate the regions of DNase I protection. The -35 and -10 sites were predicted by softberry website (http://www.softberry.com/) **C.** Analysis of the PccD-binding motif of *pccA* using MEME. The

standard code of the Weblogo server is shown at the top. **D.** Verification of PccD-binding motif by EMSA. Synthetic probes M-*pccBC* and M-*pccA* containing PccD-binding site in promoters of *pccBC* and *pccA* operons respectively. The DNA probe was incubated with a protein concentration of 5 μM.

Figure 5. The metabolite methylmalonic acid is an inhibitor of PccD. A. EMSAs of His-PccD protein binding to the upstream promoter regions of *pccBC*. The biotin labeled DNA probe (about 15 ng, reaction system was 10 μL) was incubated with 5 μM His-PccD. Propionate, propionyl-CoA, methylmalonic acid, methylmalonyl-CoA or succinyl-CoA was added as a cofactor to a final concentration of 1.0 mM. **B.** Total RNAs of *S. erythraea* NRRL2338 were extracted after 36 h of growth in TSB medium or TSB supplied with 10 mM methylmalonic acid. Relative transcript levels were normalized to the 16S rRNA. The transcription value of *pccBC* in *S. erythraea* WT cultivated in TSB medium was arbitrarily normalized to 1. Error bars represent the standard deviations from three biological replicates.

Figure 6. Apparent increase of erythromycin production in the *pccD* deletion strain compared to wild type. A. Intracellular propionyl-CoA and methylmalonyl-CoA concentration of *S. erythraea* WT and Δ*pccD* strains grown in TSB medium with 1% (v/v) n-propanol. Cells were harvested at 36 h (Fig. S5B). **B.** Inhibition tests of *S. erythraea* WT and Δ*pccD* fermentation broths, collected after culturing for 84 hours, against *Bacillus subtilis*. **C.** Erythromycin concentration of *S. erythraea* WT and Δ*pccD* strains grown in TSB medium with 1% (v/v) n-propanol. Supernatants were collected after culture for 84 hours. Turbidimetric method in microbiological assay of antibiotics was used to quantify the erythromycin levels as described in material and methods. Three independent experiments were performed to calculate standard deviation.

654 **Table 1.** Bacterial strains and plasmids used in this work

Strains or plasmids	Characteristics	Source or reference
Strains		
S. erythraea NRRL2338	Used as parental strain, wild type	DSM 40517
Δ*pccD*	*S. erythraea pccD* null mutant, thiostrepton resistance	This work
WT/PIB-*pccD*	*pccD* overexpression strain, WT carrying pIB-*pccD*	This work
Rosetta (DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* λ(DE3)pRARE^2(Cam^R)	Novagen
Plasmids		
pET19b	Expression vector, Amp^r	Novagen
pET-*pccD*	pET19b derivative carrying *pccD*	This work
pMD-18T	TA-cloning vector	Takara
pUC19-tsr	pUC18 derivative containing a 1.36 kb fragment of a thiostrepton resistance cassette in the BamHI/SmaI sites	[35]
pUC-*pccD*	pUC19-tsr, with the 1.5 kb DNA fragments upstream and downstream of *pccD* gene inserted upstream and downstream of tsr correspondingly	This work
pIB139	*E. coli–S. erythraea* integrative shuttle vector containing a strong constitutive *ermE** promoter, apramycin resistance	[36]
pIB-*pccD*	pIB139 carrying an extra *pccD* for the gene overexpression	This work

655