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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 ($\Delta pccD$) and downregulated 3-fold in the *pccD* overexpression strain (WT/pIB-*pccD*), indicating that PccD was a negative transcriptional regulator of SACE_3398-3400. $\Delta pccD$ 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 $\Delta pccD$ 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.

46

47 **Importance:**

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

55

56 Introduction

57 *Saccharopolyspora erythraea*, a Gram-positive filamentous soil bacterium, is used for
58 production of the antibiotic erythromycin. Erythromycin and its derivatives play a
59 vital role in medicine, with annual sales reaching several billions of dollars annually
60 [1]. Historically, multiple rounds of random mutagenesis and selection have been
61 used to obtain overproducing mutants for industrial production. Nowadays, genetic
62 engineering of the pathways involved in biosynthesis of secondary metabolites also
63 holds promises to enhance production [2-4].

64 Production of one unit of erythromycin requires one propionyl coenzyme A
65 (propionyl-CoA) to provide a starter unit and six (2S)-methylmalonyl-CoA to provide
66 extender units to form 6-deoxyerythronolide B (6-dEB) [5]. Reeves' experiments
67 indicated that methylmalonyl-CoA was a limiting factor for erythromycin biosynthesis
68 [6]. Engineering of the methylmalonyl-CoA metabolic flux of *S. erythraea* through
69 knockout of the methylmalonyl-CoA mutase operon resulted in a 50% increase in
70 erythromycin production in an oil-based fermentation medium [7]. Integrating
71 genomics, transcriptomics, and proteomics comparison between the wild-type strain
72 and the industrial high-producing strain of *S. erythraea* revealed that the industrial
73 strain has a high flux of metabolites towards erythromycin biosynthesis and
74 degradation pathway of branched-chain amino acids [8]. Because branched-chain
75 amino acids are a source of methylmalonyl-CoA, the significantly higher expression
76 levels of *ilvB* (acetolactate synthase large subunit, SACE_4565), *acd* (acyl-CoA
77 dehydrogenase, SACE_4125 and SACE_5025), and *mmsA*
78 (methylmalonate-semialdehyde dehydrogenase, SACE_4672) in the high-producing
79 strain may lead to an increased supply of propionyl-CoA and methylmalonyl-CoA, key
80 precursors for erythromycin synthesis. This is also supported by an increased
81 erythromycin yield when different genes of the branched-chain amino acid
82 metabolism were overexpressed [9]. The quantitative analysis of metabolic carbon
83 flux indicated that high consumption rate of propanol increased propionyl-CoA and
84 methylmalonyl-CoA concentrations. The addition of n-propanol at a concentration of

85 1% (v/v) increased the erythromycin production by 20% [10]. Analysis of [¹⁸O]
86 n-propanol metabolic carbon flux suggested that the oxygen atoms of 6-dEB are
87 largely derived from n-propanol [11]. Propanol is converted into propionate, which is
88 subsequently metabolized to propionyl-phosphate or propionyl-CoA by propionate
89 kinase or acetyl/propionyl-CoA synthetase. However, propionyl-CoA is an inhibitor of
90 CoA-dependent enzymes such as pyruvate dehydrogenase, succinyl-CoA synthetase,
91 and ATP citrate lyase [12-16]. Therefore, the assimilation of propionyl-CoA needs to
92 be precisely regulated to prevent its accumulation within the cell.

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

106

107 Results

108 The *pccBC* and *pccA* operons are induced by propanol and propionate in *S.* 109 *erythraea*

110 It was reported that propionyl-CoA carboxylases were upregulated when propanol or
111 propionate was added to microorganisms [18,19]. Propanol is converted into
112 propionate, which is subsequently metabolized to propionyl-phosphate or
113 propionyl-CoA by propionate kinase or acetyl/propionyl-CoA synthetase (Fig. 1A). The
114 quantitative analysis of metabolic carbon flux indicated that a high consumption rate
115 of propanol increased intracellular concentration of propionyl-CoA and
116 methylmalonyl-CoA [11] to enhance the biosynthesis of erythromycin. In the genome
117 of *S. erythraea*, at least six possible acetyl-CoA/propionyl-CoA carboxylases are
118 annotated, namely SACE_0026–0028, SACE_3241–3242, SACE_3398–3400,
119 SACE_7038–7039, SACE_4237, and SACE_6509 (Fig. S1). The co-transcription of
120 genes in each locus was investigated (Fig. S2). We found that gene *pccB* (SACE_3398)
121 was cotranscribed with the *pccC* (SACE_3399) (form an operon: *pccBC*), and wasn't
122 cotranscribed with the *pccA* (SACE_3400). So far, it is still unclear which of the
123 possible propionyl-CoA carboxylases make a contribution to erythromycin
124 biosynthesis. To investigate which genetic locus were responsive to the addition of
125 n-propanol or propionate, transcript levels of all locus encoding
126 acetyl-CoA/propionyl-CoA carboxylases were examined using qRT-PCR (Fig. 1B). The
127 results showed that transcriptional levels of *pccBC* operon and *pccA* gene increased
128 4- or 14-fold and 5- or 16-fold, respectively, by addition of propanol or propionate.
129 No changes were observed in transcriptional levels of the other locus coding for
130 acetyl-CoA/propionyl-CoA carboxylases (Fig. 1B). *pccB* encodes a β -subunit (PccB)
131 that has a carboxyl transferase (CT) activity, and *pccA* codes for a larger α -subunit
132 (PccA) comprising the biotin carboxylase (BC) and biotin carboxy carrier protein
133 (BCCP). *pccC* encodes the small ϵ -subunit [17]. These results indicate that *pccBC* and
134 *pccA* operons perform a major function in propionate metabolism and propionyl-CoA
135 assimilation.

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

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

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

147 To further investigate the regulatory effect of PccD on the *pccBC* and *pccA* operons,
148 we constructed the *pccD*-deleted mutant ($\Delta pccD$) and *pccD*-overexpressed strain
149 (WT/PIB-*pccD*), and performed transcriptional analysis by qRT-PCR of wild-type (WT),
150 $\Delta pccD$ and WT/PIB-*pccD* strains. These strains were cultivated in TSB medium, and
151 RNA was extracted at the late exponential growth/early stationary phase. Results
152 show that deletion of *pccD* resulted in a 15-fold (for *pccA*) and 37-fold (for *pccBC*)
153 upregulation of the *pccA* and *pccBC* operons, and overexpression (approximately 5
154 folds) of *pccD* inhibited the expression of two operons by 3-fold as compared to that
155 in the WT strain (Fig. 3A). Next, we examined the growth of WT, $\Delta pccD$, and
156 WT/PIB-*pccD* strains cultivated in Evans media with 20 mM sodium propionate as the
157 sole carbon source. As shown in Figure 3B, differences in growth behavior were
158 observed among these strains. On one hand, the deletion of *pccD* significantly
159 promoted propionate metabolism and propionyl-CoA assimilation by inducing the
160 transcription of *pccBC* and *pccA* operons, and increased the growth rate of *S.*
161 *erythraea* on propionate medium (Fig. S5). On the other hand, the WT/PIB-*pccD*
162 strain overexpressing *pccD* revealed a decrease in growth rate on propionate
163 compared to the wild-type strain due to the low levels of *pccBCA* transcripts (Fig. 3A).
164 Meanwhile, the WT/PIB-*pccD* strain showed a decrease in growth yield probably

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

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

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

188 The metabolite methylmalonic acid is an effector of PccD

189 TetR/AcrR family transcriptional repressors are homodimeric DNA-binding proteins.
190 Their DNA-binding activity is allosterically inactivated by the binding of
191 small-molecule ligands (effectors). PccD protein from *S. erythraea* contains two TetR
192 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*.

$\Delta pccD$ 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 $\Delta pccD$ 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 $\Delta pccD$ strain will facilitate the biosynthesis of erythromycin. Indeed, the $\Delta pccD$ strain revealed a higher erythromycin yield than the WT strain (Fig. 6B and 6C).

223 Discussion

224 Common and distinctive features of propionyl-CoA assimilation in actinobacteria

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

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

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

261 BldD, a key developmental regulator in actinobacteria, was identified to directly
 262 activate the transcription of all genes involved in the biosynthesis of erythromycin
 263 [29]. More recently, it was found that two TetR family regulators (SACE_7301 and
 264 SACE_3986) were associated with biosynthesis of erythromycin in *S. erythraea* A226.
 265 SACE_7301 activated the transcription of erythromycin biosynthetic gene *eryAI* and
 266 the resistance gene *ermE* by interacting with their promoter regions [30]. SACE_3986
 267 negatively controlled erythromycin biosynthesis by reducing the transcription of its
 268 adjacent gene SACE_3985 coding for a short-chain dehydrogenase/reductase [31].
 269 Herein, PccD, a TetR family regulator, was found to negatively regulate erythromycin
 270 biosynthesis by repressing feeder pathways.

271 **The important role of propionyl-CoA carboxylation for high production of** 272 **erythromycin with n-propanol supplementation**

273 In *S. erythraea*, branched-chain amino acids (BCAA) are a source of propionyl-CoA
 274 and methylmalonyl-CoA, key precursors in erythromycin biosynthesis. As shown in
 275 Figure 1A, two degradation pathways of valine maybe exist for the generation of
 276 methylmalonyl-CoA from valine: MPMS pathway and the predicted MMMS pathway
 277 [32,33]. Methylmalonyl-CoA is a limiting factor for erythromycin biosynthesis. In
 278 actinobacteria, at least four pathways have been found to generate
 279 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 methylmalonyl-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 $\Delta pccD$ strain

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

312 **Material and methods**

313 **Bacterial strains, plasmids, and growth conditions**

314 Bacterial strains and plasmids used in this study are listed in [Table 1](#). *S. erythraea*
315 NRRL2338 was grown on R2YE agar plates for 5-6 days at 30°C for sporulation. An
316 agar piece of about 1 cm² was inoculated into a 150-mL flask containing 30 mL of
317 tryptone soya broth (TSB) medium and grown at 30°C and 200 rpm for 48 h for
318 seed-stock preparation. Next, about 0.5-1mL of the seed culture (before inoculation,
319 the seed was centrifuged at 2,880 × g for 10 min at 4°C to form a pellet and were
320 resuspended and washed three times with the next corresponding medium) was
321 added to a 500-mL flask containing 50 mL TSB (to make the initial OD₆₀₀ was 0.05) or
322 minimal Evans medium [34] (to make the initial OD₆₀₀ was 0.1) supplemented with
323 various carbon sources grown at 30°C and 200 rpm for genomic DNA extraction,
324 phenotype, or transcription studies.

325 **RNA preparation and qRT-PCR**

326 *S. erythraea* wild-type (WT), *pccD* deficient strain ($\Delta pccD$), and *pccD* overexpression
327 (WT/PIB-*pccD*) strains were grown to late-exponential growth/early stationary phase
328 at 30 °C in TSB liquid media, TSB supplemented with 1% (v/v) propanol, or 20 mM
329 propionate, or 10 mM methylmalonic acid. The cells were harvested at 36 h by
330 centrifugation at 4 °C, 1,500 × g for 10 min. Total RNA was prepared using the
331 RNeasy Pure Cell/Bacteria Kit (Qiagen Biotech, Beijing, China). RNA integrity was
332 analyzed by 1% agarose gel electrophoresis. RNA concentration were quantitated by
333 SynergyMx multi-mode microplate reader (BioTek, Winooski, USA). DNase digestion
334 was performed to remove genomic DNA before reverse transcription for 5 min at
335 42 °C. Total RNA (1 µg) was reverse transcribed using the PrimeScript RT Reagent Kit
336 (Takara, Kusatsu, Japan). qPCR was conducted using the SYBR Premix ExTaq GC kit
337 (Takara, Japan), and about 50 ng cDNA was added to a final PCR reaction volume of
338 20 µL. PCR was performed using the primers ([Table S1](#)) with final concentration of
339 0.25 µM. PCR assays were carried out using a CFX96 Real-Time System (Bio-Rad, CA,
340 USA), and the thermocycling conditions were as follow: 95 °C for 5 min, followed by

341 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s, with a final extension cycle
342 at 72 °C for 10 min. 16S_rRNA is used for reference gene. qRT-PCR validation
343 information on amplification efficiency, calibration curves with slope and R^2 , and
344 specificity (melt) were shown in DATASET S1. Data analysis of qRT-PCR were shown in
345 DATASET S2, DATASET S3, and DATASET S4. STDEV indicate standard deviation from
346 three independent experiments.

347 **Protein overexpression and purification**

348 To produce the PccD protein, the *pccD* gene was amplified from *S. erythraea*
349 NRRL2338 by PCR using *pccD*-F/-R primers (Table S1) and cloned into pET-19b,
350 generating the recombinant plasmid pET-*pccD* (Table 1). The correct gene sequences
351 were confirmed by sequencing analysis and the recombinant plasmids were
352 introduced into *E. coli* Rosetta (DE3) competent cells. The recombinant cells selected
353 by ampicillin were grown in 5 mL LB medium with 100 mg L⁻¹ ampicillin in an orbital
354 shaker (200 rpm, 37°C) for 12 hrs. Next, 2.5 mL of the seed culture was added to a
355 250-mL flask containing 50 mL TB medium (24 g L⁻¹ yeast extract, 12 g · L⁻¹ tryptone,
356 0.4% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) followed by incubation at 20°C for 20–
357 24 hrs. His₆-tagged PccD protein (His-PccD) was purified from Rosetta
358 (DE3)-harboring pET-*pccD* as previously described [37]. Purity of the purified protein
359 was checked by SDS-PAGE (Fig. S4). The fractions G, H, I were pooled and used in the
360 assay. The protein concentration was determined using the Bradford assay.

361 **Electrophoretic mobility shift assay (EMSA)**

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

371 6% non-denaturing PAGE gels in ice-cold 0.5× Tris-borate-EDTA at 160 V, and bands
372 were detected by BeyoECL Plus (Beyotime Biotechnology, China).

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

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

379 For an insertional deletion of the *pccD* gene, a previously described homologous
380 recombination strategy was used [35]. 1.5 kb DNA fragments upstream and
381 downstream of *pccD* gene locus were amplified from *S. erythraea* NRRL2338 genomic
382 DNA by PCR using the primer pair pUC3396upF/R, pUC3396dwF/R (Table S1). The
383 PCR products were digested with HindIII/XbaI and KpnI/EcoRI, and subsequently
384 inserted into the corresponding sites of pUC19-*tsr*, creating pUC-*pccD* knockout
385 plasmids (Table 1). The thiostrepton-resistance cassette amplified from pUC-*pccD*
386 knockout plasmids by PCR using the primer pair pUC3396upF/ pUC3396dwR was
387 transferred into *S. erythraea* by polyethylene glycol (PEG)-mediated transformation.
388 The mutants were selected by thiostrepton on R₃M agar plates. The selected mutants
389 were verified by PCR and DNA sequencing (Fig. S6). Primers are listed in Table S1.

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

396 Erythromycin assay

397 Turbidimetric method in microbiological assay of antibiotics was used to quantify the
398 erythromycin levels as previously described [38], with modifications. Briefly, the
399 supernatant (cultivated in TSB with 1% (v/v) n-propanol) were collected after culture
400 for 84 hours of *S. erythraea* wild-type (WT), *pccD* deficient strain (*ΔpccD*) 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 (OD₆₀₀ 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 KH₂PO₄, pH 5.5) and buffer B

431 (800 mL of 75 mM KH₂PO₄, pH 5.0 mixed with 200 mL of 20% acetonitrile). The
432 chromatographic separations were performed at room temperature at a flow rate of
433 0.8 mL /min⁻¹ on an ODS-C18 column, and the samples was monitored at 254 nm.
434 The mobile phase composition profile was divided into several linear gradient
435 segments, with sequential segment end-points of 10 min (when buffer B reached 28%
436 from 10%), 15 min (buffer B reached 30% from 28%), 25 min (buffer B 40% reached
437 from 30%), 26 min (buffer B reached 42% from 40%), and 35 min (buffer B reached
438 54% from 42%), 36 min (buffer B was reduced from 54% to 10%), and after a 14-min
439 constant buffer B level of 10%, data collection was stopped at 50 min. Three
440 independent experiments was performed to calculate standard deviation.

441 **DNase I footprinting assay**

442 The promoter region of *pccB* (SACE_3398) was amplified by PCR with primers
443 pMD18T-3398F/R (Table S1), and the amplicon was cloned into the T-vector
444 pMD-18T (Takara). For preparation of fluorescent FAM-labeled probes, the promoter
445 region of *pccB* was PCR-amplified from the obtained plasmids using primers of
446 M13R-48 (FAM) and M13F-47. The FAM-labeled probes were purified using a
447 QIAquick gel extraction kit (Qiagen) and quantified using a NanoDrop 2000C (Thermo
448 Fisher). DNase I footprinting assays were performed as previously described [40]. For
449 each assay, 500 ng of probes were incubated with different amounts of His-PccD in a
450 total volume of 40 µL. After incubation for 30 min at 25°C, a 10 µL solution containing
451 about 0.015-unit DNase I (Promega), and 100 nmol freshly prepared CaCl₂ was added
452 and further incubated for 1 min at 25°C. The reaction was stopped by adding 140 µL
453 DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15%
454 SDS). Samples were first extracted with phenol/chloroform and precipitated with
455 ethanol, and the pellets were dissolved in 30 µL MiniQ water. The preparation of the
456 DNA ladder, electrophoresis and data analysis were previously described [40], except
457 that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

458

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461 National Key Technologies R&D Programs (2014AA021502). The authors have no
462 conflict of interest to declare.

463 **Supporting information**

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

466

467 **References**

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589 Figure Legends

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

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

617 **Figure 3. PccD is a repressor of *pccBC* and *pccA* operons. A.** qPCR analysis of the relative
618 transcription levels of *pccD*, *pccBC*, *pccA* genes between the WT, $\Delta pccD$ and WT/PIB-*pccD* strains.
619 Total RNAs of these strains were extracted after 36 h of growth in TSB medium (Fig. S5A). The
620 expression levels of genes in the WT strain were arbitrarily set to 1.0. Error bars indicate SD from
621 three independent experiments. **B** and **C.** Growth curves of *S. erythraea* strains WT, $\Delta pccD$, and
622 WT/PIB-*pccD* grown on the Evans medium supplemented with 20 mM sodium propionate (B) or
623 2.5% glucose (C) as the sole carbon source. Error bars indicate standard deviation from three
624 biological replicates.

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

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

635 **Figure 5. The metabolite methylmalonic acid is an inhibitor of PccD.** **A.** EMSAs of His-PccD
636 protein binding to the upstream promoter regions of *pccBC*. The biotin labeled DNA probe (about
637 15 ng, reaction system was 10 μ L) was incubated with 5 μ M His-PccD. Propionate, propionyl-CoA,
638 methylmalonic acid, methylmalonyl-CoA or succinyl-CoA was added as a cofactor to a final
639 concentration of 1.0 mM. **B.** Total RNAs of *S. erythraea* NRRL2338 were extracted after 36 h of
640 growth in TSB medium or TSB supplied with 10 mM methylmalonic acid. Relative transcript levels
641 were normalized to the 16S rRNA. The transcription value of *pccBC* in *S. erythraea* WT cultivated
642 in TSB medium was arbitrarily normalized to 1. Error bars represent the standard deviations from
643 three biological replicates.

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

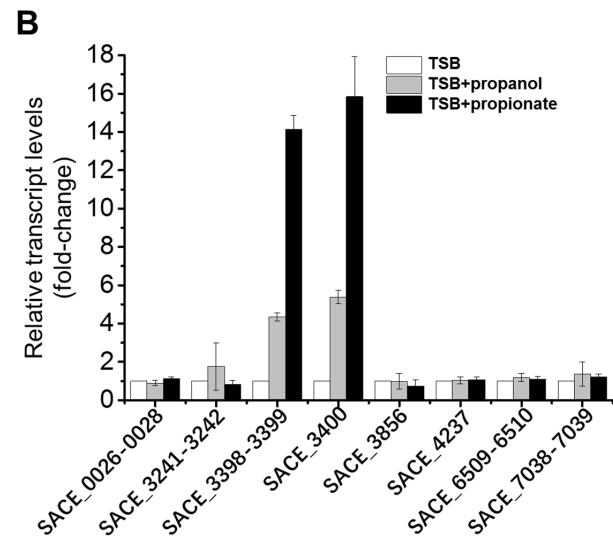
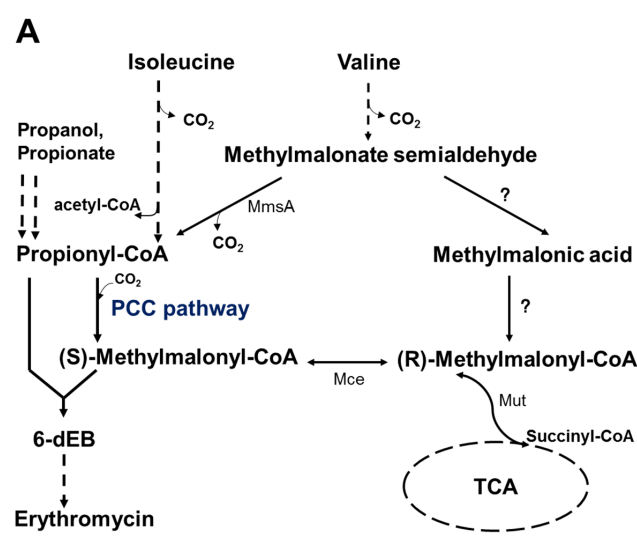
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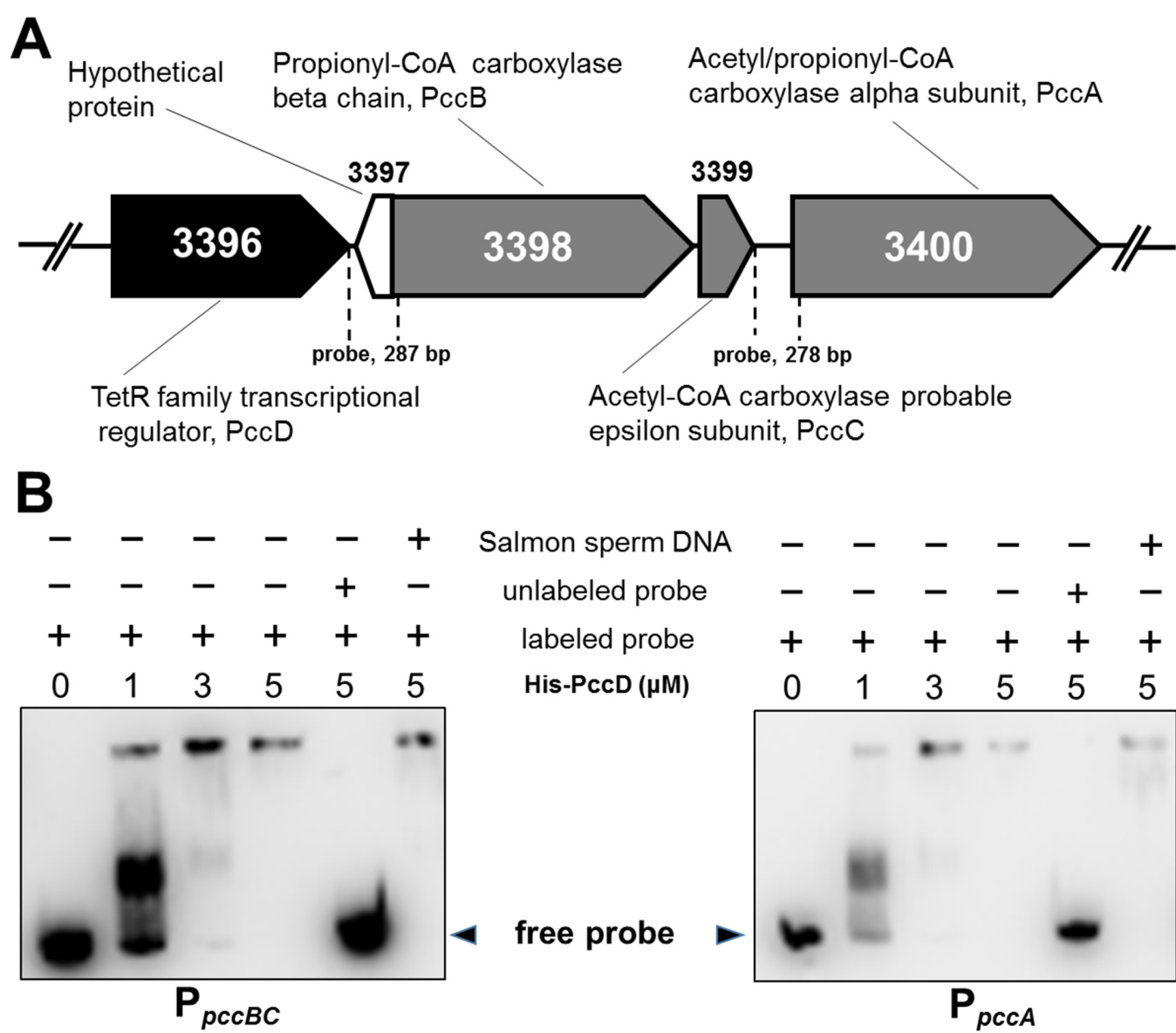
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Table 1. Bacterial strains and plasmids used in this work

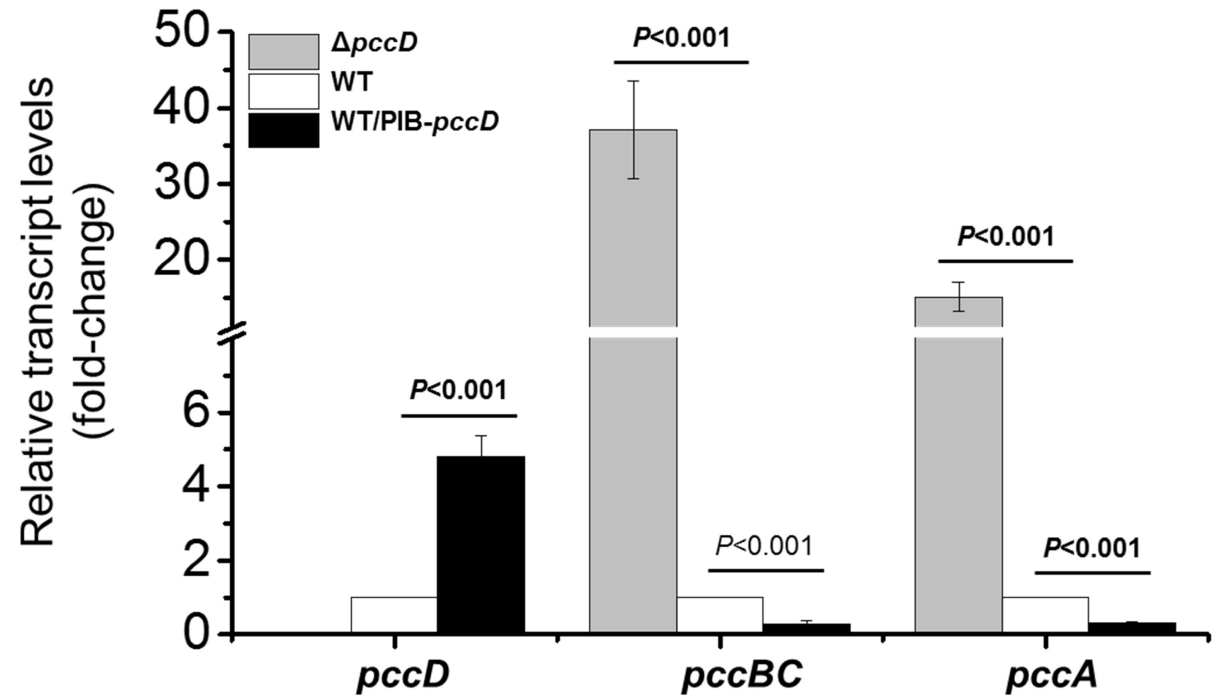
Strains or plasmids	Characteristics	Source or reference
Strains		
<i>S. erythraea</i> NRRL2338	Used as parental strain, wild type	DSM 40517
$\Delta pccD$	<i>S. erythraea pccD</i> null mutant, thiostrepton resistance	This work
WT/PIB- <i>pccD</i>	<i>pccD</i> overexpression strain, WT carrying PIB- <i>pccD</i>	This work
Rosetta (DE3)	$F^- ompT hsdS_B(r_B^- m_B^-) gal dcm \lambda(DE3)pRARE^2(Cam^R)$	Novagen
Plasmids		
pET19b	Expression vector, Amp ^r	Novagen
pET- <i>pccD</i>	pET19b derivative carrying <i>pccD</i>	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- <i>pccD</i>	pUC19-tsr, with the 1.5 kb DNA fragments upstream and downstream of <i>pccD</i> gene inserted upstream and downstream of tsr correspondingly	This work
plB139	<i>E. coli</i> - <i>S. erythraea</i> integrative shuttle vector containing a strong constitutive <i>ermE</i> * promoter, apramycin resistance	[36]
plB- <i>pccD</i>	plB139 carrying an extra <i>pccD</i> for the gene overexpression	This work

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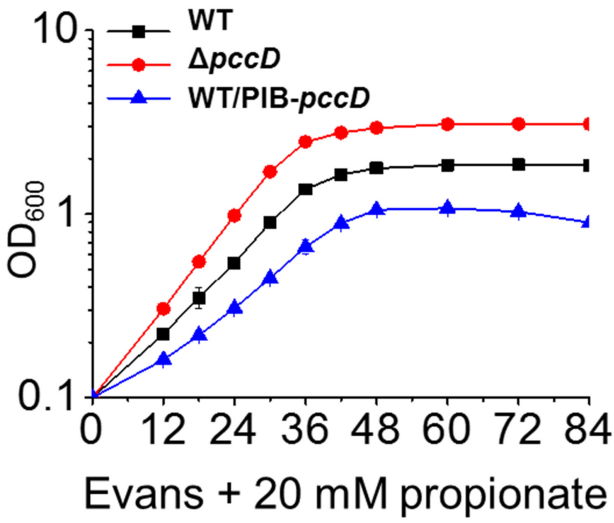




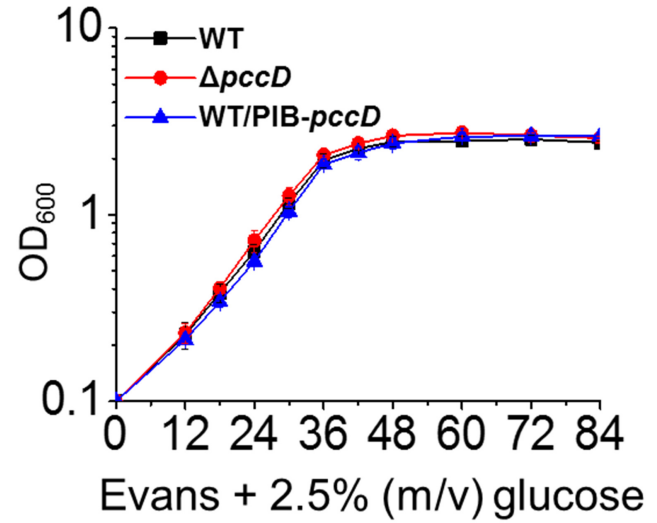
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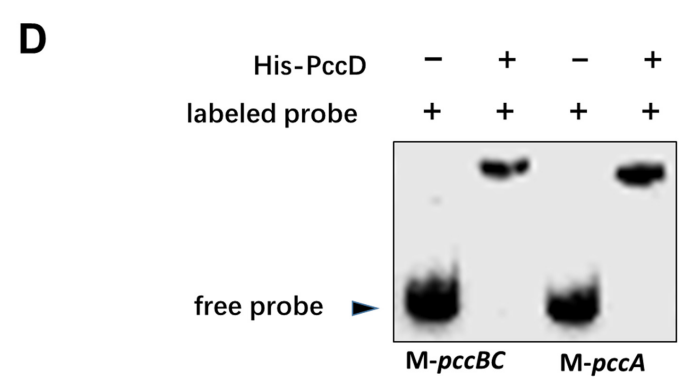
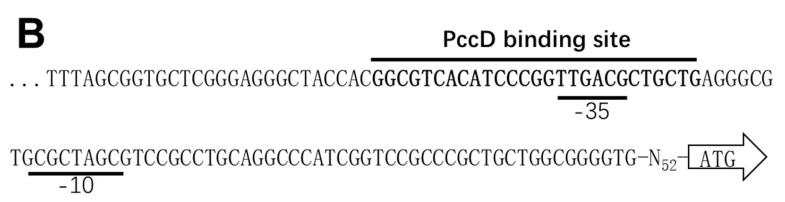
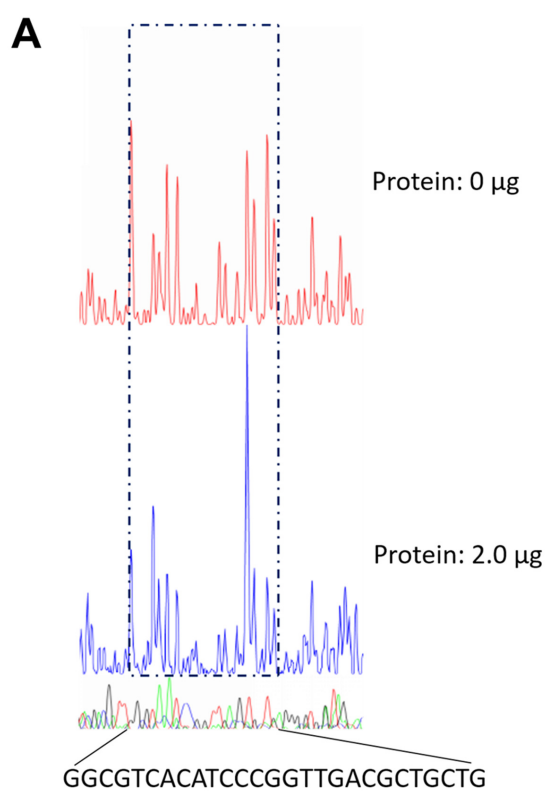


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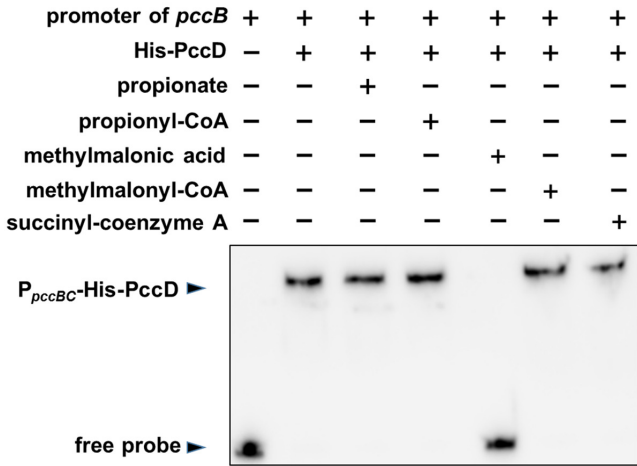


C





A



B

