1 The roles of diol dehydratase from *pdu* operon on glycerol catabolism in *Klebsiella pneumoniae*

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18 **Abstract**

19 The *dha* operon of *Klebsiella pneumoniae* is responsible for glycerol catabolism and 1,3-propanediol
20 formation. Subunits of glycerol dehydratase and the large subunit of glycerol dehydratase reactivating
21 factor are encoded by *dhaBCE* and *dhaF*, respectively. Proteins of *pdu* operon form a microcompartment
22 (bacteria organelle) and responsible for 1,2-propanediol catabolism. In this operon, *pduCDE* and *pduG*
23 encode subunits of diol dehydratase and its reactivating factor. Diol dehydratase is an isofunctional
24 enzyme of glycerol dehydratase, but its role in glycerol catabolism was not entirely clear. In this study,
25 *dhaBCE*, *pduCDE*, *dhaF*, and *pduG* in *K. pneumoniae* were knocked out individually or combinedly.
26 These strains were cultured with glycerol as a substrate, and dehydratase activities in the cytoplasm and
27 microcompartment were detected. Results showed that glycerol dehydratase and diol dehydratase were
28 simultaneously responsible for glycerol catabolism in *K. pneumoniae*. Besides being packaged in
29 microcompartment, large amounts of diol dehydratase was also presented in the cytoplasm. However,
30 the Pdu microcompartment reduced the accumulation of 3-hydroxypropionaldehyde in the fermentation
31 broth. PduG can cross reactivate glycerol dehydratase instead of DhaF. However, DhaF is not involved
32 in reactivation of diol dehydratase. In conclusion, diol dehydratase and Pdu microcompartment play
33 important roles in glycerol catabolism in *K. pneumoniae*.

34

35 **Key words:** Glycerol dehydratase; diol dehydratase; microcompartment; 1,3-propanediol; *Klebsiella*
36 *pneumoniae*

37

38 **1. Introduction**

39 *Klebsiella pneumoniae* is an important industrial microorganism. It is a native 1,3-propanediol producer
40 with high efficiency [1, 2]. 1,3-Propanediol is an important chemical and a monomer in the synthesis of
41 polyesters for use in carpet and textile fibers [3]. Glycerol is used as a starting material for the production
42 of 1,3-propanediol. The glycerol catabolism and 1,3-propanediol synthesis is a dismutation pathway. In
43 the oxidative branch, glycerol is oxidized to dihydroxyacetone under the catalysis of glycerol
44 dehydrogenase, and dihydroxyacetone is then phosphorylated to dihydroxyacetone phosphate and
45 directed into glycolysis. 2,3-Butanediol, lactic acid, ethanol, acetic acid and succinic acid are synthesized
46 in this oxidative branch. In a coupled reductive pathway, glycerol is converted to 3-
47 hydroxypropionaldehyde with the catalysis of glycerol dehydratase. 3-Hydroxypropionaldehyde is
48 further reduced to 1,3-propanediol and this reaction is catalyzed by 1,3-propanediol oxidoreductase.
49 Genes encoding these enzymes are located in the *dha* regulon, which is induced by dihydroxyacetone
50 [4]. Glycerol dehydratase contains three subunits, and uses adenosylcobalamin (vitamin B12) as a
51 coenzyme. The large, medium, and small subunits of glycerol dehydratase are encoded by *dhaBCE*
52 (*dhaB123*), respectively [5]. *dhaF* (*gdrA*) and *dhaG* (*gdrB*) encode the large and small subunits of
53 glycerol dehydratases reactivating factor. Subunits of reactivating factor form a tight complex whose
54 subunit structure is $DhaF_2DhaG_2$.

55 In addition to glycerol dehydratase, a diol dehydratase has also been found in some strains of *K.*
56 *pneumoniae* [6]. Subunits of diol dehydratase and its reactivating factor are encoded by *pduCDEGH*, and
57 these genes are arranged continuously in the *pdu* operon. The *pdu* operon is responsible for 1,2-
58 propanediol catabolism, and has been studied in detail in *Salmonella enterica*. Similar to the pathway of
59 glycerol catabolism. This metabolic pathway begins with the conversion of 1,2-propanediol to
60 propionaldehyde by diol dehydratase. Subsequently, propionaldehyde is converted to propionyl-CoA or
61 1-propanol [7]. The expression of the *pdu* operon in *S. enterica* was induced by the presence of 1,2-
62 propanediol, but not by other carbon sources [8].

63 Glycerol and diol dehydratases are isofunctional enzymes, but the former prefers glycerol over 1,2-
64 propanediol, while the latter prefers 1,2-propanediol over glycerol as a substrate [9]. Both glycerol
65 dehydratase and diol dehydratase have high initial velocities with glycerol as substrate. These enzymes
66 are simultaneously irreversibly inactivated by glycerol during catalysis. This inactivation is due to a
67 mechanism, in which the coenzyme is modified by irreversible cleavage of its Co-C bond, leading to
68 inactivation of the enzyme by tight binding of the modified coenzyme to the active site [10]. The

69 reactivating factor reactivates the inactivated holoenzyme in the presence of ATP and Mg^{2+} by mediating
70 the exchange of tightly bound damaged cofactor for free intact coenzyme [11].
71 Bacterial microcompartments (MCPs) contain a set of metabolic enzymes encapsulated within a
72 protein shell [12]. Carboxysomes were the first bacterial MCP to be discovered. They were observed in
73 the cytoplasm of *cyanobacteria* more than 50 years ago. In the early years, however, they were mistaken
74 for viruses [13]. In the investigation of the *pdu* operon, several proteins were found in this operon that
75 are homologous to the structural proteins of the carboxysome. Finally, the MCP responsible for 1,2-
76 propanediol catabolism was uncovered and termed the "microcompartment" [8]. MCPs use their protein
77 shell as a diffusion barrier to help channel toxic or volatile intermediates to internal metabolic pathways.
78 Propionaldehyde, formed in the 1,2-propanediol catabolism pathway, is a toxic chemical. The Pdu MCP
79 consists of catabolic enzymes encased in a protein shell, and its function is to sequester propionaldehyde
80 [14]. The 18 N-terminal amino acids of PduD have been identified be a single peptide that guild
81 packaging diol dehydratase into the lumen of Pdu MCP [15].
82 *K. pneumoniae* CGMCC 1.6366 was a strain isolated for 1,3-propanediol production [1]. The *pdu*
83 operon was found in the genome of this strain. Genes encoding glycerol dehydratase and diol dehydratase
84 were located in different operons and the expression of the two operons were regulated in different ways.
85 Diol dehydratase was packaged in the lumen of Pdu MCP in *S. enterica*. Glycerol dehydratase was a
86 cytoplasmic enzyme. The relationship between glycerol dehydratase and diol dehydratase seems to be
87 interesting, and it has been supposed that diol dehydratase is involved in glycerol catabolism in *K.*
88 *pneumoniae*. The aim of this study is to determine the role of diol dehydratase and the Pdu MCP in
89 glycerol catabolism in *K. pneumoniae* (Fig. 1).
90

91 ## 2. Materials and methods

92 ### 2.1 Strains, plasmids, and primers

93 Bacterial strains and plasmids used in this study are listed in Table 1. Primers used for PCR are listed in
94 Table S1.

95 ### 2.2 Construction of mutants of *K. pneumoniae*

96 For mutant constructions, *K. pneumoniae* and *E. coli* were grown in Luria-Bertani (LB) media at 37 °C.
97 The antibiotics used in the selective medium were ampicillin (50 μg/mL), kanamycin (50 μg/mL),
98 apramycin (50 μg/mL), and streptomycin (25 μg/mL).
99 *K. pneumoniae* Δ*dhaBCE* construction
100 Mutants construction was generated using a Red recombinase associated gene replacement method,

101 which was previously described with some modifications [18]. Briefly, *dhaBCE* in the chromosome of
102 *K. pneumoniae* and flanking sequences were amplified by PCR using the primer pair dhaBCE s1/
103 dhaBCE a1. The PCR product was ligated into the pMD18-T-simple to generate pMD18-T-*dhaBCE*. A
104 linear DNA with 39 and 40 nt homologous extensions flanking the streptomycin resistance gene *aadA*
105 was amplified with plasmid pIJ778 as the template using the primer pair dhaBCE s2/ dhaBCE a2.
106 pMD18-T-Δ*dhaBCE* was constructed by replacing the *dhaBCE* in plasmid pMD18-T-*dhaBCE* with the
107 *aadA* cassette using the Red system in *E. coli*. A linear DNA containing *aadA* with 500-bp homologous
108 regions was amplified from pMD18-T-Δ*dhaBCE*. Finally, the linear DNA was transformed into *K.*
109 *pneumoniae*. Homologous recombination between the linear DNA and the chromosome was facilitated
110 by Red recombinase and resulted in the deletion of *dhaBCE* in the chromosome of *K. pneumoniae*. *dhaF*,
111 *pduCDE*, *pduG* knocked out strains were constructed in the same manner as *K. pneumoniae* Δ*dhaBCE*.

112 **2.3 Medium and culture conditions**

113 The fermentation medium contained 30 g/L glycerol, 1.5 g/L yeast extract, 4 g/L $(NH_4)_2SO_4$, 0.69 g/L
114 $K_2HPO_4 \cdot 3H_2O$, 0.25 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL of trace element
115 solution. One liter of trace element solution contained 200 mg $CoCl_2 \cdot 6H_2O$, 100 mg $MnSO_4 \cdot 4H_2O$, 70
116 mg $ZnCl_2$, 60 mg H_3BO_3, 35 mg $Na_2MoO_4 \cdot 2H_2O$, 29.28 mg $CuSO_4 \cdot 5H_2O$, 25 mg $NiCl_2 \cdot 6H_2O$ and 0.9
117 mL 37% HCl.

118 *K. pneumoniae* strains were inoculated into 250 mL flasks containing 50 mL medium and incubated
119 overnight at 37 °C and 120 rpm on a rotary shaker. The seed culture was inoculated into 5 L bioreactors
120 (BIOSTAT-A plus Sartorius) with a working volume of 3 L. The pH of the culture was automatically
121 controlled to 7. Fermentations were operated in microaerobic conditions. The agitation speed was 250
122 rpm, and 2 L/min of air was supplied through a filter (0.2μm PTFE, satorius stedim). All experiments
123 were done in triplicate, and data are expressed as mean ± standard error.

124 **2.4 Analytical methods**

125 The biomass yield at specific time intervals was determined by optical density (OD600) using a
126 spectrometer (WFJ2100 UNICO, Shanghai). Water was used as the blank.

127 Chemical compounds in the broth were quantified using a Shimadzu 20AVP high performance liquid
128 chromatography system (HPLC) equipped with a RID-20A refractive index detector. An Aminex HPX-
129 87H column (300×7.8 mm) (Bio-Rad, USA) was used and the column temperature was set at 65 ºC. The
130 mobile phase was a 0.005 mol/L H_2SO_4 solution with a flow rate of 0.8 mL/min.

131 **2.5 Purification of the Pdu MCP**

132 After cell grew to a density of 5 OD units, cells were harvested from 50 ml broth by centrifugation. Pellet
133 cells were washed twice and then resuspended in 10 ml of 50 mM Tris-HCl (buffer A). Sonication or
134 lysozyme treatment was used to prepare the cell lysate for the purification of Pdu MCP.
135 The treatment with lysozyme was carried out according to the method developed by the group of
136 Thomas A. [19]. Briefly, the 10 mL suspension was mixed with 15 mL BPER-II, supplemented with 5
137 mM β-mercaptoethanol, 0.4 mM protease inhibitor AEBSF, 10 mL cells, 25 mg lysozyme (Macklin
138 Biochemical, Shanghai), and 2 mg DNase I. The suspension was incubated at room temperature with 60
139 rpm shaking for 30 min and then placed on ice for 5 min. Cell debris was removed by centrifugation
140 twice at 12,000 × g for 5 min at 4 °C, and the Pdu MCP pellet was obtained by centrifugation at 25,000
141 × g for 30 min at 4 °C. The supernatant was collected as the cytoplasm free of Pdu MCP. The Pdu MCP
142 pellet was washed once with a mixture of 4 mL buffer A and 6 mL BPER-II containing 0.4 mM AEBSF
143 and then resuspended in 0.5 mL buffer B (50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 1% 1,2-
144 propanediol) containing 0.4 mM AEBSF.
145 Sonication cell lysis: Sonication was performed in a tube immersed in ice-water with a pulse duration
146 of 3 s on 3 s off, for a total of 99 cycles. Purification of the Pdu MCP followed the same protocol as
147 lysozyme treatment method, with all buffers used were buffer A.
148 **2.6 Enzyme assay**
149 Diol dehydratase or glycerol dehydratase activities were measured as described by Ahrens et al [19]. This
150 method was based on the reaction of aldehyde with 3-methyl-2-benzothiazolinone-hydrazone (MBTH).
151 The assay mixture contained (in a total volume of 1 mL) 0.05 M KCl, 0.2 M 1,2-propanediol, 15 mM
152 coenzyme B12, and eluate in 0.035 M potassium phosphate buffer solution (pH 7.0). The assay was
153 started by adding the enzyme samples and incubated at 37 °C. After 5 or 10 min of incubation, the
154 reaction was stopped by adding 1 mL of 0.1 M potassium citrate buffer (pH 3.6). To develop the color,
155 the mixture was incubated again for 15 min at 37 °C, after the addition of 0.5 mL of 0.1% MBTH solution.
156 The colored azin formed was detected at 305 nm after addition of 1 mL of water with a NanoDrop-2000C
157 spectrophotometer.
158 **3. Results**
159 **3.1 Glycerol dehydratase and diol dehydratase in glycerol catabolism**
160 *K. pneumoniae ΔdhaBCE, K. pneumoniae ΔpduCDE,* and *K. pneumoniae ΔdhaBCEΔpduCDE* were
161 constructed. Glycerol dehydratase and diol dehydratase were disrupted in these strains individually and
162 in combination. These strains and the wild-type strain were cultured in 5 L bioreactors, and fermentation

163 results are shown in Fig. 2.

164 Glycerol depletions of *K. pneumoniae* Δ*dhaBCE* and *K. pneumoniae* Δ*pduCDE* were similar to that
165 of the wild-type strain. Glycerol was exhausted by these strains after 12 hours of cultivation. 1,3-
166 Propanediol was produced by these strains at titers of 11.1, 15.6, and 13.7 g/L, respectively. Glycerol
167 consumed by *K. pneumoniae* Δ*dhaBCE*Δ*pduCDE* was very slow, 3 g/L of glycerol was not yet consumed
168 by the cell after 16 hours of cultivation. No 1,3-propanediol was detected in the broth of this strain. 3-
169 Hydroxypropionaldehyde is an intermediate of 1,3-propanediol synthesis from glycerol. Its accumulation
170 by these strains were different. A peak level of 2.6 mmol/L 3-hydroxypropionaldehyde was achieved
171 after 6 hours of cultivation of the wild type strain. The peak value of 3-hydroxypropionaldehyde
172 increased to 8.4 mmol/L in *K. pneumoniae* Δ*pduCDE* after 10 hours of cultivation. The 3-
173 hydroxypropionaldehyde concentration increased continuously, and a final titer of 1.5 mmol/L was
174 achieved for *K. pneumoniae* Δ*dhaBCE*. No 3-hydroxypropionaldehyde was detected in the broth of the
175 double genes knocked out strain.

176 2,3-Butanediol, succinic acid, lactic acid, acetic acid and ethanol are by-products of 1,3-propanediol
177 production. Their titers were 0.6, 1.1, 0.9, 4.7 and 0.1 g/L for the wild type strains. The concentration of
178 most of these by-products increased in *K. pneumoniae* Δ*dhaBCE*, and with titers of 1.2, 0.9, 1.6, 4.1 and
179 1.2 g/L. While only 1.2 g/L of lactic acid was detected in the broth of *K. pneumoniae* Δ*pduCDE*, this
180 strain produced almost no 2,3-butanediol and ethanol. Since *K. pneumoniae* Δ*dhaBCE*Δ*pduCDE* didn't
181 produce 1,3-propanediol, these by-products became major products, with titers of 1.7 g/L of 2,3-
182 butanediol, 7.1 g/L of lactic acid, and 5.4 g/L of ethanol. Except acetic acid produced by this strain was
183 decreased to 2.0 g/L.

184 **3.2 Glycerol dehydratase and diol dehydratase location between the cytoplasm and the Pdu MCP**

185 Pdu MCP of *K. pneumoniae* Δ*dhaBCE, K. pneumoniae* Δ*pduCDE, K. pneumoniae* Δ*dhaBCE*Δ*pduCDE* ,
186 and the wild-type strain were purified by sonication and lysozyme treatment. Glycerol dehydratase
187 activities in the Pdu MCP, and cytoplasm free of Pdu MCP were detected. Results are shown in Fig. 3.

188 In the purified Pdu MCP, 0.55 U of dehydratase was detected, with a specific activity of 0.37 U/mg of
189 protein. The activity of dehydratase in cytoplasm, free of Pdu MCP, was 41.3 U or 0.55 U/mg protein.
190 Although the specific activity of dehydratase in the Pdu MCP was close to that in the cytoplasm, the total
191 protein of the Pdu MCP was very low. The dehydratase was mainly located in the cytoplasm.

192 Dehydratase activities of the Pdu MCP and the cytoplasm of *K. pneumoniae* Δ*dhaBCE* were all lower
193 than those of the wild type strain. The Pdu MCP contained 0.27 U dehydratase or 0.20 U/mg protein.

194 Dehydratase activity in the cytoplasm was 27 U or 0.32 U/mg protein. Apparently, *dhaBCE* was not
195 expressed in *K. pneumoniae* Δ*dhaBCE*, thus PduCDE was the sole dehydratase in this strain. Dehydratase
196 activity of this strain was equivalent to the activities of PduCDE. Dehydratase activity in the cytoplasm
197 equates to PduCDE in the cytoplasm. Thus PduCDE was mainly located in the cytoplasm, rather than in
198 the Pdu MCP.

199 For *K. pneumoniae* Δ*pduCDE*, the dehydratase activities of Pdu MCP and cytoplasm were 0.02 and
200 12.0 U, respectively. The specific activities were 0.01, and 0.16 U/mg protein. PduCDE was not
201 expressed by *K. pneumoniae* Δ*pduCDE*, and it's logical the dehydratase activity in the Pdu MCP was
202 erased.

203 Neither DhaBCE nor PduCDE was expressed in *K. pneumoniae* Δ*dhaBCE*Δ*pduCDE*. It is reasonable
204 that dehydratase activities were not detected in samples of this strain.

205 The dehydratase activities of these samples prepared by the lysozyme treatment method showed a
206 similar trend to the data for samples prepared by sonication. The specific activities of the Pdu MCP
207 obtained by the lysozyme treatment method were higher than the data obtained by sonication. This
208 indicates that the lysozyme treatment method better preserved dehydratase activities in the Pdu MCP.
209 But the whole activities of these samples obtained by lysozyme treatment method were lower than those
210 obtained by sonication method. This indicates that the ratio of protein recovery was low in the lysozyme
211 treatment method. The sonication method saves time and money and was selected for the following
212 research.

213 **3.3 The exchangeable of reactivating factors of glycerol dehydratase and diol dehydratase**

214 Both glycerol dehydratase and diol dehydratase have their reactivating factors. *dhaF* and *pduG* encode
215 the large subunit of glycerol dehydratase and the diol dehydratase reactivating factors, respectively.
216 *dhaBCE*, *pduCDE*, *dhaF* and *pduG* were knocked out combinedly to obtain five double genes knocked
217 out strains. These strains were cultured in 5 L bioreactors, and fermentation results are shown in Fig. 4.
218 Pdu MCP was purified from the cells of these strains at the same time and results are shown in Table 2.

219 *K. pneumoniae* Δ*dhaBCE*Δ*dhaF* was similar to the strain of *K. pneumoniae* Δ*dhaBCE* (shown in
220 Fig.2). After 12 hours of cultivation, 30 g/L of glycerol was consumed and 11.2 g/L of 1,3-propanediol
221 was produced. The production of 2,3-butanediol, succinic acid, lactic acid, acetic acid, and ethanol were
222 1.2, 0.9, 1.1, 3.5 and 1.1 g/L, respectively.

223 *K. pneumoniae* Δ*dhaBCE*Δ*pduG* losted the activity to synthesise 1,3-propanediol, and 8.2 g/L of
224 glycerol was unused after 14 hours of cultivation. Lactic acid and ethanol concentrations in the broth of

225 this strain were significantly increased.

226 After 10 hours of cultivation, 17 g/L of glycerol was consumed and 8.8 g/L of 1,3-propanediol was 227 produced by *K. pneumoniae* Δ*pduCDE*Δ*pduG*. However, the fermentation process was ceased at this 228 time. Since 11 mmol/L of 3-hydroxypropionaldehyde was accumulated in the broth. This is a lethal level 229 of 3-hydroxypropionaldehyde for the cell. By-products produced by this strain were all in low levels.

230 In contrast to *K. pneumoniae* Δ*dhaBCE*Δ*pduG*, *K. pneumoniae* Δ*pduCDE*Δ*dhaF* had the ability to 231 synthesise 1,3-propanediol. The glycerol consumption rate of this strain was weaker than that of the wild 232 type strain (shown in Fig. 2). After 12 hours of cultivation, 8.4 g/L 1,3-propanediol was produced and 233 26.1 g/L of glycerol was consumed.

234 The cell growth and catabolites synthesis of *K. pneumoniae* Δ*dhaF*Δ*pduG* were similar to that of *K.* 235 *pneumoniae* Δ*dhaBCE*Δ*pduG*. This strain is unable to synthesise 1,3-propanediol. After 14 hours of 236 cultivation, 10.9 g/L glycerol remained in the broth. The major catabolites of this strain were ethanol, 237 lactic acid, and 2,3-butanediol.

238 All of these strains have dehydratase activities in the cytoplasm. Strains in which the *pduCDE* were 239 knocked out (*K. pneumoniae* Δ*pduCDE*Δ*pduG* and *K. pneumoniae* Δ*pduCDE*Δ*dhaF*) had no 240 dehydratase activities in the Pdu MCP. Other strains all had dehydratase activities in the Pdu MCP.

241 *K. pneumoniae* Δ*dhaBCE*Δ*pduG* and *K. pneumoniae* Δ*dhaE*Δ*pduG* had lost the ability to synthesise 242 1,3-propanediol, but both possess dehydratase activities *in vitro*. This suggests that the *in vivo* and *in* 243 *vitro* activities of dehydratase do not match.

244 4. Discussion

245 4.1 *pduCDE* encoded diol dehydratase is involved in glycerol catabolism and 1,3-propanediol 246 synthesis in *K. pneumoniae*

247 Under anaerobic conditions or glycerol abundant conditions, glycerol is catabolic through *dha* pathway. 248 The *dha* pathway is a dismutation pathway and contains an oxidative branch and a reductive pathway. 249 The genes in this pathway form a *dha* operon, whose expression is induced by dihydroxyacetone [4]. The 250 genes in the *dha* operon are located nearly in the chromosome. *dhaR* encodes a regulation protein and 251 positively regulates the expression of *dha* operon. The dihydroxyacetone kinases II are involved in the 252 regulation of *dha* operon expression [21]. In our recent research, a high level of dihydroxyacetone were 253 shown to inhibit the expression of the *dha* operon [22]. The *pdu* operon is responsible for 1,2-propanediol 254 catabolism in *S. enterica*, and the expression of this operon is induced by 1,2-propanediol [8]. In nature, 255 1,2-propanediol is likely to be present in the intestinal environment. It is a catabolite of some deoxy

256 sugars [23]. Disruption of *pduCDE* or *dhaBCE* has no clear effect on glycerol catabolism and 1,3-
257 propanediol production, indicating that they are both expressed at considerable levels under the condition
258 of glycerol as a carbon source. Pdu MCP was purified from cells of *K. pneumoniae* strains indicating that
259 the entire *pdu* operon was expressed with glycerol induction. Glycerol was found to induce the expression
260 of *pdu* operon in *S. enterica*, but under the conditions of growth on poor carbon sources [24].

261 **4.2 *pduCDE* encoded diol dehydratase mainly located in the cytoplasm rather than in the Pdu MCP**

262 The enzymes of the MCP are located in the lumen of the MCP. Individual peptides have been identified
263 that control the packaging of these enzymes. PduCDE are homologous to DhaBCE, but PduD has an
264 additional single peptide at the N-terminal. PduC and PduE do not have single peptides. PduD mediated
265 the packaging of itself and other subunits of diol dehydratase into the Pdu MCP in *S. enterica* [15]. The
266 dehydratase activities in the cytoplasm of *K. pneumoniae* Δ*dhaBCE* contained 97- 99% (lysozyme
267 treatment or sonication method) of the total dehydratase activities (shown in Fig 3). Thus PduCDE was
268 mainly located in the cytoplasm rather than in the Pdu MCP (around 1-3%). PduC, PduD, and PduE
269 contained 4.7%, 3.6%, and 2.4% total protein of Pdu MCP, respectively, in *S. enterica* [25]. If the Pdu
270 MCP structure of *K. pneumoniae* was similar to that of *S. enterica*. The PduCDE were too highly
271 expressed to be packaged in the Pdu MCP in *K. pneumoniae*.

272 **4.3 The Pdu MCP affect the 3-hydroxypropionaldehyde accumulation in the broth of *K.***
273 ***pneumoniae***

274 3-Hydroxypropionaldehyde is a toxic intermediate in the 1,3-propanediol biosynthesis from glycerol.
275 The imbalance between the formation and consumption of 3-hydroxypropionaldehyde leads to its
276 accumulation. High levels of 3-hydroxypropionaldehyde inhibit the activities of cells and cease the
277 fermentation process. To prevent accumulation of 3-hydroxypropionaldehyde accumulation, the glycerol
278 level in the broth was precisely controlled [26]. Metabolic engineering works that over expression of
279 *dhaT* was done to reduce the 3-hydroxypropionaldehyde content in the broth [27].

280 The native role of Pdu MCP in *S. enterica* was to retain propionaldehyde in the lumen of MCPs, and
281 prevent its toxicity to the cell [28]. A mutant MCP of *S. enterica* accumulated 10-fold higher amount of
282 propionaldehyde than that of the wild type, and caused growth arrest [29]. However, the dehydratase
283 activities in the Pdu MCP contain only 1-3% of the total dehydratase activities. 3-
284 Hydroxypropionaldehyde accumulated to a higher extent in the broth of *K. pneumoniae* Δ*pduCDE* than
285 in the wild type strain and *K. pneumoniae* Δ*dhaBCE*. Typically, the 3-hydroxypropionaldehyde content
286 in *K. pneumoniae* Δ*pduCDE*Δ*pduG* was too high and lead to the fermentation process cease. These

287 phenomena suggest that Pdu MCP can reduce the accumulation of 3-hydroxypropionaldehyde.

288 **4.4 *pduG* encoded diol dehydratase reactivating factor can be used to cross reactive glycerol**
289 **dehydratase**

290 Diol dehydratase and glycerol dehydratase both have their reactivating factors to keep their activities in
291 catalyzing the reaction of converting glycerol to 3-hydroxypropionaldehyde. *K. pneumoniae*
292 Δ*pduCDE*Δ*dhaF* had the ability to synthesise 1,3-propanediol, whereas *K. pneumoniae*
293 Δ*dhaBCE*Δ*pduG* could not. Thus, PduG can be used as a reactivating factor for DhaBCE, but DhaF can
294 not matching PduCDE. This is consistent with a report that the reactivating factor of diol dehydratase
295 from *Klebsiella oxytoca* efficiently cross reactivated the inactivated glycerol dehydratase from *K.*
296 *pneumoniae*, whereas the reactivating factor of glycerol dehydratase hardly cross reactivated the
297 inactivated diol dehydratase [30]. The specificities of reactivating factors were determined by the
298 capability of reactivating factors to form complexes with apoenzymes [31]. *dhaF* and *pduG* encode the
299 large subunit of the reactivating factors, and it was found that the large subunit of the reactivating factor
300 mainly determines the specificity for a dehydratase substrate [30].

301 **4.5 The *in vivo* and *in vitro* activities of diol dehydratase were not coincide.**

302 *K. pneumoniae* Δ*dhaBCE*Δ*pduG* and *K. pneumoniae* Δ*dhaE*Δ*pduG* do not produce 1,3-propanediol, but
303 dehydratase activities were detected in the cytoplasm and the Pdu MCP. In these two strains, *pduCDE*
304 was wild-type. Thus, PduCDE has the dehydratase activity without the reactivating factors *in vitro*, but
305 not *in vivo*. It has been reported that the dehydration of 1,2-propanediol by diol and glycerol dehydratases
306 proceeds linearly with time, but the enzymes undergo mechanism-based inactivation by glycerol [32].
307 Glycerol dissimilation was blocked in the two strains for the absence of reactivating factors. Since 1,2-
308 propanediol was used as substrate for the dehydratase assay, both strains both showed dehydratase
309 activities *in vitro*. Interestingly, although the native role of *pdu* operon is responsible for 1,2-propanediol
310 catabolism, the PduGH seems redundant for 1,2-propanediol catabolism but for glycerol catabolism.

311 **5. Conclusion**

312 It has long been known that diol dehydratase and glycerol dehydratase are isofunctional enzymes. But
313 the role of diol dehydratase in glycerol catabolism was not clear. In last 20 years, bacterial MCPs were
314 funded and Pdu MCP was investigated in detail, and the functions of PduCDE in 1,2-propanediol
315 catabolism were clear. The structure of 1,2-propanediol catabolism pathway was similar to glycerol
316 catabolism pathway, and the molecule of 1,2-propanediol was similar to glycerol. It is suggested that the
317 catabolism pathway of 1,2-propanediol and glycerol interact with each other. Here, it is shown that the

318 *pduCDE* encoded diol dehydratase and the Pdu MCP are involved in glycerol catabolism. These findings
319 provide guidance for metabolic engineering modification of glycerol catabolism and 1,3-propanediol
320 synthesis in *K. pneumoniae*.

321 **Compliance with Ethical Standards:** This article does not contain any studies with human participants
322 or animals performed by any of the authors.

323 **Authors' contributions**

324 JH designed this study. LS, QW, WJ, JG, YW and DW conducted the research. MT, BO,JP, GL, FB, DW
325 and JH analysed the data. LS and JH wrote the manuscript. All authors read and approved the final
326 manuscript.

327 **Author agreement**

328 All authors have seen and approved the final version of this submission. This article is the authors'
329 original work, has not received prior publication and is not under consideration for publication elsewhere.

330 **CRediT authorship contribution statement**

331 **Lin Shu**: Investigation, Formal analysis, Writing original draft. **Qinghui Wang**: Investigation. **Weiyan**
332 **Jiang**: Investigation. **Marina Tišma**: Formal analysis. **Beakrock Oh**: Formal analysis. **Jiping Shi**:
333 Formal analysis. **Gary J. Lye**: Formal analysis. **Frank Baganz**: Formal analysis. **Dong Wei**:
334 Investigation, Formal analysis, Writing original draft. **Jian Hao**: Conceptualization, Formal analysis,
335 Writing - review & editing.

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341

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343

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424
425

426 Tables

427 Table 1 Strains and plasmids

Strains or plasmids	Relevant genotype and description	Reference or source
E. coli DH5α	Host of plasmid	Lab stock
K. pneumoniae CGMCC 1.6366	TUAC01 Wild type, Amp^r	[1]
Δ*dhaBCE*	Δ*dhaBCE*	This work
Δ*pduCDE*	Δ*pduCDE*	This work
Δ*dhaF*	Δ*dhaF*	This work
Δ*pduG*	Δ*pduG*	This work
Δ*dhaBCE* Δ*dhaF*	Δ*dhaBCE* Δ*dhaF*	This work
Δ*dhaBCE* Δ*pduCDE*	Δ*dhaBCE* Δ*pduCDE*	This work
Δ*dhaBCE* Δ*pduG*	Δ*dhaBCE* Δ*pduG*	This work
Δ*pduCDE* Δ*pduG*	Δ*pduCDE* Δ*pduG*	This work
Δ*pduCDE* Δ*dhaF*	Δ*pduCDE* Δ*dhaF*	This work
Δ*dhaF* Δ*pduG*	Δ*dhaF* Δ*pduG*	This work
Plasmids		
pIJ773	Apr^r, *aac(3)IV* with FRT sites	[16]
pIJ778	Str^r, *aadA* with FRT sites	[16]
pDK6	Kan^r, lacI^Q, tac,	[17]
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo)	[18]
pDK6-flp	Kan^r, carries the yeast FLP recombinase gene	[18]
pMD18-T-simple	Amp^r, TA cloning vector, 2692 bp	Takara
pMD18-T-Δ*dhaBCE*	Amp^r, Apr^r, carries part of *dhaBCE*	This work
pMD18-T-Δ*dhaF*	Amp^r, Str^r, carries part of *dhaF*	This work
pMD18-T-Δ*pduCDE*	Amp^r, Apr^r, carries part of *pduCDE*	This work
pMD18-T-Δ*pduG*	Amp^r, Apr^r, carries part of *pduG*	This work

428

429 Table 2. Dehydratase activities of strains that knocked out reactivating factors of dehydratases

Strains	Dehydratase activities (U/ 50 ml broth)		Dehydratase activities (U/ mg pterion)	
	Cytoplasm	Pdu MCP	Cytoplasm	Pdu MCP
WT	41.4±0.4	0.55±0.02	0.78±0.01	0.52±0.03
Δ*dhaBCE*Δ*dhaF*	10.7±0.3	0.3±0.01	0.34±0.01	0.44±0.02
Δ*dhaBCE*Δ*pduG*	35.5± 1.1	1.4±0.03	0.89±0.03	1.21±0.04
Δ*pduCDE*Δ*pduG*	6.8±0.04	0.01±0.001	0.16±0.002	0.01±0.001
Δ*pduCDE*Δ*dhaF*	16.2±1.0	0.01±0.002	0.28±0.02	0.01±0.001
Δ*dhaF*Δ*pduG*	40.5± 0.8	1.5±0.05	0.81±0.02	1.26±0.05

430 **Figure captions**

431 Fig. 1 Diol dehydratase involved in glycerol catabolism in *K. pneumoniae*. Glycerol dehydratase and diol
432 dehydratase function simultaneously for glycerol catabolism. A small part of diol dehydratase is located
433 in the lumen of Pdu MCP, large amounts of diol dehydratase are located in the cytoplasm. PduG can
434 cross reactivate glycerol dehydratase instead of DhaF. However, DhaF do not hold the ability to reactivate
435 diol dehydratase. The native role of Pdu MCP is catabolism of 1,2-propanediol. 3-HPA: 3-
436 Hydroxypropionaldehyde; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; PEP:
437 phosphoenolpyruvate.

438

439 Fig. 2. The cell growth, glycerol depletion, and products formation during *K. pneumoniae* strains knocked
440 out glycerol dehydratase, diol dehydratase, or both. Data points are the average of n = 3; error bars
441 represent standard error about the mean.

442

443 Fig. 3. Glycerol dehydratase activities of Pdu MCP and the cytoplasm free of Pdu MCP of strains knocked
444 out of glycerol dehydratase or diol dehydratase or both glycerol dehydratase and diol dehydratase. A, C:
445 dehydratase activities of whole 50 mL fermentation broth; B, D: dehydratase activities per weight protein.
446 A, B: Pdu MCP was purified by the sonication method; C, D: Pdu MCP was purified by the lysozyme
447 treatment method.

448

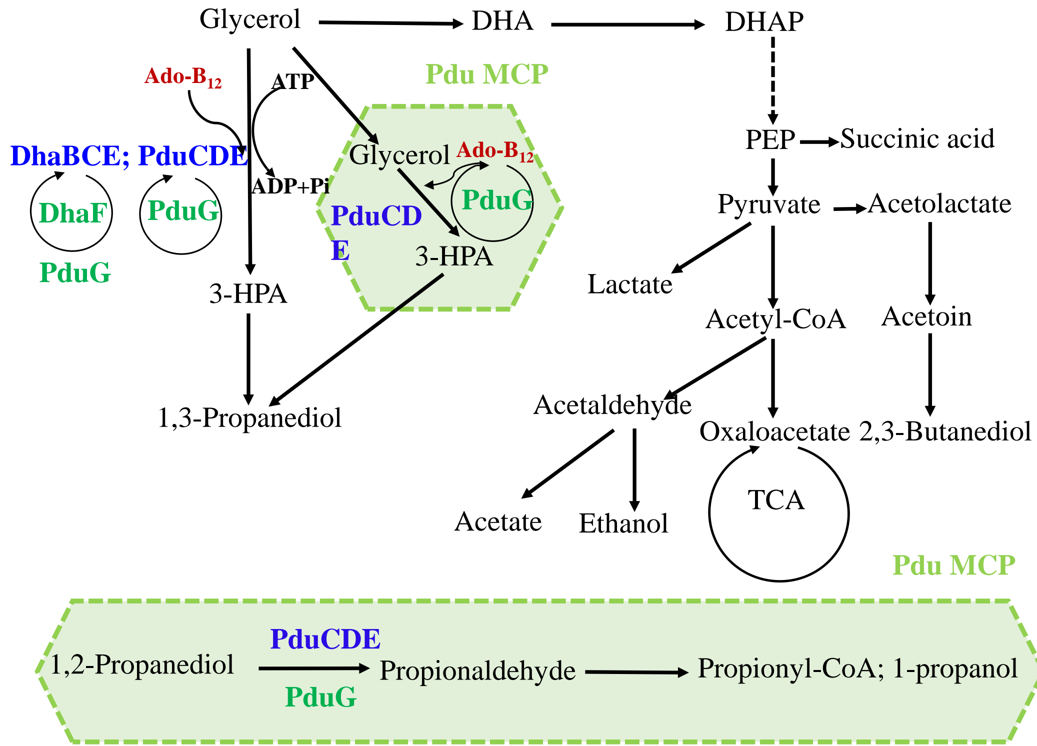
449 Fig. 4. The cell growth, glycerol depletion, and products formation during *K. pneumoniae* strains knocked
450 out dehydratase activating factors. Data points are the average of n = 3; error bars represent standard
451 error about the mean.

452

453

454 **Figures**

455 Fig.1



456

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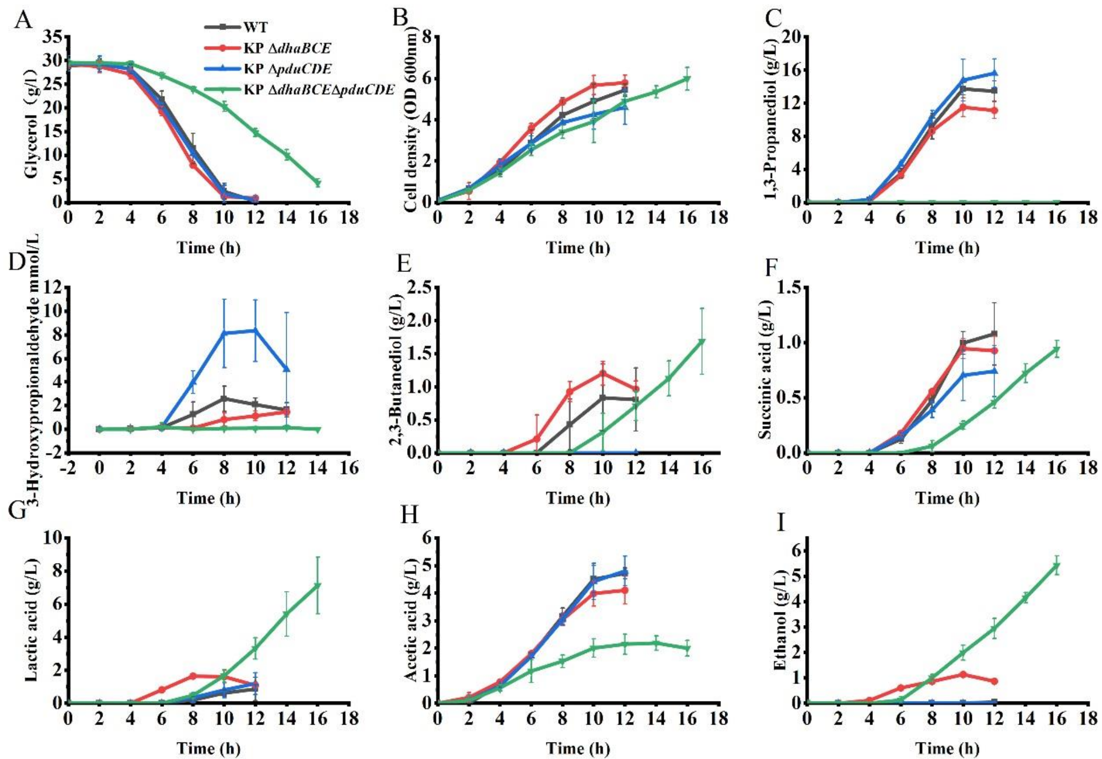
458 Fig.2

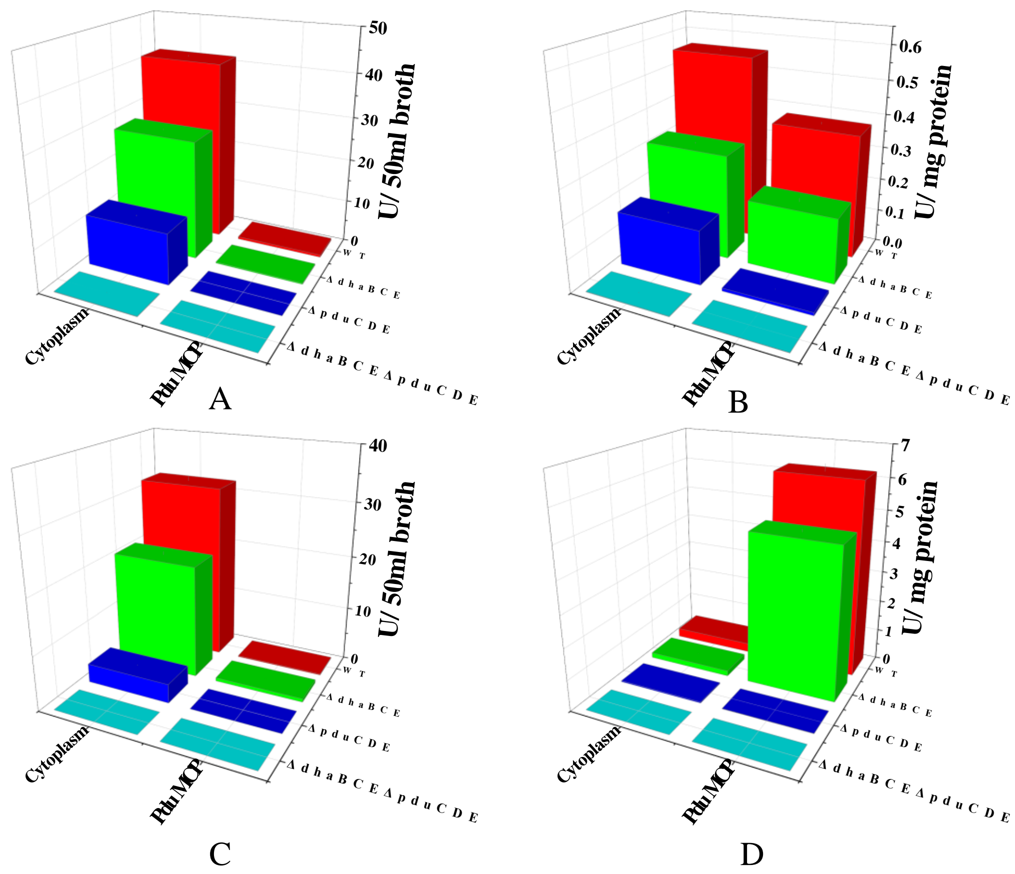


459

460

461

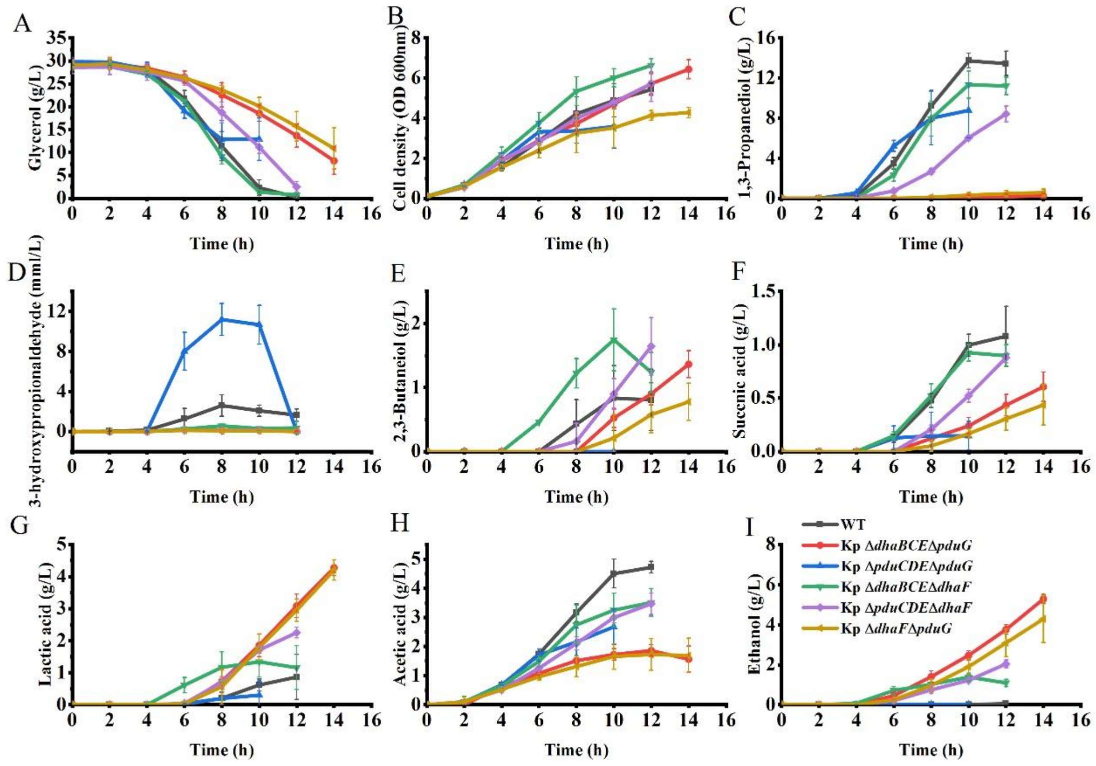
462 Fig.3



463

464

465 Fig.4



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