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3 **Significance of the Cgl1427 gene encoding cytidylate kinase in microaerobic**

4 **growth of *Corynebacterium glutamicum***

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Abstract

The Cgl1427 gene was previously found to be relevant to the microaerobic growth of *Corynebacterium glutamicum* (Ikeda et al. 2009). In the present work, Cgl1427 was identified as a cytidylate kinase gene (*cmk*) by homology analysis of its deduced amino acid sequence with that of other bacterial cytidylate kinases (CMP kinases), and on the basis of findings that deletion of Cgl1427 results in loss of CMP kinase activity. Deletion of the *cmk* gene significantly impaired the growth of *C. glutamicum* in oxygen-limiting static culture, and the impaired growth was restored by introducing a plasmid containing the *cmk* gene, suggesting that this gene plays an important role in microaerobic growth of *C. glutamicum*. On the other hand, in the main culture with aerobic shaking, a prolonged lag phase was observed in the *cmk* disruptant, despite an unchanged growth rate, compared to the behavior of the wild-type strain. The prolongation was observed when using seed culture grown to later growth stages in which oxygen limitation occurred, but not observed when using seed culture grown to an earlier growth stage in which oxygen remained relatively plentiful. Since nucleotide biosynthesis in *C. glutamicum* requires oxygen, we hypothesized that the ability of the

cmk disruptant to synthesize nucleotides was influenced by oxygen limitation in the later growth stages of the seed culture, which caused the prolongation of the lag phase in the following shaken culture. To verify this hypothesis, a plasmid containing genes encoding all components of a homologous ribonucleotide reductase, a key enzyme for nucleotide synthesis that requires oxygen for its reaction, was introduced into the *cmk* disruptant, which significantly ameliorated the lag phase prolongation. Furthermore, this experimental setup almost completely restored the growth of the *cmk* disruptant in the oxygen-limiting static culture. These results indicate that CMP kinase plays an important role in normal nucleotide biosynthesis under an oxygen-limiting environment.

Key words: cytidylate kinase, nucleotide synthesis, oxygen, microaerobic growth, *Corynebacterium glutamicum*

Introduction

An aerobic microorganism, *Corynebacterium glutamicum*, has been used for production of various amino acids (Kinoshita and Nakayama 1978). For *C. glutamicum* as well as

55 for most industrial aerobic microorganisms, oxygen is a very important factor for
56 growth and production. Under oxygen limitation, production strains usually accumulate
57 undesirable organic acids, which results in damaged fermentation with decreased
58 production yields. For this reason, considerable effort and expense have been devoted to
59 ensure that such production environments maintain a high oxygen supply (Oka 1999;
60 Ikeda 2003). If, however, we can develop so-called low-O_2 adapted strains that produce
61 amino acids efficiently even under oxygen limitation, this will be industrially significant
62 from the viewpoint of cost savings. Such technology does not yet exist, but a few
63 studies have adopted a common method in research, namely, the use of *Vitreoscilla*
64 hemoglobin (VHb). Research on biotechnological applications using VHb has described
65 increased production of antibiotics (Priscila et al. 2008; Liu et al. 2010; Zhu et al. 2011),
66 biopolymers (Horng et al. 2010; Chien et al. 2006), and biochemicals (Liu et al. 2008;
67 Li et al. 2010). The positive effects are generally presumed to be due to VHb-enhanced
68 oxygen delivery to the respiratory chain, which facilitates respiration under hypoxic
69 conditions. These studies are examples showing that oxygen availability is associated
70 with respiration.

71 In a previous study, we revealed that wild-type *C. glutamicum* can grow even
72 in environments with low oxygen concentration, whereas other aerobic bacteria cannot

73 (Takeno et al. 2007). Therefore, this bacterium is speculated to have certain functions

74 for adaptation to low oxygen environments. Elucidation and subsequent improvement of

75 such functions may lead to new technologies for creating productive strains. For this

76 purpose, we obtained a number of high-oxygen-requiring mutants of *C. glutamicum*,

77 and identified genes that enable these mutants to grow under low oxygen conditions

78 (Ikeda et al. 2009). While we could provide relevant discussion for most of the genes,

79 concerning adaptability to low-oxygen environments, the Cgl1427 that is annotated as a

80 putative cytidylate kinase (CMP kinase) has not yet been satisfactorily explained. In this

81 report, we identified Cgl1427 as a CMP kinase gene (*cmk*) and investigated the effects

82 of Cgl1427 disruption on the growth of *C. glutamicum*. We describe the importance of

83 the cytidylate kinase in normal nucleotide biosynthesis under low oxygen environments.

84 Our findings indicate that the nucleotide synthetic pathway is particularly important

85 when seeking to develop low-O_2 adapted strains (Fig. 1).

86

87 **Materials and methods**

88

89 Bacterial strain, growth conditions, and plasmids

90

91 The wild-type *C. glutamicum* strain ATCC 31833 was used in this study. Complete
92 medium BY (Takeno et al. 2007) and minimal medium MM (Takeno et al. 2007), each
93 containing 20 μg/ml of kanamycin, were used for the cultivation of ATCC 31833 and its
94 derivative recombinants. MM medium contained 1% (wt/vol) glucose as a sole carbon
95 source. Seed culture was grown in 5 mL of BY medium in test tubes at 30°C subjected
96 to reciprocal shaking at 120 strokes/min. Chronological assessment of oxygen depletion
97 under the shaking conditions set for seed cultures was done using 5 mL of MM medium
98 containing 1.0 mg/L of resazurin (Takeno et al. 2007). For the aerobic growth test,
99 liquid culture was carried out at 30°C in L-type test tubes shaken with a Monod shaker
100 at 48 strokes/min. For the growth test under oxygen-limiting conditions, liquid culture
101 was carried out statically at 30°C in test tubes (Ikeda et al. 2009). *Escherichia coli*
102 DH5α was used as a host for DNA manipulation. LB medium (Sambrook and Russell
103 2001) was used to grow *E. coli*. Plasmid pESB30 (Mitsuhashi et al. 2004), which is
104 nonreplicative in *C. glutamicum*, was used for gene disruption in *C. glutamicum*.
105 Plasmid pCS299P (Mitsuhashi et al. 2004) was used for the expression of *C.*
106 *glutamicum* ribonucleotide reductase genes. Plasmid pRNR, which contains the *C.*
107 *glutamicum nrdHIE* operon and the *nrdF* gene, was constructed as diagramed in Fig. 5.
108 Plasmid pCcmk, which was previously designated as pBam1.8 (Ikeda et al. 2009), was

109 used for the plasmid-mediated expression of the *C. glutamicum cmk* gene. Plasmid

110 pEcmk, for expression of the *E. coli cmk* gene, was constructed as follows. The genomic

111 region comprising the *cmk* gene from *E. coli* K-12 W3110 was amplified by PCR using

112 two primers, cmkup200FBamHI and cmkdown400RBamHI. The resulting fragment

113 was digested with *Bam*HI and then ligated to *Bam*HI-digested pCS299P to yield pEcmk.

114 Both pCcmk and pEcmk contain the corresponding *cmk* gene, with no other intact

115 genes.

116

117 Recombinant DNA techniques

118

119 Standard protocols (Sambrook and Russell 2001) were used for the construction,

120 purification, and analysis of plasmid DNA and for the transformation of *E. coli*.

121 Chromosomal DNA was extracted from *C. glutamicum* as described by Saito and Miura

122 (1963). Transformation of *C. glutamicum* by electroporation was carried out using the

123 method described by van der Rest et al. (1999), using the Gene Pulser II electroporation

124 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR was performed using a

125 DNA thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City,

126 CA, USA), using Phusion High-Fidelity DNA Polymerase (New England Biolabs,

127 Ipswich, MA, USA). Sequencing to confirm the nucleotide sequences of relevant DNA

128 regions was performed using an ABI PRISM 377 DNA sequencer from Applied

129 Biosystems, with an ABI PRISM BigDye Terminator cycle sequencing kit (Applied

130 Biosystems). The subsequent electrophoresis analysis was carried out using Pageset

131 SQC-5ALN 377 (Toyobo, Osaka, Japan).

132

133 Strain construction

134

135 The sequences of the primers used in this study are listed in Table 1. All primers were

136 designed based on the genomic sequences of *C. glutamicum* (BA000036) and *E. coli*

137 (AP009048), which are publicly available at http://www.genome.jp/kegg/genes.html.

138 For the chromosomal deletion of the *cmk* gene, plasmid pΔcmk that contains

139 the internally deleted *cmk* gene was constructed as follows. The 5’ region of the *cmk*

140 gene was amplified by PCR using two primers, Pr8 and Cgl1427FusR, with pBam1.8

141 (pCcmk in the present work) as a template. Similarly, the 3’ region of the gene was

142 amplified using two primers, Cgl1427FusF and Pr7. Fusion PCR was then performed

143 using the purified 5’ and 3’ region fragments as templates, and the Pr8 and Pr7 primers.

144 The resulting 1.2-kb fragment containing the deleted *cmk* gene, shortened by in-frame

145 deletion of the inner sequence, was digested with *Bam*HI and then ligated to

146 *Bam*HI-digested pESB30 to yield pCΔcmk. Defined chromosomal deletion of the *cmk*

147 gene was accomplished using pCΔcmk via two recombination events as described

148 previously (Ohnishi et al. 2002). The strain carrying the *cmk* gene deletion in a

149 wild-type background was designated strain Δcmk. Strain Δcmk contained the deleted

150 *cmk* gene which was shortened from 693 bp to 78 bp.

151

152 Preparation of soluble fraction and enzyme assay

153

154 *C. glutamicm* strains were grown to late-log phase in 200 ml of MM medium in 2-L

155 Sakaguchi flasks reciprocally shaken at 120 strokes/min. Cells were harvested by

156 centrifugation at 10,000 × *g* for 10 min and then washed twice with 50 mM Tris-HCl

157 buffer (pH7.4). The cells were suspended in 4 volumes of the same buffer and sonicated

158 on ice for 5 min using a UD-200 ultrasonic disruptor (Tomy Seiko Co., Ltd., Tokyo,

159 Japan). Cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and the

160 supernatant was further ultracentrifuged at 100,000 × *g* for 60 min using an Optima TL

161 ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The resulting supernatant

162 was dialyzed against the same buffer, and then used in the enzyme assay. All steps were

163 done at 4°C unless otherwise stated. CMP kinase activity was spectrophotometrically

164 measured at 30°C according to the methods described by Blondin et al. (1994) using an

165 Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

166 One unit of enzyme corresponds to 1 μmol of product formed per minute.

167

168 Analysis

169

170 Bacterial growth was monitored by measuring the optical density at 660 nm (OD_{660}) of

171 the culture broth, using a Miniphoto 518R spectrophotometer (Taitec, Saitama, Japan).

172 Protein content was determined using a Bio-Rad protein assay kit (Bio-Rad

173 Laboratories). Homology analysis was performed using GENETYX-WIN ver. 5.1.1

174 software (Genetyx Co., Tokyo, Japan).

175

176 **Results**

177

178 Homology analysis of the deduced amino acid sequence of Cgl1427, and CMP kinase

179 activity in a Cgl1427 disruptant

180

181 Although Cgl1427 is annotated as a putative CMP kinase, this has not been confirmed
182 experimentally. The deduced amino acid sequence of Cgl1427 shows 58.2, 39.7, and
183 37.3% identity with well-defined CMP kinases of *Mycobacterium tuberculosis* (Thum et
184 al. 2009), *E. coli* (Fricke et al. 1995), and *Bacillus subtilis* (Schultz et al. 1997),
185 respectively. Cgl1427 is located in the *C. glutamicum* chromosome between Cgl1426
186 (probable RNA pseudouridylate synthase) and Cgl1428 (GTP-binding protein). The
187 same arrangement is found in *M. tuberculosis*, a species related to *Corynebacterium*. No
188 other putative CMP kinase gene is found in the *C. glutamicum* genomes (Ikeda and
189 Nakagawa 2003; Yukawa et al. 2007).
190 We constructed a Cgl1427 deletion mutant, strain Δcmk, through in-frame
191 deletion of the Cgl1427 inner sequence from a wild *C. glutamicum* ATCC 31833 strain.
192 The soluble fraction from strain Δcmk/pCS299P grown in MM medium exhibited a
193 reduced level of CMP kinase activity (2.34 mU/mg) compared with that of strain ATCC
194 31833/pCS299P (28.4 mU/mg). Bacterial CMP kinase also catalyzes the
195 phosphorylation of dCMP to dCDP (Thum et al. 2009). A reduced level of dCMP kinase
196 activity was also found in strain Δcmk/pCS299P (3.68 mU/mg) compared with that of
197 strain ATCC 31833/pCS299P (12.7 mU/mg). These results indicate that Cgl1427
198 encodes the CMP kinase in *C. glutamicum*. Cgl1427 is referred to as the *cmk* gene in the

199 sections that follow. It should be noted that the data represent mean values obtained

200 using the soluble fraction from two independent cultures, which showed < 10%

201 differences between each other.

202

203 Effect of *cmk* gene disruption on the growth of *C. glutamicum*

204

205 Our previous results that plasmid-mediated expression of the *cmk* gene restores the

206 growth defects of a variety of high-oxygen-requiring mutants of *C. glutamicum* in

207 oxygen-limiting static culture (Ikeda et al. 2009) indicate that the *cmk* gene is relevant

208 to microaerobic growth. In the present work, we examined the effects of *cmk* gene

209 disruption on the growth of *C. glutamicum* under oxygen-limiting conditions.

210 Under oxygen-limiting static conditions, the growth of strain Δcmk/pCS299P

211 was markedly damaged, but this impaired level of growth was restored to that of strain

212 ATCC 31833/pCS299P by introducing pCcmk (Fig. 2), suggesting that the CMP kinase

213 is relevant to microaerobic growth of *C. glutamicum*. Plasmid pEcmk that contains the

214 *E. coli cmk* gene also restored the growth of strain Δcmk to near the level of strain

215 Δcmk/pCcmk (Fig. 2), supporting that Cgl1427 is the *cmk* gene in *C. glutamicum*.

216 If the *cmk* gene was involved only in microaerobic growth, gene disruption

217 should have no effect on the aerobic growth of *C. glutamicum*. The effect of *cmk* gene

218 disruption on aerobic growth was examined by cultivation using routine methods; 5 mL

219 of the main medium, to which 50 μl of seed culture grown to the late-exponential phase

220 had been transferred, was subjected to aerobic shaking (Fig. 3a). The growth rate of

221 strain Δcmk/pCS299P was almost comparable to that of strains ATCC 31833/pCS299P

222 and Δcmk/pCcmk. However, the growth curve of strain Δcmk/pCS299P seemed to be

223 only slightly shifted backward, compared to that of strain ATCC 31833/pCS299P,

224 raising the possibility that the lag phase in strain Δcmk/pCS299P was prolonged. To

225 verify this, we decreased the inoculum volume from 50 μl to 5 μl and then 0.5 μl, and

226 performed the same cultivation (Fig. 3b and 3c). The results showed that a prolonged

227 lag phase did emerge in strain Δcmk/pCS299P with 5 μl of inoculum and, furthermore,

228 the prolongation was more pronounced with 0.5 μl of inoculum, as compared with that

229 of strain ATCC 31833/pCS299P, under each condition tested. The phenotype was fully

230 complemented by pCcmk to the level of strain ATCC 31833/pCS299P, and partially

231 complemented by pEcmk (Fig. 3b). These data show that the disruption of the *cmk* gene

232 causes prolongation of the lag phase in the main culture with aerobic shaking.

233

234 Effects of different seed culture growth phases on the lag phase of the *cmk* disruptant in

235 the main culture with aerobic shaking.

236

237 As described above, prolongation of the lag phase occurred in the *cmk* disruptant, with

238 no significant changes in growth rates observed in the main culture with aerobic shaking.

239 Our hypothesis for this puzzling phenomenon is that the *cmk* disruptant, in which its

240 ability to grow under microaerobic conditions was markedly damaged (Fig. 2), suffered

241 from limitation of oxygen in the later stage of the previous seed culture due to increased

242 cell mass, and an intracellular carryover of the resulting disadvantage was reflected in

243 the main culture as a prolongation of the lag phase. As the first step to test this

244 hypothesis, shaking conditions set for cultivation of seed culture were checked for

245 residual oxygen (Fig. 4a). The environment shifted from aerobic to oxygen-deprivation

246 states; the early-exponential phase (OD_{660} = approximately 1.0) was barely aerobic

247 (Photograph d inset in Fig 4a), and oxygen deprivation occurred in the mid- (OD_{660} =

248 approximately 2.0) and late-exponential phases (OD_{660} = approximately 4.0)

249 (Photographs c and b inset in Fig 4a, respectively). Based on our hypothesis,

250 prolongation of the lag phase demonstrated by the *cmk* disruptant should occur only

251 when using seed cultures grown to later growth stages during which oxygen deprivation

252 occurs, and prolongation should not occur when using seed cultures grown to a growth

253 stage where oxygen remains. Accordingly, we performed the same cultivation as that

254 shown in Fig. 3b, using seed cultures grown to each growth phase (Fig. 4b, 4c, and 4d).

255 Whereas prolongation of the lag phase was observed when using seed culture grown to

256 mid- and late-exponential phases (Fig. 4c and 4b, respectively), no prolongation was

257 observed when using seed culture grown to the early-exponential phase, as expected

258 (Fig. 4d). These results suggest that the prolonged lag phase observed in strain

259 Δcmk/pCS299P in the main culture with aerobic shaking was attributed to

260 oxygen-limitation in the seed culture.

261

262 Effect of amplification of homologous ribonucleotide reductase genes on the growth of

263 the *cmk* disruptant

264

265 Although nucleotide synthesis is speculated to be damaged by the *cmk* gene disruption,

266 the mechanism by which this damage influences the microaerobic physiology of this

267 strain remains unclear. We hypothesized that the damage limits the performance of a

268 certain oxygen-dependent metabolic step in the nucleotide synthetic pathway. In this

269 regard, we focused on a ribonucleotide reductase (RNR) of this strain, a key enzyme of

270 nucleotide biosynthesis and an oxygen-requiring enzyme responsible for the conversion

271 of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphate,

272 *e.g*., CDP to dCDP (Fig. 1) (Oehlmann et al. 1999; Sjoberg 2010; Kolberg et al. 2004).

273 *C. glutamicum* RNR is a class I RNR composed of four components; NrdH,

274 NrdI, NrdE, and NrdF (Oehlmann et al. 1999). NrdE and NrdF are large and small

275 subunits of the RNR, respectively (Hofer et al. 2012). NrdE contains the active site and

276 carries out the actual reduction of the ribonucleoside diphosphates. For this reaction,

277 radical transfer from the tyrosyl radical of NrdF to the cysteine in the active site of NrdE

278 is required. The tyrosyl radical of NrdF was recently proposed to be formed by an

279 oxygen-requiring reaction of NrdI (Cotruvo and Stubbe 2010). NrdH is a redoxin

280 protein that reduces the disulfide cysteine at the active site, which is concomitantly

281 formed by ribonucleotide diphsophate reduction.

282 If the prolongation of the lag phase and impaired growth in oxygen-limiting

283 static culture is due to a limitation in RNR function, an increased dosage of RNR should

284 complement both phenotypes of the *cmk* disruptant. Plasmid pRNR that contains genes

285 encoding all components of a homologous ribonucleotide reductase was constructed as

286 diagramed in Fig. 5 (in which the arrangement of RNR genes in the *C. glutamicum*

287 genome is also diagramed). The effects of amplification of RNR genes on the growth

288 properties of the *cmk* disruptant were analyzed using pRNR (Fig. 6 and 7). In the main

289 culture under shaken conditions, the prolonged lag phase, observed in cases where

290 strain Δcmk/pCS299P was applied, was abolished to the level observed in strains ATCC

291 31833/pCS299P and Δcmk/pCcmk by introduction of pRNR (with 5μl of inoculum, Fig.

292 6a). The ameliorating effect of pRNR on the prolongation of lag phase was evident with

293 0.5 μl of inoculum (Fig. 6b). Furthermore, in oxygen-limiting static culture, the growth

294 level of strain Δcmk/pRNR was comparable to that of strains ATCC 31833/pCS299P

295 and Δcmk/pCcmk (Fig. 7). These results demonstrate that RNR is a determining factor

296 for growth and physiology of the *cmk* disruptant in an oxygen-limiting environment.

297

298 **Discussion**

299

300 In the present study, we found that deletion of the Cgl1427 gene encoding CMP kinase

301 reduced the ability of *C. glutamicum* to grow in oxygen-limiting static culture, and

302 caused a prolonged lag phase in the main culture with aerobic shaking when using seed

303 culture grown to a later growth stage in which oxygen limitation was observed. Since

304 both phenomena were concurrently resolved by amplification of genes encoding RNR, a

305 key enzyme in nucleotide synthesis that requires oxygen for its reaction, it was

306 suggested that Cgl1427 has a great impact on the efficiency of nucleotide synthesis

307 under microaerobic conditions. These findings indicate that the dispensable CMP kinase,
308 a nucleotide salvage pathway enzyme, plays an important role in the adaptability of an
309 aerobic microorganism *C. glutamicum* to low oxygen environment. These findings
310 direct our attention to the nucleotide synthetic pathway when seeking to improve the
311 adaptability to low oxygen environments in which producer strains are prone to fall.

312 The present study concentrated on CMP kinase and RNR. Deletion of the *cmk*
313 gene reduced enzymatic activities to trace amounts. The extensive deletion of the *cmk*
314 gene should not allow any enzymatic activities. Furthermore, no isozyme of CMP
315 kinase is found in the *C. glutamicum* genome. Therefore, the remaining activities are
316 now supposed to be due to the other unspecified enzyme(s) that catalyzes the
317 phosphorylation of CMP to CDP and/or dCMP to dCDP. However, because of the low
318 level of the remaining activity, such unspecified enzyme(s) hardly contributes to CDP
319 supply. The physiological role of CMP kinase is conversion of CMP, produced from
320 hydrolytic cleavage of mRNA or during synthesis of phospholipids, to the CDP required
321 for dCDP synthesis by RNR (Thum et al. 2009). *C. glutamicum* has only one RNR, a
322 so-called class I RNR due to its oxygen dependency. In *E. coli*, where only a single
323 copy of the *cmk* gene is present, *cmk* gene disruption decreased dCTP and dTTP pools,
324 most probably due to the decline in the CDP supply (Fricke et al. 1995). Moreover, it

325 has been reported that reduction of class I RNR activity progresses rapidly with

326 increasing hypoxia (Climploy et al. 2000). Taken together, low oxygen availability and

327 a decline in CDP supply synergistically limit the function of the RNR that is necessary

328 for nucleotide synthesis in *C. glutamicum*, which results in the impaired growth

329 observed in strain Δcmk in the oxygen-limiting static culture (Fig. 2 and 7), and which

330 influences the physiology of the cells in the later growth stage where oxygen

331 deprivation is severe. The physiological influence appears as a prolonged lag phase in

332 the following shaken culture (Fig. 3 and 4). The lag phase is a period during which cells

333 adjust their metabolism and prepare for cell proliferation (Folch-Mallol et al. 2004). It

334 was reported with *Lactococcus lactis* subsp. *lactis* (Larsen et al. 2006) and

335 *Saccharomyces cerevisiae* (Brejning et al. 2003) that higher expression levels of genes

336 involved in nucleotide synthesis are observed in the lag phase, rather than in

337 exponential or stationary phases. A reliable explanation for this observation is that the

338 cells accumulate nucleotides for DNA, RNA, and ATP synthesis before they start to

339 divide (Koistinen et al. 2007). The prolonged lag phase observed in strain Δcmk here

340 may be explained by reduced pools of nucleotides, due to low oxygen availability and

341 the decline in CDP supply. This condition of reduced nucleotide pool is carried into the

342 following culture, so more time is required to accumulate nucleotides to the

343 concentrations required for cellular proliferation. Considering that the increased level of
344 RNR by introducing pRNR complemented the both phenotypes of the *cmk* disruptant
345 (Fig. 6 and 7), it seems that oxygen is still barely present even in such low-oxygen
346 conditions. Therefore, a limited ability of RNR caused by low oxygen availability is not
347 attributed to shortage of oxygen required for radical formation in RNR. We assume that
348 low concentrations of oxygen reduce the reaction rate of RNR and thereby disable the
349 maintenance of a desirable level of nucleotide synthesis. Therefore, it is thought that the
350 enhanced level of RNR increases the total reaction rate for the ribonucleotide reduction
351 and thereby alleviates the impaired ability of the nucleotide synthesis under the
352 low-oxygen environments.

353 From an application standpoint, enhancement of CDP supply and/or
354 improvement of the RNR's functions might enable the development of low O_2-adapted
355 strains. We introduced pCcmk or pRNR into the wild-type strain, but no substantial
356 improvement in the ability to grow under oxygen-limiting static conditions was seen
357 (data not shown). It appears that such application is difficult for the strains retaining a
358 normal copy of these genes. It is now worthy proposing other possible ways to lead low
359 O_2-adapted strains. Utilization of oxygen-independent RNRs from other microbial
360 sources (Kolberg et al. 2004) is one of such candidates. Furthermore, in addition to

361 CMP kinase, utilization of polynucleotide phosphorylase, which phosphorolytically

362 cleaves mRNA to nucleoside diphosphates containing CDP (Favaro and Deho 2003),

363 might be effective for increased CDP supply. The corresponding gene is present in the *C.*

364 *glutamicum* genome, but the functional analysis of the gene has yet to be performed.

365 However, more importantly, industrial strains that have traditionally been constructed

366 by multiple rounds of mutagenesis generally have weak constitutions, showing

367 sensitivities to various stresses, including limited oxygen availability, as compared to

368 the ancestral wild-type strains (Ikeda et al. 2009). Although the genetic backgrounds of

369 such traits remain undefined, Cgl1427 is a promising target for cellular engineering of

370 industrial strains to improve their performance under low oxygen conditions, because of

371 its cross-complementation ability between the different high-oxygen-requiring mutants

372 of *C. glutamicum* derived by random mutagenesis (Ikeda et al. 2009).

373

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375

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378

379 **References**

380

381 Blondin C, Serina L, Wiesmuller L, Gilles AM, Barzu O (1994) Improved

382 spectrophotometric assay of nucleoside monophosphate kinase activity using the

383 pyruvate kinase/lactate dehydrogenase coupling system. Anal Biochem 220:219-221

384 Brejning J, Jespersen L, Arneborg N (2003) Genome-wide translational changes during

385 the lag phase of *Saccharomyces cerevisiae*. Arch Microbiol 179:278-294

386 Chien LJ, Chen HT, Yang PF, Lee CK (2006) Enhancement of cellulose pellicle

387 production by constitutively expressing *Vitreoscilla* hemoglobin in *Acetobacter*

388 *xylinum*. Biotechnol Prog 22:1598-1603

389 Climploy K, Tassotto ML, Mathews CK (2000) Ribonucleotide reductase, a possible

390 agent in deoxyribonucleotide pool asymmetries induced by hypoxia. J Biol Chem

391 275:39267-39271

392 Cotruvo JA Jr. and Stubbe J (2010) An active dimanganese(III)-tyrosyl radical cofactor

393 in *Escherichia coli* class Ib ribonucleotide reductase. Biochemistry 49:1297-1309

394 Favaro R, Deho G (2003) Polynucleotide phosphorylase-deficient mutants of

395 *Pseudomonas putida*. J Bacteriol 185:5279-5286

396 Folch-Mallol JL, Martinez LM, Casas SJ, Yang R, Martinez-Anaya C, Lopez L,

397 Hernandez A, Nieto-Sotelo J (2004) New roles for *CDC25* in growth control,

398 galactose regulation and cellular differentiation in *Saccharomyces cerevisiae*.
399 Microbiology 150:2865-2879
400 Fricke J, Neuhard J, Kelln RA, Pedersen S (1995) The *cmk* gene encoding cytidine
401 monophosphate kinase is located in the *rpsA* operon and is required for normal
402 replication rate in *Escherichia coli*. J Bacteriol 177:517-523
403 Hofer A, Crona M, Logan DT, Sjoberg BM (2012) DNA building blocks: keeping
404 control of manufacture. Crit Rev Biochem Mol Biol 47:50-63
405 Horng YT, Chang KC, Chien CC, Wei YH, SunYM, Soo PC (2010) Enhanced
406 polyhydroxybutyrate (PHB) production via the coexpressed *phaCAB* and *vgb* genes
407 controlled by arabinose P-BAD promoter in *Escherichia coli*. Lett Appl Microbiol
408 50:158-167
409 Ikeda M (2003) Amino acid production processes. In: Faurie R, Thommel J (eds) Adv
410 Biochem Eng Biotechnol, vol. 79, Microbial production of L-amino acids. Springer,
411 Berlin, Heidelberg, New York, pp 1-35
412 Ikeda M, Baba M, Tsukamoto N, Komatsu T, Mitsuhashi S, Takeno S (2009)
413 Elucidation of genes relevant to the microaerobic growth of *Corynebacterium*
414 *glutamicum*. Biosci Biotechnol Biochem 73:2806-2808

415 Ikeda M, Nakagawa S (2003) The *Corynebacterium glutamicum* genome: features and
416 impacts on biotechnological processes. Appl Microbiol Biotechnol 62:99-109
417 Kinoshita S, Nakayama K (1978) Amino acids. In: Rose AH (ed) Primary products of
418 metabolism. Academic, London, pp 209-261
419 Koistinen KM, Plumed-Ferrer C, Lehesranta SJ, Karenlampi SO, von Wright A (2007)
420 Comparison of growth-phase-dependent cytosolic proteomes of two *Lactobacillus*
421 *plantarum* strains used in food and feed fermentations. FEMS Microbiol Lett
422 273:12-21
423 Kolberg M, Strand KR, Graff P, Andersson KK (2004) Structure, function, and
424 mechanism of ribonucleotide reductases. Biochim Biophys Acta 1699:1-34
425 Larsen N, Boye M, Siegumfeldt H, Jakobsen M (2006) Differential expression of
426 proteins and genes in the lag phase of *Lactococcus lactis* subsp. *lactis* grown in
427 synthetic medium and reconstituted skim milk. Appl Environ Microbiol
428 72:1173-1179
429 Li M, Wu J, Lin J, Wei D (2010) Expression of *Vitreoscilla* hemoglobin enhances cell
430 growth and dihydroxyacetone production in *Gluconobacter oxydans*. Curr Microbiol
431 61:370-375

432 Liu Q, Zhang J, Wei XX, Ouyang SP, Wu Q, Chen GQ (2008) Microbial production of
433 L-glutamate and L-glutamine by recombinant *Corynebacterium glutamicum*
434 harboring *Vitreoscilla* hemoglobin gene *vgb*. Appl Microbiol Biotechnol
435 77:1297-1304
436 Liu Y, Gong G, Xie L, Yuan N, Zhu C, Zhu B, Hu Y (2010) Improvement of
437 cephalosporin C production by recombinant DNA integration in *Acremonium*
438 *chrysogenum*. Mol Biotechnol 44:101-109
439 Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M (2004) A gene homologous to β-type
440 carbonic anhydrase is essential for the growth *Corynebacterium glutamicum* under
441 atmospheric conditions. Appl Microbiol Biotechnol 63:592-601
442 Oehlmann W, Auling G (1999) Ribonucleotide reductase (RNR) of *Corynebacterium*
443 *glutamicum* ATCC 13032–genetic characterization of a second class IV enzyme.
444 Microbiology 145:1595-1604
445 Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M (2002) A
446 novel methodology employing *Corynebacterium glutamicum* genome information to
447 generate a new L-lysine-producing mutant. Appl Microbiol Biotechnol 58:217-223

448 Oka T (1999) Amino acids, production processes. In: Flickinger MC, Drew SW (eds)
449 Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation.
450 Wiley, New York, pp 89-100
451 Priscila G, Fernandez FJ, Absalon AE, Suarez MR, Sainoz M, Barrios-Gonzalez J,
452 Mejia A (2008) Expression of the bacterial hemoglobin gene from *Vitreoscilla*
453 *stercoraria* increases rifamycin B production in *Amycolatopsis mediterranei*. J Biosci
454 Bioeng 106:493-497
455 Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol
456 treatment. Biochim Biophys Acta 72:619-629
457 Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold
458 Spring Harbor Laboratory Press, Cold Spring Harbor, New York
459 Schultz CP, Ylisastigui-Pons L, Serina L, Sakamoto H, Mantsch HH, Neuhard J, Barzu
460 O, Gilles AM (1997) Structural and catalytic properties of CMP kinase from *Bacillus*
461 *subtilis*: a comparative analysis with the homologous enzyme from *Escherichia coli*.
462 Arch Biochem Biophys 340:144-153
463 Sjoberg BM (2010) A never-ending story. Science 329:1475-1476

464 Takeno S, Ohnishi J, Komatsu T, Masaki T, Sen K, Ikeda M (2007) Anaerobic growth
465 and potential for amino acid production by nitrate respiration in *Corynebacterium*
466 *glutamicum*. Appl Microbiol Biotechnol 75:1173-1182
467 Thum C, Schneider CZ, Palma MS, Santos DS, Basso LA (2009) The Rv1712 locus
468 from *Mycobacterium tuberculosis* H37Rv codes for a functional CMP kinase that
469 preferentially phosphorylates dCMP. J Bacteriol 191:2884-2887
470 Yukawa H, Omumasaba CA, Nonaka H, KosP, Okai N, Suzuki N, Suda M, Tsuge Y,
471 Watanabe J, Ikeda Y, Vertes AA, Inui M (2007) Comparative analysis of the
472 *Corynebacterium glutamicum* group and complete genome sequence of strain R.
473 Microbiology 153:1042-1058
474 Zhu H, Sun S, Zhang S (2011) Enhanced production of total flavones and
475 exopolysaccharides via *Vitreoscilla* hemoglobin biosynthesis in *Phellinus igniarius*.
476 Bioresour Technol 102:1747-1751
477 van der Rest ME, Lange C, Molenaar D (1999) A heat shock following electroporation
478 of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol
479 Biotechnol 52:541-545
480

481 **Figure Legends**

482

483 **Fig. 1** Metabolic pathway of pyrimidine nucleotides. CMP is produced by the hydrolytic
484 cleavage of mRNA or during synthesis of phospholipids (dotted arrows). CMP kinase
485 catalyzes phosphorylation of CMP to CDP that is required for dCDP synthesis by
486 ribonucleotide reductase (RNR). *C. glutamicum* has only one RNR and it is an
487 oxygen-dependent enzyme.

488

489 **Fig. 2** Growth of the *cmk* disruptant in oxygen-limiting static culture. Strains were
490 statically cultivated in 5 mL of MM medium in test tubes at 30°C. Cultivation was
491 started by the addition of 50 μL of seed culture grown in BY medium. Symbols: *solid*
492 *circles*, *C. glutamicum* ATCC 31833 carrying pCS299P; *solid squares*, strain Δcmk
493 carrying pCS299P; *solid triangles*, strain Δcmk carrying pCcmk; *solid diamonds*, strain
494 Δcmk carrying pEcmk. Values are means for triplicate cultures.

495

496 **Fig. 3** Growth of the *cmk* disruptant in the main culture with aerobic shaking. Strains
497 were cultivated in 5 mL of MM medium in L-type test tubes subjected to Monod
498 shaking at 48 strokes/min. The cultivation was started by the addition of 50 μL (a), 5 μL

499 (b), or 0.5 μL (c) of seed culture grown in BY medium to the late-exponential phase.

500 Symbols: *solid circles*, *C. glutamicum* ATCC 31833 carrying pCS299P; *solid squares*,

501 strain Δcmk carrying pCS299P; *solid triangles*, strain Δcmk carrying pCcmk; *solid*

502 *diamonds*, strain Δcmk carrying pEcmk. Values are means for triplicate cultures.

503

504 **Fig. 4** Effect of different seed culture growth phases on lag phase interval for *cmk*

505 disruptant in the main culture. (a) Progress in oxygen depletion under the shaking

506 conditions set for the seed culture was assessed by monitoring changes in the color of

507 the oxygen indicator resazurin. Resazurin changes color from blue to pink in response

508 to gradual oxygen depletion, and is colorless under severe oxygen depletion. Cultivation

509 was carried out at 30°C in 5 mL of MM medium in test tubes subjected to reciprocal

510 shaking at 120 strokes/min. Inset color photographs showing the progression of oxygen

511 depletion in (a) were obtained from strain Δcmk carrying pCS299P. The same results

512 were obtained from strains ATCC 31833 carrying pCS299P and Δcmk carrying

513 pCS299P. (b, c, d) Strains were cultivated in 5 mL of MM medium in L-type test tubes

514 subjected to Monod shaking at 48 strokes/min. Seed culture used for each experiment

515 were grown in BY medium grown to the late-exponential phase (OD_{660} = 4.0) (b),

516 mid-exponential phase (OD_{660} = 2.0) (c), and early-exponential phase (OD_{660} = 1.0) (d),

517 respectively. Symbols: *solid circles*, *C. glutamicum* ATCC 31833 carrying pCS299P;

518 *solid squares*, strain Δcmk carrying pCS299P; *solid triangles*, strain Δcmk carrying

519 pCcmk. Values are means for triplicate cultures.

520

521 **Fig. 5** Arrangement of RNR genes in *C. glutamicum* genome and construction of

522 plasmid pRNR for amplification of RNR genes. The genomic regions comprising the

523 *nrdF* gene and *nrdHIE* operon from *C. gluamicum* ATCC 31833 were amplified by PCR

524 using pairs of primers, nrdFf (primer 2) and nrdFr (primer 1), and nrdHIEf (primer 4)

525 and nrdHIEr (primer 3), respectively. Restriction sites: *B*, *Bam*HI; *K*, *Kpn*I.

526

527 **Fig. 6** Effect of amplification of RNR genes on growth of the *cmk* disruptant in shaken

528 culture. Strains were cultivated at 30°C in 5 mL of MM medium in L-type test tubes

529 subjected to Monod shaking at 48 strokes/min. Cultivation was started by the addition

530 of 5 μL (a) or 0.5 μL (b) of seed culture grown in BY medium to the late-exponential

531 phase (OD_{660} = 4.0). Symbols: *solid circles*, *C. glutamicum* ATCC 31833 carrying

532 pCS299P; *solid squares*, strain Δcmk carrying pCS299P; *solid triangles*, strain Δcmk

533 carrying pCcmk; *open circles*, strain Δcmk carrying pRNR. Values are means for

534 triplicate cultures.

535

536 **Fig. 7** Effect of amplification of RNR genes on growth of the *cmk* disruptant in

537 oxygen-limiting static culture. Strains were statically cultivated in 5 mL of MM medium

538 in test tubes at 30°C. Cultivation was started by the addition of 50 μL of seed culture

539 grown in BY medium. Symbols: *solid circles*, *C. glutamicum* ATCC 31833 carrying

540 pCS299P; *solid squares*, strain Δcmk carrying pCS299P; *solid triangles*, strain Δcmk

541 carrying pCcmk; *open circles*, strain Δcmk carrying pRNR. Values are means for

542 triplicate cultures.

543

544 Table 1. Sequences of primers used in this study

Primer	Sequence[a]
nrdHIEf	5’-CTGGGTACCGGAGTGTTTTGGGTTGT-3’
nrdHIEr	5’-CACGGTACCAGCGGAATTCGCGGAAA-3’
nrdFf	5’-ATG*GGATCC*TAGTGGCGATAATTTAGG-3’
nrdFr	5’-CTT*GGATCC*AAAGGTGTGAAGGGGTT-3’
Pr8	5’-AGCGGATAACAATTTCACACAGGAAAC-3’
Cgl1427FusR	5’-AGGTGGATGAGGTGATCAAGTACTTGGAGGCCACCGGCAGGCATGTTGG-3’
Cgl1427FusF	5’-CCAACATGCCTGCCGGTGGCCTCCAAGTACTTGATCACCTCATCCACCT-3’
Pr7	5’-CGCCAGGGTTTTCCCAGTCACGAC-3’
cmkup200FBamHI	5’-AAC*GGATCC*GTGATCGTATCGCCTCTG-3’
cmkdown400RBamHI	5’-GCG*GGATCC*AGGCTTCGTGACGTTTAG-3’

545 [a]*Kpn*I sites are underlined, and *Bam*HI sites are italicized.

546

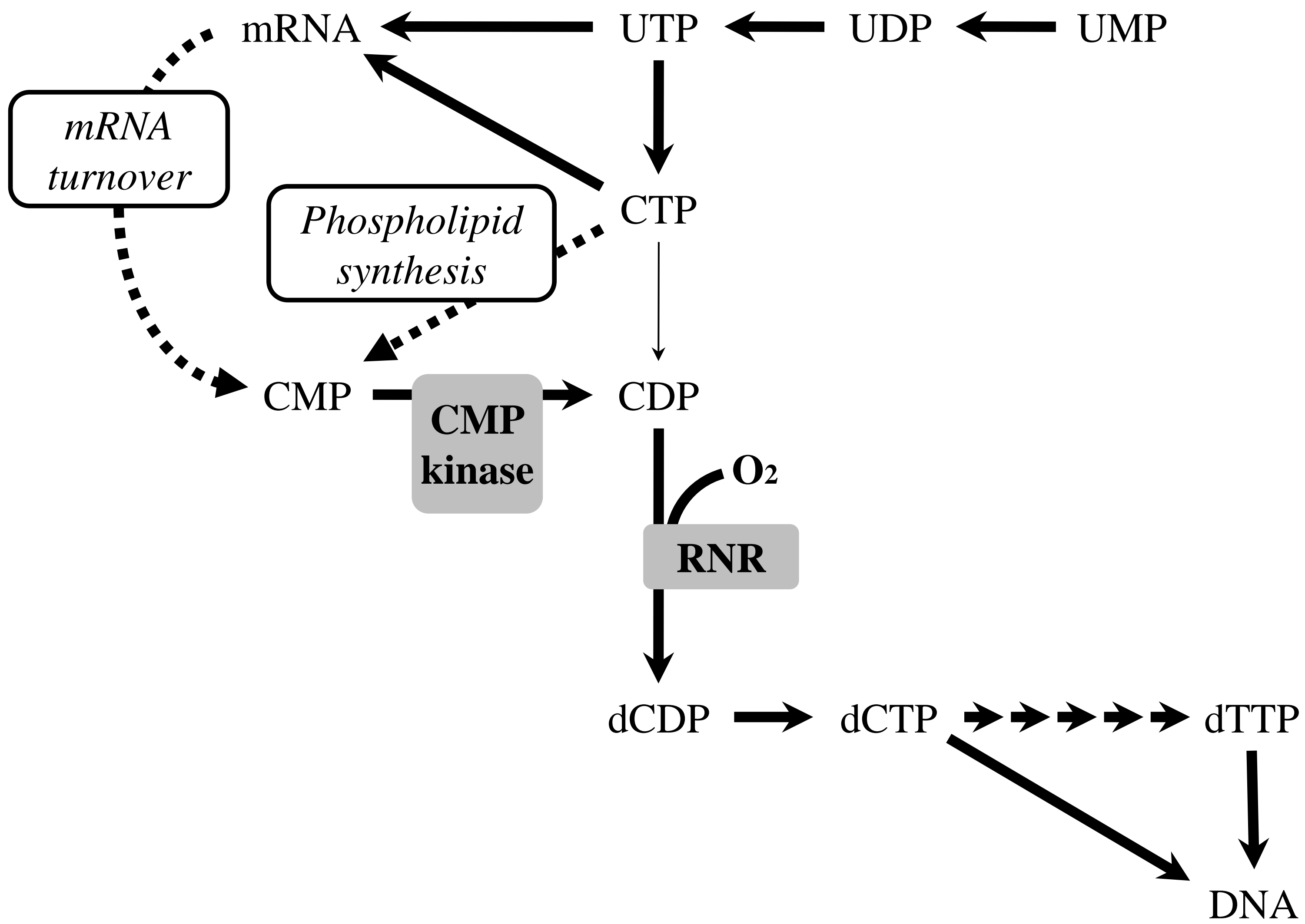
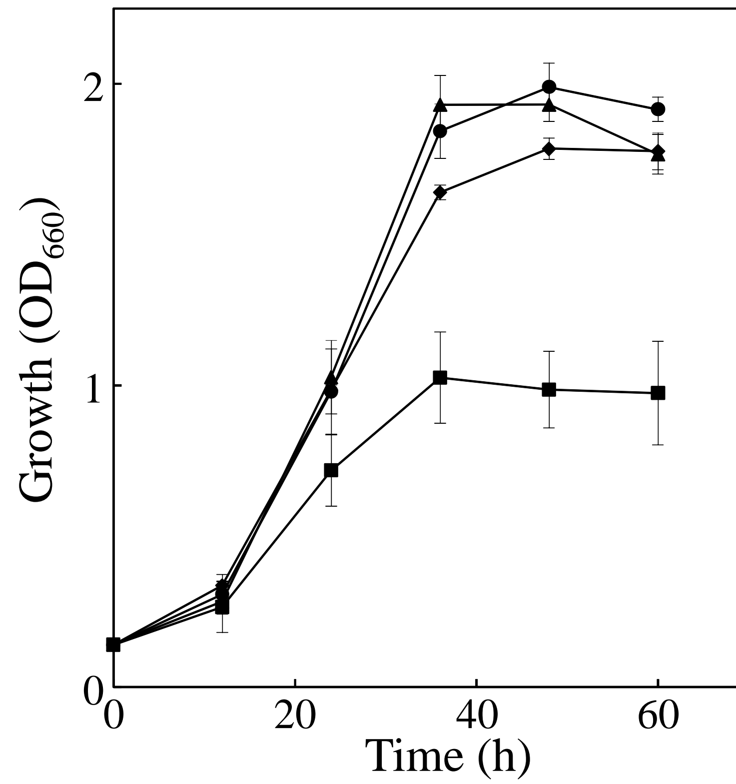


Fig. 1 Takeno et al.



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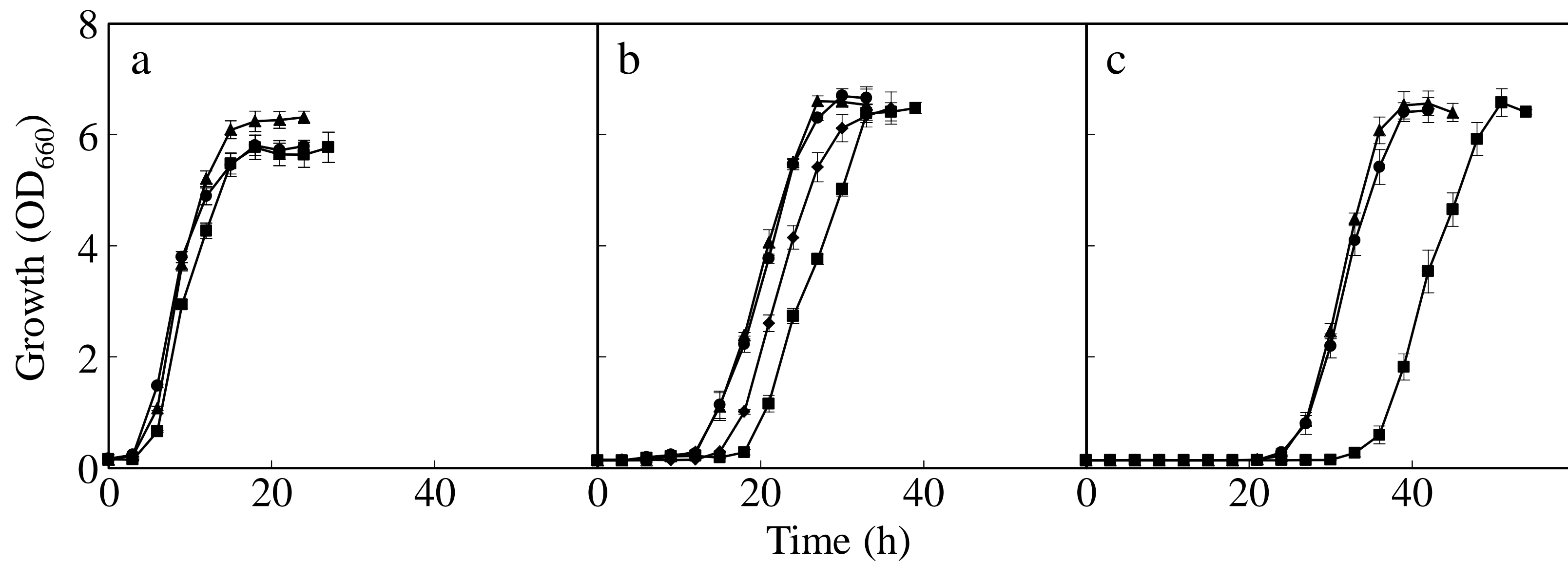

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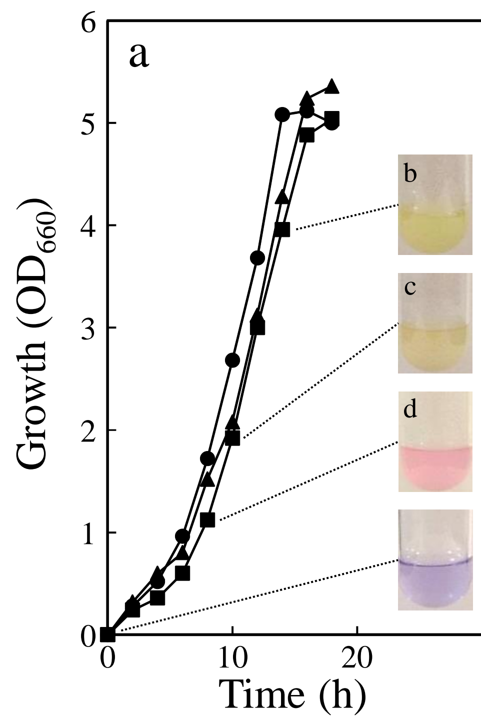

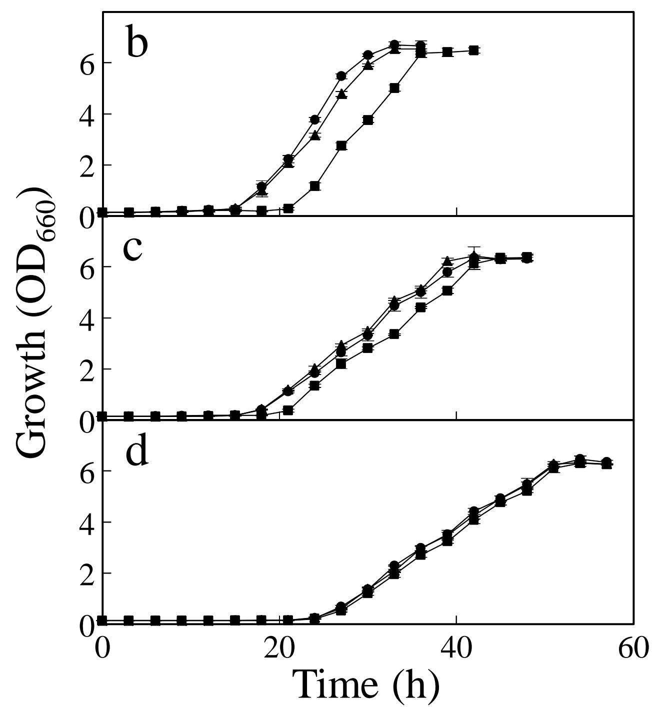

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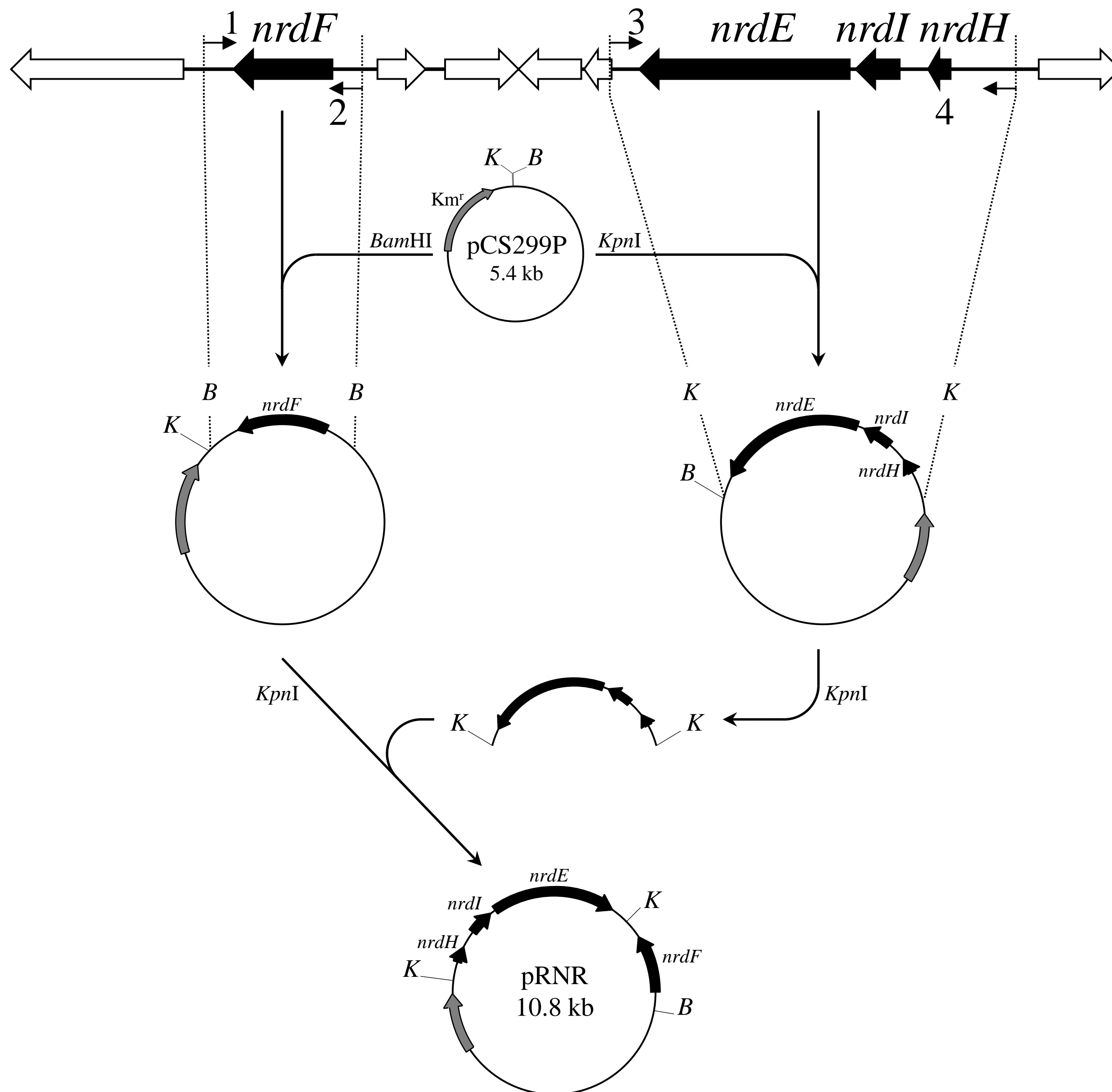

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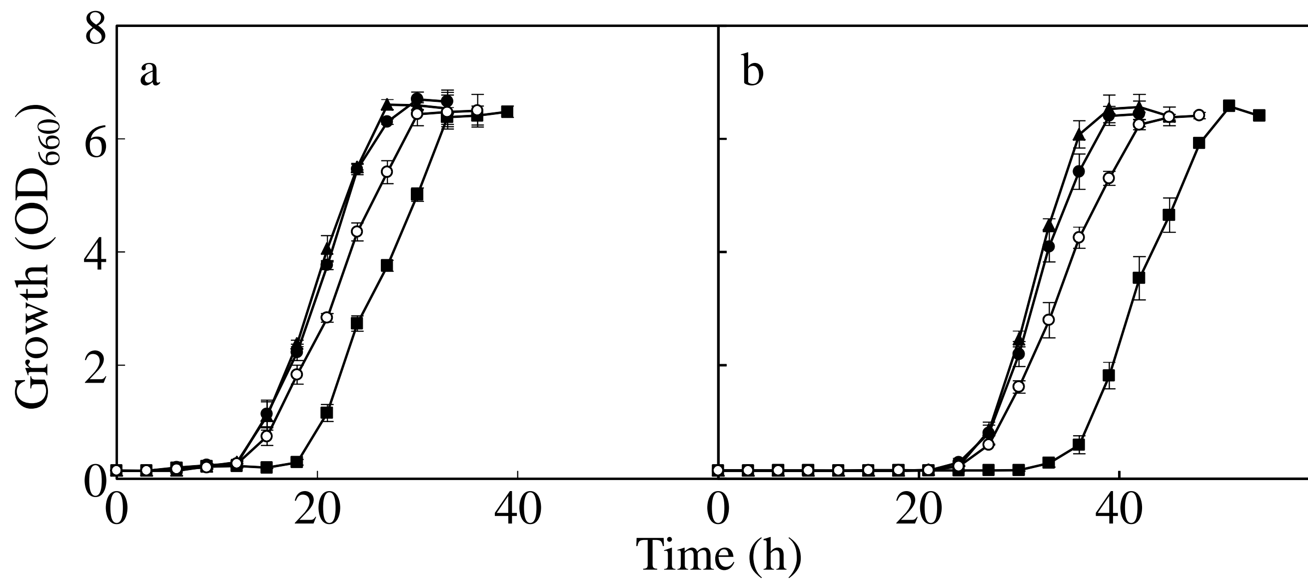

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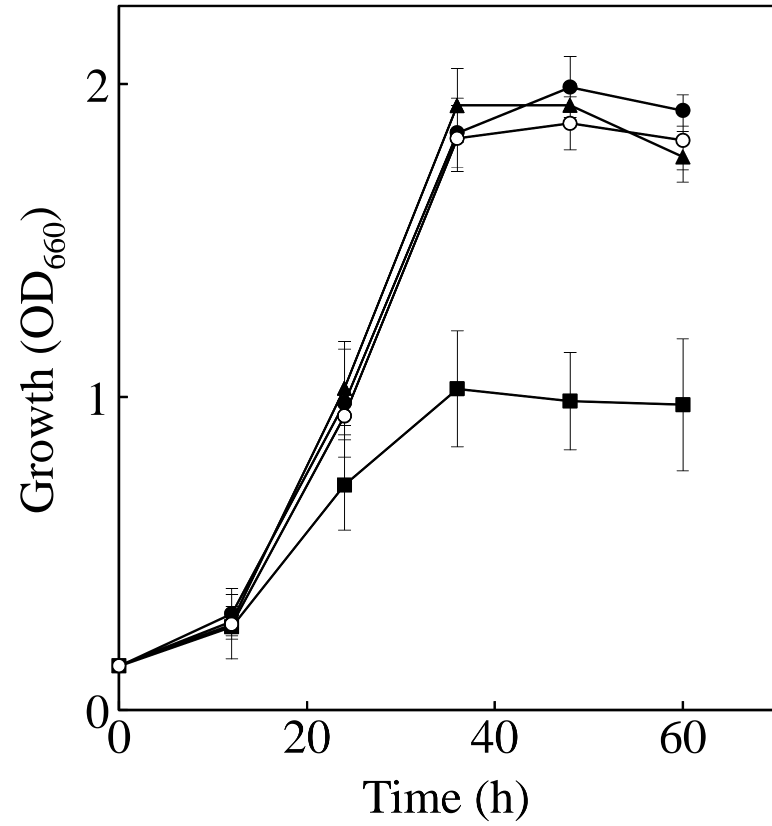

Fig. 7   Takeno et al.