1   **High energy-charged cell factory for heterologous protein synthesis.**

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3   Yeong Deok Kwon[1], Hye Jung Kim[1], Sang Yup Lee[2], and Pil Kim[1]

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5   *[1]Department of Biotechnology, Catholic University of Korea, Bucheon, Gyunggi 420-*

6   *743, Korea;*

7   *[2]Department of Chemical and Biomolecular Engineering, KAIST, Daejon 305-701,*

8   *Korea.*

9

10

11   Correspondence to

12   Pil Kim, Ph.D., Asso. Prof.

13

14   Department of Biotechnology

15   Catholic University of Korea,

16   Bucheon, Gyunggi 420-743, Korea

17

18   T. +82-2-2164-4922

19   F. +82-2-2164-4865

20   E. kimp@catholic.ac.kr

21

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23   **Overexpression of gluconeogenic phosphoenolpyruvate carboxykinase (PCK)**

24   **under glycolytic conditions enables *Escherichia coli* to maintain a greater**

25   **intracellular ATP concentration and, consequently, to up-regulate genes for amino**

26   **acid and nucleotide biosynthesis. To investigate the effect of a high intracellular**

27   **ATP concentration on heterologous protein synthesis, we studied the expression of**

28   **a foreign gene product, enhanced green fluorescence protein (eGFP), under control**

29   **of the T7 promoter in *E. coli* BL21(DE3) strain overexpressing PCK. This strain**

30   **was able to maintain twice as much intracellular ATP and to express two times**

31   **more foreign protein than the control strain. These results indicate that a high**

32   **energy-charged cell can be beneficial as a protein-synthesizing cell factory. The**

33   **potential uses of such a cell factory are discussed.**

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Introduction

Adenosine triphosphate (ATP) is the universal energy currency of the cell, transferring the chemical energy from metabolism to various biological activities in all life forms on earth[1]. ATP is produced during the processes of photosynthesis, fermentation, and respiration, and it is consumed by many processes, such as biosynthesis, motility, cell signaling, and cell division. ATP consists of adenosine and three phosphates and chemical energy is released by cleavage of a phosphate to produce adenosine diphosphate (ADP) + phosphate. Since ATP is an unstable molecule, it is hydrolyzed spontaneously and therefore is continuously recycled in living organisms; i.e., a human turns over the equivalent of its body weight in ATP every day[2]. It is well known that the intracellular ATP concentration is one of the most important factors for cellular physiology, since it regulates many cellular events, such as growth[3], ribosomal RNA synthesis[4], and cellular metabolic pathways[5].

Biomaterial synthesis is an age-old knowledge involved in the manufacturing of bread, beer, wine, and cheese. The recent concept of biomaterial synthesis, however, is the use of live cells as factories for manufacturing highly valued biomaterials, such as proteins, amino acids, alcohols, organic acids, solvents, as well as bioplastics used in molecular biology and bioinformatics[6]. Many living cell factory models have been studied for the

55   synthesis of a variety of biomaterials: *Escherichia coli*[7], *Bacillus subtilis*[8],

56   *Corynebacterium glutamicum*[9], yeast[10], lactic acid bacteria[11], thermophile[12],

57   actinomycetes[13], filamentous fungi[14], plant cells[15], and mammian cells[16].

58   Some reports have described reduced macromolecular synthesis in the presence of a

59   lower intracellular concentration of ATP[17]. Conversely, one might expect that a higher

60   than normal intracellular ATP level would be beneficial for biomaterial synthesis in cell

61   factories. Enhanced production of intracellular ATP by overexpression of gluconeogenic

62   phosphoenolpyruvate carboxykinase (PCK) under glycolytic conditions enables *E. coli*

63   to up-regulate genes for amino acid and nucleotide synthesis as well as flagella

64   components[18] (Fig. 1), a finding which strengthens the above hypothesis. A mouse

65   whose cell constitutively express PCK has been reported to be super athlete since it ran

66   over 6 km when control mouse only ran 0.2 km[19]. Therefore, a verification of the high

67   energy-charged single cell as a factory works better for human purposed biomolecule

68   synthesis is required.

69   In this paper, we artificially expressed heterologous enhanced green fluorescence

70   protein (eGFP) in *E. coli* BL21(DE3) that maintain a high intracellular ATP to

71   determine whether this strain is eligible for use as a powerful, protein-synthesizing cell

72   factory. The potential uses of such a high energy-charged cell factory are also discussed.

73   **Results**

74   To estimate heterologous protein expression in a high energy-charged cell, enhanced

75   green fluorescence protein (eGFP) was expressed under control of the T7 promoter in

76   an *E. coli* BL21(DE3) host (Table 1). Strain BL21(DE3) was the control host while

77   BL21(DE3) expressing phosphoenolpyruvate carboxykinase (PCK) was the high

78   energy-charged host. Strain BL21(DE3)/pEGFP (BL21[DE3] expressing eGFP by the

79   T7 promoter) and BL21[DE3]/pEcPCK/pEGFP (BL21[DE3] co-expressing eGFP by the

80   T7 promoter and PCK by the *trc* promoter) were inoculated in LB-glucose medium

81   containing 50 μg/mL of antibiotics. An isopropyl-β-D-thiogalactopyranoside (IPTG, 1

82   mM) was supplemented in the log phase (optical density; O.D. = 0.6) to induce eGFP

83   expressions. The intracellular ATP concentrations after 6 h induction was 43.1 nmol/mg-

84   protein   for   the   BL21(DE3)/pEGFP   and   84.5   nmol/mg-protein   for   the

85   BL21(DE3)/pEcPCK/pEGFP, which harbored 2-times greater [ATP]. The BL21(DE3)

86   expressing PCK showed same pattern of greater [ATP] than that of BL21(DE3) (42.3 vs.

87   78.4 nmol/mg-protein). The total protein contents of BL21(DE3)/pEGFP was 0.60

88   mg/mL and that of the BL21(DE3)/pEcPCK/pEGFP was 0.55 mg/mL. The amount of

89   eGFP   expression   in   BL21(DE3)   overexpressing   PCK   was   957.8   RF(relative

90   fluorescence)/mL, which is more than 2-times greater than that observed for the control

5

91   strain (443.5 RF/mL) (Fig 2). The specific amount of eGFP produced by the unit cell of

92   high energy-charged *E. coli* (1741.5 RF/mg-protein) was 2.4-times greater than that

93   produced by the control (739 RF/mg-protein).

94

95   **Discussion**

96   The high energy-charged *E. coli* was beneficial for the expression of foreign protein

97   (Table 1, Fig. 2, Suppl. 1). One explanation for this result could be that these high

98   energy-charged cells up-regulate the genes for biosynthesis of amino acids[18], thus

99   increasing amino acid biosynthesis turnover. An increase in the rate of production of

100   ribosomes by cells at a higher energy state has been reported[4] and it is another potential

101   explanation for the observed enhancement of foreign protein synthesis (Suppl. 2). The

102   decreased growth rate observed for the high energy-charged cells were found in the LB-

103   glucose and the minimal glucose medium (data not shown) and that is reasonable

104   considering that a high energy charge has been reported to limit the cellular growth by

105   restriction of the glycolytic flux[20], under such conditions, the carbon would be re-

106   directed to foreign protein synthesis rather than to growth. *E. coli* BL21(DE3) has been

107   widely used for production of recombinant proteins and the whole genome sequence of

108   BL21(DE3) was reported lacks of genes for the motility (21 *fli* genes) as well as Lon

109 protease [21]. The amount eGFP from the high energy-charged BL21(DE3) host (957.8

110 RF/mL) was found to be about 57% more than that from the high energy-charged

111 W3110(DE3) host (610.1 RF/mL, Spuppl. 3). Lacks of motility in BL21(DE3) would

112 have saved energy for movement and produced more proteins than that in flagella

113 moving W3110(DE3) strain, which also supports that the more [ATP] contributes more

114 foreign protein expression hypothesis. Aminoacyl-tRNA synthetase requires ATP to

115 mediate amino acid-charged tRNA synthesis. The mRNA concentrations of alanyl-

116 tRNA synthetase and glutaminyl-tRNA synthetase in the high energy-charged

117 BL21(DE3)/pEcPCK was 1.5-times higher than those in BL21(DE3) control (Suppl. 4),

118 which implying greater intracellular [ATP] might have contributed the enhanced protein

119 synthesis by providing more AA-charged tRNA during translation process. In

120 combination, reduction in growth rate, the increase in the size of the amino acids

121 turnover, the enhanced production of ribosomes, and the more charged tRNA would

122 have a synergistic effect on foreign protein synthesis.

123 The use of high energy-charged cells could be beneficial to other cell factory

124 applications in addition to foreign protein synthesis. For instance, high energy-charged

125 cells might be able to enhance the production of a metabolite such as succinic acid, a

126 widely used specialty chemical[22]. When a recombinant cell directs its metabolism

7

127   toward production of a target metabolite, it tends to restrict its metabolic pathways by

128   way of feedback control. If the target metabolite remains inside the cell factory, and

129   accumulates to a high concentration, it would eventually inhibit its own synthesis.

130   Active transport the of target metabolite using the energy of the high-powered cell

131   factory could prevent feedback control from occurring, thereby enabling synthesis via

132   the desired pathways to continue unabated[23]. In addition to the export of products, the

133   high-level intracellular energy could be useful for the import of raw materials into the

134   cell factory; one could expect to increase the rate of biomaterial synthesis once the

135   regulation of the carbon influx by the high energy-charged cells is understood. Secretion

136   and surface display of proteins might also be enhanced by the availability of high-level

137   intracellular energy[24].

138   It is not clear whether high energy-charged cells other than *E. coli* would be better

139   suited as cell factories for foreign protein synthesis. Studies on the engineering and use

140   of other types of cells (i.e., yeast, mammalian cell) as high energy-charged cell factories

141   are therefore needed.

142

143   **Methods**

144   *Strain and plasmids*

145   All strains, plasmids, and oligonucleotides are summarized in Table 2. The gene for

146   eGFP was amplified by PCR using the oligonucleotides of <u>gaattc</u>atggtgagcaagggcgagga

147   (*Eco*RI site underlined; forward I primer) and <u>ctcgag</u>cttgtacagctcgtccatgcc (*Xho*I site

148   underlined; reverse primer) based on pMEGFP as a template. The resulting DNA

149   fragment (0.7 kb) was sub-cloned into an expression vector (pET41a, Novagen,

150   Darmstadt, Germany) after sub-cloning into a PCR-cloning vector (pGEM-T easy

151   vector, Promega, Madison, WI, USA), verification by DNA sequencing (Bioneer Co.,

152   Daejon, Korea), and double digestion with *Eco*RI and *Xho*I, resulting pEGFP for eGFP

153   expression. The constructed vectors were transformed into the BL21(DE3) and

154   BL21(DE3)/pEcPCK strains by electrophoration (BTX ECM, Harvard Apparatus,

155   Holliston, MS, USA).

156

157   *Media and culture*

158   Luria-Bertani medium was used for routine DNA manipulations. The LB-glucose

159   medium contained glucose (9 g/L), $NaHCO_3$ (10 g/L), yeast extract (5 g/L), tryptone

160   (10 g/L), NaCl (10 g/L), and antibiotics (ampicillin and kanamycin, 50 μg/mL)[25]. Single

161   colony was inoculated into a 15-mL tube containing 4 mL medium and maintained at

162   37ºC and 250 rpm for 12h. Four hundred microliter of culture was transferred into a

163 250-mL Erlenmeyer flask containing 50 mL medium and maintained at 37ºC and 250

164 rpm. When the optical density (O.D.) of culture reached 0.6, isopropyl-β-D-

165 thiogalactopyranoside (IPTG, 1 mM) was supplemented to allow eGFP expression.

166 After 6 h after IPTG induction, culture was withdrawn for the analysis.

167

168 *Analysis*

169 Biomass was estimated by measuring O.D. at 600 nm. Intracellular [ATP] and PCK

170 enzyme activity were determined as described previously[26]. The protein concentration

171 of the enzyme solution was determined using a protein assay kit (Bio-Rad, La Jolla, CA,

172 USA) with bovine serum albumin as a standard after the sonic disruption of the sample

173 for 5 min at 140 W on ice using a UP200S ultrasonic processor (Hielscher Ultrasonics

174 Co., Teltow, Germany). To estimate the amount of eGFP expression, a 700 µL-sample

175 was subjected to fluorescence spectroscopy (RF-5301PC, Shimadzu, Kyoto, Japan) with

176 excitation at 395 nm and emission at 509 nm. Cells were observed using phase contrast

177 and fluorescence microscopy (AX-70, Olympus Co, Tokyo, Japan).

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184   **References**

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234   **Table 1. Effect of intracellular ATP level on eGFP expression.**

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240   **Figure 1. Biochemical reactions between PEP and OAA in high energy-charged *E.***

241   ***coli.***

242   Biochemical reactions under glycolytic and gluconeogenic conditions occurring in

243   BL21(DE3) and high energy-charged BL21(DE3).

244   *pck*: PEP carboxykinase, *ppc*: PEP carboxylase. Superscripts *p* and *c* indicate promoter-

245   origin and chromosome-origin, respectively.

246

247   **Figure 2. Microscopic images of *E. coli* BL21(DE3) expressing eGFP.**

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249

250 **Table 2. Effect of intracellular energy level on eGFP expression[a].**

251

Strain	Biomass		Intracellular [ATP] (nmol/mg-protein)	Volumetric eGFP expression[b] (fluorescence intensity/mL)	Specific eGFP expression[d] (fluorescence intensity/mg-protein)
	O.D.$_{600nm}$	Total protein (mg/mL)			
BL21(DE3)	1.98 $_{\pm0.01}$	0.62 $_{\pm0.01}$	42.3 $_{\pm1.5}$	-	-
BL21(DE3)/pEcPck	2.06 $_{\pm0.03}$	0.64 $_{\pm0.05}$	78.4 $_{\pm6.3}$	-	-
BL21(DE3)/pTGFP	1.98 $_{\pm0.01}$	0.60 $_{\pm0.03}$	43.1 $_{\pm4.6}$	443.5 $_{\pm21.9}$	739.2 $_{\pm36.5}$
BL21(DE3)/pEcPck/pEGFP	1.77 $_{\pm0.09}$	0.55 $_{\pm0.01}$	84.5 $_{\pm4.7}$	957.8 $_{\pm30.3}$	1741.5 $_{\pm55.1}$

252

253 [a]All experiments were repeated at least 5 times. Samples were harvested at 6 h of IPTG

254 induction. Cells were cultured in a 250-mL Erlenmeyer flask containing 5 mL medium

255 at 250 rpm, and 37°C.

256 [b]A culture sample was directly subjected to fluorescence spectrophotometry (excitation

257 at 395 nm, emission at 509 nm) for estimation of eGFP expression.

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259

Table 2. Strains and plasmids

Strains	Description	Source
Oligonucleotides		
Forward eGFP	<u>gaattc</u>atggtgagcaagggcgagga (*Eco*RI site underlined)	This study
Reverse eGFP	<u>ctgcag</u>cttgtacagctcgtccatgcc (*Xho*I site underlined)	This study
Plasmids		
pGEM-T	T&A cloning vector, Ap^R	Promega
pET41a	Expression vector, p$_{T7}$, Km^R	Novagen
pMEGFP	eGFP expression vector, p$_{tac}$, Ap^R	Donated by Dr. Seong Goo Lee, (KRIBB) [26]
pEcPck	pTrc99A containing PCK gene	
pEGFP	pET41a containing eGFP at *Eco*RI-*Xho*I sites	This study
Strains		
DH5α	F- Φ80 *dlacZ* ΔM15 Δ (*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (rK-mK+) *deoR thi-1 phoA supE44 λ- gyrA96 relA1*	Invitrogen
BL21(DE3)	F⁻ *ompT gal dcm lon hsdS$_B$* (r$_B^-$ m$_B^-$; *E. coli* B strain), with DE3, a λ prophage carrying the T7 RNA *pol* gene	

265
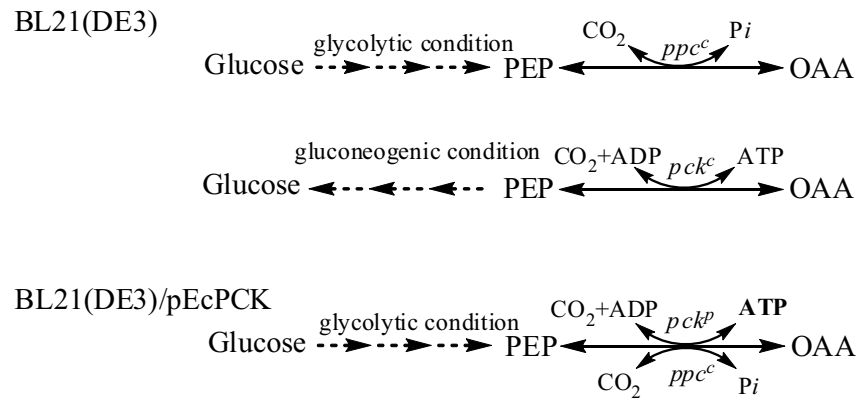
266

BL21(DE3)

Glucose $\cdots\blacktriangleright\cdots\blacktriangleright\cdots\blacktriangleright$ PEP $\longleftarrow$ CO_2 $\underset{ppc^c}{\curvearrowright}$ Pi $\longrightarrow$ OAA

Glucose $\blacktriangleleft\cdots\blacktriangleleft\cdots\blacktriangleleft\cdots$ PEP $\longleftarrow$ CO_2+ADP $\underset{pck^c}{\curvearrowright}$ ATP $\longrightarrow$ OAA

gluconeogenic condition

BL21(DE3)/pEcPCK

Glucose $\cdots\blacktriangleright\cdots\blacktriangleright\cdots\blacktriangleright$ PEP $\longleftarrow$ CO_2+ADP pck^p **ATP** $\longrightarrow$ OAA
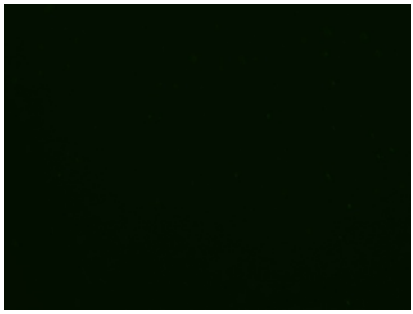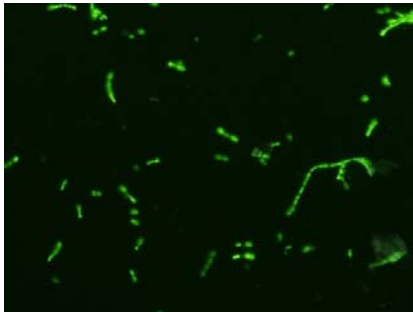
CO_2 ppc^c Pi

267

268

Figure 1. Biochemical reactions between PEP and OAA in high energy-charged *E. coli*.

Biochemical reactions under glycolytic and gluconeogenic conditions occurring in BL21(DE3) and high energy-charged BL21(DE3).

pck: PEP carboxykinase, *ppc*: PEP carboxylase. Superscripts *p* and *c* indicate promoter-origin and chromosome-origin, respectively.

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276

Strains	Phase contrast microscopy (×1000)	Fluorescence microscopy (×1000)
BL21(DE3) /pEGFP		
BL21(DE3) /pEcPCK/pEGFP/		

277

Figure 2. Microscopic images of *E. coli* BL21(DE3) expressing eGFP.

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