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SHORT COMMUNICATION

N. Kamada · A. Yasuhara · M. Ikeda

Significance of the non-oxidative route of the pentose phosphate pathway for supplying carbon to the purine-nucleotide pathway in *Corynebacterium ammoniagenes*

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Abstract To evaluate the strategy of supplying ribose 5-phosphate to the purine-nucleotide pathway exclusively via the nonoxidative route, the glucose 6-phosphate dehydrogenase gene *zwf* was disrupted in inosine- and 5′-xanthylic acid-producers of *Corynebacterium ammoniagenes*. In both producers, interruption of the oxidative route caused a decrease in production yields of about 50%. Attempts to increase the capacity of the nonoxidative route through overexpression of the transketolase or transaldolase gene in the *zwf* mutants led to no discernable effects on production, indicating that, in *C. ammoniagenes*, the nonoxidative route alone cannot provide sufficient ribose 5-phosphate for high-level production, although nonoxidative synthesis of the precursor is possible.

Keywords Metabolic engineering · Pentose phosphate pathway · Nucleotide · Nucleoside · *Corynebacterium ammoniagenes*

Introduction

Many commercially important purine compounds, including inosine, guanosine, 5′-inosinic acid, and 5′-xanthylic acid, have been produced industrially by fermentation [2, 8]. Ribose 5-phosphate, an intermediate of the pentose phosphate pathway, is an essential starting precursor for biosynthesis of the nucleosides and nucleotides. Therefore, metabolism within the pathway is of key importance for improving availability of the precursor.

The pentose phosphate pathway forms a cycle and consists of an oxidative route and a nonoxidative route. The oxidative route is one-way in the direction of ribose 5-phosphate formation during glucose metabolism while carbon on the nonoxidative route can flow in both directions since the constituent enzymes are all reversible. Recently, we showed that considerable amounts of ribose 5-phosphate could be supplied through the oxidative route and be channeled into the purine-nucleotide pathway when the nonoxidative route was blocked in inosine- and 5′-xanthylic acid-producers of *Corynebacterium ammoniagenes* [5]. Although this work has certainly illustrated one strategy useful in increasing carbon flow to the purine-nucleotide pathway, the alternative strategy of supplying carbon via the nonoxidative route has not yet been evaluated in any microorganism. The nonoxidative synthesis of ribose 5-phosphate is theoretically more advantageous than the oxidative synthesis because the former is not accompanied by the release of CO_2. However, without a comparative study of oxidative and nonoxidative synthesis, one cannot conclude which is the best route with respect to efficient nucleotide production.

In this study, we disrupted the *zwf* gene coding for glucose 6-phosphate dehydrogenase, the first enzyme in the oxidative route, to examine the effectiveness of the strategy of supplying ribose 5-phosphate exclusively via the nonoxidative route in inosine- and 5′-xanthylic acid-producers of *C. ammoniagenes*. Furthermore, the effect of increased capacity of the nonoxidative route on production was investigated in the *zwf*-disrupted backgrounds. This work is the first demonstration of nucleotide and nucleoside production exclusively via the nonoxidative route in a microorganism.

N. Kamada · A. Yasuhara · M. Ikeda (✉)
Technical Research Laboratories, Kyowa Hakko Kogyo Ltd,
Hofu, 747–8522, Yamaguchi, Japan
E-mail: m.ikeda@kyowa.co.jp
Fax: +81-42-7268330

Present Address: N. Kamada
Tokyo Research Laboratories, Kyowa Hakko Kogyo Ltd,
Asahi-machi, Machida, 194–8533, Tokyo, Japan

Present Address: M. Ikeda
Tokyo Research Laboratories, Kyowa Hakko Kogyo Ltd,
Asahi-machi, Machida, 194–8533, Tokyo, Japan

Materials and methods

Bacterial strains and plasmids

An inosine producer, *C. ammoniagenes* KY13761 [7], and a 5′-xanthylic acid producer, *C. ammoniagenes* KY13203 [2] were used as the host strains for investigating the effects of *zwf* disruption on carbon flow to the purine-nucleotide pathway. *Escherichia coli* DH5α [3] was used as the host strain for genetic manipulation. Plasmids pCZX1, pCSX1, and pCAX1 [5] have the intact glucose 6-phosphate dehydrogenase, transketolase, and transaldolase genes from the wild-type strain *C. ammoniagenes* ATCC 6872, respectively, in the multicopy vector pCG116. Vectors pCG116 [6], pUC19 [11], and pHSG299 [10] were used for genetic manipulation.

Media and culture conditions

Complete medium (CM) [5], minimal medium (MM) [7], and enriched MM containing 0.1% yeast extract (MMYE) were used for cultivation of *C. ammoniagenes*. Solid plates were prepared by the addition of 1.6% (w/v) Bacto Agar (Difco, Detroit, Mich.). IS and IP media [5] were used for seed and fermentation cultures, respectively, for inosine production in test tubes. XS and XP media [5] were used for seed and fermentation cultures, respectively, for 5′-xanthylic acid production in test tubes. When required, supplements or antibiotics were added at the following final concentrations: adenine and guanine, 20 mg l^{-1} each for MM medium; ampicillin, 100 mg l^{-1} for LB medium; kanamycin, 20 mg l^{-1} for LB, CM, MM, and MMYE media; streptomycin, 50 mg^{-1} for CM, MM, and MMYE media. For inosine- and 5′-xanthylic acid-production, cultivations were performed as described previously [5].

Recombinant DNA techniques

Standard protocols [9] were used for the construction, purification and analysis of plasmid DNA, analysis of chromosomal structure of plasmid integrates by PCR, and transformation of *E. coli*. Transformation of *C. ammoniagenes* by electroporation was carried out as described previously [5].

Enzyme assays

Cell-free extracts were prepared by sonic disruption of cells grown in MMYE medium [4]. Protein quantity was determined by the method of Bradford [1]. Glucose 6-phosphate dehydrogenase, transketolase, and transaldolase activities in cell-free extracts were measured spectrophotometrically as described previously [5].

Analysis

Cell growth, glucose concentration, and titers of inosine and 5′-xanthylic acid were measured as described previously [5].

Results and discussion

Site-specific disruption of the *zwf* gene

First, to locate the *zwf* internal region within the 3.8 kb *Xho*I fragment originally cloned in plasmid pCZX1 [5], several subclones constructed with vector pCG116 were examined for glucose 6-phosphate dehydrogenase activity in the wild-type strain *C. ammoniagenes* ATCC 6872. Results indicated that the 0.8 kb *Kpn*I-*Bam*HI fragment was within the *zwf* gene. Thus, the internal 0.8 kb fragment was recloned into *E. coli* vector pHSG299 to generate pHGD2, which was used to construct *zwf* mutants of an inosine producer KY13761 and a 5′-xanthylic acid producer KY13203 as well as the wild type strain ATCC 6872. Since plasmid pHGD2 cannot replicate in *C. ammoniagenes*, transformation of the three *C. ammoniagenes* strains with the plasmid and subsequent selection for the plasmid marker kanamycin resistance yielded transformants that had integrated plasmid DNA into the genome via single-crossover homologous recombination. The chromosomal structure of the plasmid integrates was confirmed by PCR (data not shown). One of the isolated kanamycin-resistant derivatives of each strain, strain DGD872 from strain ATCC 6872, strain DGD761 from strain KY13761, or strain DGD203 from strain KY13203, was assayed for glucose 6-phosphate dehydrogenase activity, showing that each kanamycin-resistant derivative had completely lost the enzyme activity as expected while each parent exhibited an essentially normal level of activity (Table 1). We also confirmed that the *zwf* disruption did not affect the activities of transketolase and transaldolase (data not shown). Growth experiments showed that the *zwf* mutants did not require any other nutrients for growth on MM plates than their original requirements (adenine and guanine in the case of the production

Table 1 Effectof glucose 6-phosphate dehydrogenase deficiency on production of inosine and 5′-xanthylic acid. Glucose 6-phosphate dehydrogenase activities from cells grown inenriched minimal medium (MMYE) were expressed in units per milligram (nmol-product min^{-1} mg $protein^{-1}$) as the mean values from three measurements. Production was carried out in test tubes as described in Materials and methods. The concentration of 5′-xanthylic acid-$Na_2 \cdot 7H_2O$ was expressed as free 5′-xanthylic acid. Results are the mean values offive independent cultures. Standard deviation was < 5%

Strain	Glucose 6-phosphate dehydrogenase (U mg^{-1})	Growth (OD_{660})	Purine compounds produced (g l^{-1})	
			Inosine	5′-Xanthylic acid
ATCC6872	40	NT[a]	NT	NT
DGD872	ND[b]	NT	NT	NT
KY13761	39	51	10.0	ND
DGD761	ND	52	5.4	ND
KY13203	40	49	ND	30.6
DGD203	ND	48	ND	15.9

[a]Not tested
[b]Not detected

Table 2 Effect of amplified transketolase andtransaldolase activities on production of inosine by DGD761. The enzyme activities from cells grown in enriched minimal mediumMMYE were expressed in units permilligram (nmol product min^{-1} mg $protein^{-1}$) as the mean valuesfrom three measurements. Production was carried out in test tubes as describedin Materials and methods. Results are the mean values of five independentcultures. Standard deviation was < 5%

Strain (plasmid)	Specific activity (U mg^{-1})			Growth (OD_{660})	Inosine (g l^{-1})
	Glucose 6-phosphate dehydrogenase	Transketolase	Transaldolase		
KY13761	39	22	58	51	10.0
DGD761	ND[a]	22	59	52	5.4
DGD761 (pCG116)	ND	21	59	52	5.5
DGD761 (pCSX1)	ND	218	57	51	5.3
DGD761 (pCAX1)	ND	23	231	52	5.5

[a]Not detected

strains). This finding suggests that *C. ammoniagenes* has adequate sources of NADPH for normal growth on glucose besides the oxidative route of the pentose phosphate pathway. Such possible sources may involve the malic enzyme and isocitrate dehydrogenase.

Effect of *zwf* disruption on production of inosine and 5′-xanthylic acid

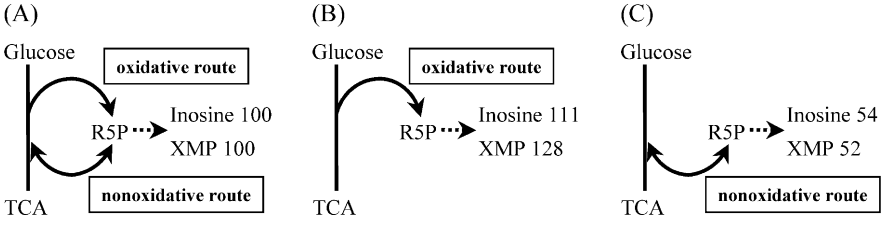
The inosine producer KY13761, the 5′-xanthylic acid producer KY13203, and the corresponding *zwf* mutants DGD761 and DGD203 were tested for production of these purine compounds by test-tube cultivations with IP medium (5% glucose) for inosine production and XP medium (8% glucose) for 5′-xanthylic acid production. As shown in Table 1, both *zwf* mutants grew to almost the same levels as the parents. In addition, their glucose consumption rates were almost the same as those of their parents. Under these conditions, the titer of each product was significantly lower in the *zwf* mutant than in the parent. The yield decreases for inosine and 5′-xanthylic acid were 46% and 48%, respectively. Five independent cultures showed that the effect was reproducible and statistically significant. Therefore, it was concluded that blockage of the oxidative route results in decreased production of purine compounds.

Fig. 1A–C Schematic illustration of the relationship between the biosynthetic routes of ribose 5-phosphate (*R5P*) and production yields of inosine and 5′-xanthylic acid (*XMP*). The values are the production yields of purine compounds by transketolase-deficient strains having only the oxidative route [5] (**B**), and the glucose 6-phosphate dehydrogenase-deficient strains DGD761 and DGD203 with the nonoxidative route (**C**) relative to those of the parental strains, KY13761 and KY13203, which have both routes (**A**)

Effect of overexpressed transketolase or transaldolase activity on production in the *zwf*-disrupted background

Plasmids pCSX1 and pCAX1, which contained the intact transketolase and transaldolase genes from *C. ammoniagenes* ATCC 6872, respectively, were introduced into the *zwf*-disrupted, inosine producer DGD761, and their effects on both enzyme activities and production yields were examined. As shown in Table 2, the presence of pCSX1 and pCAX1 in strain DGD761 caused increased transketolase and transaldolase activities, respectively, but no effects on inosine yields were observed. There were no substantial differences either in the cell growth or in the rate of glucose consumption during fermentations with the plasmid carriers and the control host strain. These results show that the overexpressed transketolase and transaldolase activities did not contribute to inosine production by the *zwf* mutant. In addition, similar results were obtained in the case of the *zwf*-disrupted, 5′-xanthylic acid producer DGD203 when transketolase or transaldolase was overproduced (data not shown). Based on these results, it is likely that the limited capacity of nonoxidative production might be due to the catalytic properties of transketolase and/or transaldolase, rather than a shortage of their specific activities. Unfortunately, the properties of these enzymes from *C. ammoniagenes*, such as K_m values toward substrates, are not available at present, and we cannot evaluate the preferential flux of carbon in the *zwf* mutants.

Based on both the results presented here and our previous data [5], the relationship between the route of ribose 5-phosphate synthesis and the production yield of purine compounds is schematically summarized in Fig. 1. Taking all of these findings into consideration, we conclude that oxidative synthesis of ribose 5-phosphate has an advantage over nonoxidative synthesis of



the precursor for efficient production of purine compounds in *C. ammoniagenes*.

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