Note

gsk Disruption Leads to Guanosine Accumulation in *Escherichia coli*

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We tried some improvement of inosine production using an inosine-producing mutant of *Escherichia coli* which is deficient in *purF* (phosphoribosylpyrophosphate (PRPP) amidotransferase gene), *purA* (succinyl-adenosine 5'-monophosphate (AMP) synthetase gene), *deoD* (purine nucleoside phosphorylase gene), *purR* (purine repressor gene) and *add* (adenosine deaminase gene), and harboring the desensitized PRPP amidotransferase gene as a plasmid.

The *guaB* (inosine 5'-monophosphate (IMP) dehydrogenase gene) disruption brought about a slightly positive effect on the inosine productivity. Alternatively, the gsk (guanosine-inosine kinase gene) disruption caused a considerable amount of guanosine accumulation together with a slight increase in the inosine productivity.

The further addition of *guaC* (guanosine 5'-monophosphate (GMP) reductase gene) disruption did not lead to an increased guanosine accumulation, but brought about the decrease of inosine accumulation.

Key words: gueB; gsk; gueC gene; *Escherichia coli*; inosine; guanosine production

Purine nucleosides such as inosine and guanosine are important intermediates in the manufacture of IMP and GMP, respectively. The sodium salts of IMP and GMP together with sodium glutamare are known as taste enhancers, which are manufactured and used as food additives world-wide.

The studies on the microbial production of purine nucleosides have been done almost exclusively in mutants of *Bacillus subtilis*1-9 and *Corynebacterium ammoniagenes*.5,6 However, there have been hardly any reports of the overproduction of these substances by *Escherichia coli* to date, although the biosynthetic pathway for purine nucleotides and the enzymes involved in their biosynthesis in *E. coli* have been studied in detail.7

Therefore, in a previous report, we derived inosine-producing mutants from *E. coli* W3110 (wild type), using genetic manipulation during each step, in order to analyze the genes and functions involved in inosine production.9 A mutant strain FADradd/pKFQ deficient in *purF*,9 *purA*,10 *deoD*,10 *purR*,12 and *add*13 was produced, as well as harboring the desensitized PRPP amidotransferase gene (*purF*) as plasmid.8 That is, this strain is an adenine auxotroph, deficient in purine nucleosides phosphorylase activity, derepressed in the regulation of purine nucleotides biosynthesis, deficient in adenosine deaminase activity, and has lost the feedback inhibition of PRPP amidotransferase activity by AMP and GMP.

The pKFQ is an expression plasmid for a PRPP amidotransferase desensitized to synergistic inhibition by AMP and GMP, in which Lys-326 was replaced by Gln.8,14 The *purF* on the pKFQ contains the ribosome binding site and coding region excluding the promoter region, and the transcriptional expression is read with the lac promoter.

The FADradd/pKFQ strain accumulated about 1.1 g/l inosine from 40 g/l glucose.8 This inosine accumulation remained a very low level compared with 16-20 g/l from 80 g/l glucose in *B. subtilis* mutants2,3 or 31 g/l from 150 g/l glucose in *C. ammoniagenes* mutants.9

Thus, further studies were continued to increase the inosine accumulation using the FADradd/pKFQ strain of *E. coli*. In this report, we focused on the metabolism of IMP and inosine. The de novo biosynthetic pathway of IMP and GMP, and furthermore, the salvage synthesis system of IMP and GMP are shown in Fig. 1. IMP is formed via PRPP from ribose-5-phosphate (R-5-P) on the pentose phosphate cycle, and GMP is formed via XMP from IMP (de novo synthesis). In the salvage synthesis system, IMP and GMP are irreversibly dephosphorylated to inosine and guanosine by 5'-nucleotidase (UshA etc.), and inosine and guanosine are irreversibly converted...
to IMP and GMP by guanosine-inosine kinase (Gsk), or reversibly to hypoxanthine and guanine by purine nucleoside phosphorylase (DeoD), respectively.

First, the effects of gubA (IMP dehydrogenase gene)\(^{10}\) disruption which blocks the conversion to XMP from IMP, and second, of gsk (guanosine-inosine kinase gene)\(^{10}\) disruption, which blocks the salvage synthesis to IMP from inosine, were investigated. The former is expected to cause the increase of inosine accumulation by preventing the conversion from IMP to XMP, and the latter by the block of the inosine recycling. In fact, the effects of on inosine accumulation IMP dehydrogenase deficiency had been found in \textit{B. subtilis}\(^{5}\) and \textit{C. ammnoniagenes}\(^{6}\) mutants. On the other hand, its effect on inosine kinase deficiency has never been reported.

A gubA or gsk disrupted mutant was derived from strain FADRadd as follows. The chromosomal DNA of \textit{E. coli} W3110 (ATCC27325)\(^{17}\) was isolated using a Genomic DNA Purification Kit (Edge BioSystems). The gubA and gsk genes were amplified by PCR with the combination of primers No. 1 and No. 2, and No. 3 and No. 4, respectively. The sequence for No. 1 was 5’-ctc gag ctc atg caa tca gcg gtt aca ctc tg-3’ and No. 2 5’-ctc gca tgc tca gga gcc cag a CG gta gtt c-3’, while for No. 3 was 5’-ctc ggt acc ctc tgg cgt taa ggc tgc atc cca ga-3’ and No. 4 5’-ctc gca tgc caa cgt acc gta taa acc ta-3’. These sequences were based on the published nucleotide sequences for gubA\(^{13}\) and gsk,\(^{16,30}\) respectively.

PCR was done with the reaction mixture of about 2.5 µg of chromosomal DNA, 10 pmol of primers, and the LA Taq DNA polymerase system (Takara Shuzo) per 50 µl for 30 cycles at 94°C for 30 sec, 55°C for 1 min, 72°C for 2 min, using a GeneAmp PCR system 2400 (Perkin-Elmer).

For gubA, the amplified DNA fragment of about 1.55 kb was digested with SaeI and Sphi, inserted into the pUC18 plasmid, and cloned to produce the JM109/pUCgubA strain. As shown in Fig. 2, the gubA gene in the pUGuaB plasmid was disrupted by digesting the \textit{HincII} site on the 750th bp and the 800th bp of the cloned fragment with \textit{HincII}. The small \textit{HincII} fragment was then deleted and the plasmid was ligated with \textit{BgII} linker (8 mer) using T4 DNA ligase.

For gsk, the amplified DNA fragment of about 2.9 kb was digested with \textit{KpnI} and Sphi, inserted
into the pUC18 plasmid and cloned to produce the JM109/pUCgsk strain. The gsk gene in the pUCgsk plasmid was disrupted by digesting the *Aro51HI* and *BglII* sites on the 900th bp and the 1640th bp of the cloned fragment with *Aro51HI* and *BglII* (Fig. 2). The 740-bp fragment was then deleted and then filled in by T4 DNA polymerase before being ligated with T4 DNA ligase.

Disruption of the *guaB* or *gsk* gene on the chromosome was done as follows. *E. coli* FADRadd-competent cells were transformed using pMAN4guaB or pMAN4gsk and grown at 30°C overnight on LB agar plates containing 25 μg/ml ampicillin. The resulting colonies were spread onto fresh LB agar plates containing 25 μg/ml ampicillin and grown at 30°C overnight. The cells were then diluted appropriately, replated onto LB agar plates containing 25 μg/ml ampicillin, and grown at 42°C overnight. The last stage was repeated once more to obtain single colonies. These consisted of cells in which the plasmid had been incorporated into the chromosome by homologous recombination. The colonies were spread onto fresh LB agar plates without ampicillin and grown at 30°C overnight. Appropriately harvested cells were then grown at 42°C for about 4 h with shaking in a test tube containing 4 ml LB medium without ampicillin. They were then diluted to approximately 10^-6, spread onto LB agar plates without ampicillin and grown at 42°C overnight. The resulting colonies (clones) were picked up and grown at 37°C overnight on LB agar plates with or without ampicillin. At this point, some ampicillin-sensitive clones were selected, and *guaB*- or *gsk*-containing fragments were amplified from their chromosomes by PCR using the No. 1 and No. 2, or No. 3 and No. 4 primers described above, and for *guaB*, followed by digestion with the restriction enzyme *BglII*, and for *gsk*, followed by digestion with the restriction enzyme *Aro51HI* or *BglII*. The clone with a *guaB* fragment that had been digested by *BglII* was selected as a strain carrying a disrupted *guaB* gene on its chromosome, and was designated FADRaddguaB. Alternatively, the clone with a *gsk* fragment that had not been digested by *Aro51HI* or *BglII* was selected as a strain carrying a disrupted *gsk* gene on its chromosome, and was designated FADRaddgsk.

The inosine productivity of *guaB*- or *gsk*-disrupted mutants were investigated as follows. *E. coli* FADRadd, FADRaddguaB, and FADRaddgsk were transformed using the PRPP amidotransferase expression plasmid pKFKQ. The transformed cells were grown overnight on LB medium containing 50 μg/ml kanamycin, then inoculated into 20 ml MS medium containing 40 g glucose, 16 g (NH₄)₂SO₄, 1 g KH₂PO₄, 1 g MgSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 10 mg MnSO₄·4H₂O, 2 g yeast extract (Difco), and 30 g CaCO₃ per liter in a 500-ml flask. After cultivation at 37°C for 72 h with shaking, the culture broth was centrifuged at 12,000 × g for 10 min, and the supernatant was used to analyze the purine compounds produced.

The measurement of the inosine and other purine compounds formed was done by high-performance liquid chromatography (HPLC) using an Asahipak GS-220 column (internal diameter [ID] 7.6 mm × 500 mm long) with detection at 254 nm. The mobile phase was 200 mM NaH₂PO₄ (pH 3.98) and the flow rate was 1.5 ml/min at 55°C. Cell growth was measured as a factor of the optical density (OD) by measuring the absorbance produced by the cell density at 600 nm. The sugar consumed was measured by a glucose analyzer (Toyobo Diagluca HEK-60).

The inosine productivity of the *guaB*-disrupted mutant is shown in Fig. 3. The inosine accumulation of FADRaddguaB/pKFKQ strain decreased as compared with that of FADRadd/pKFKQ strain. Especially, in FADRaddguaB/pKFKQ strain, which is a guanine auxotrophic mutant, the cell growth and the sugar consumption was still slightly wrong with the
Table 1. Inosine and Guanosine Productivity of gsk and guaC Disrupted Mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>Adenine added (mg/l)</th>
<th>Cell growth (OD 600nm)</th>
<th>Productivity</th>
<th>Yield (Inosine /consumed sugar) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADRadd/pKFKQ</td>
<td>5</td>
<td>2.8</td>
<td>1100</td>
<td>0</td>
</tr>
<tr>
<td>FADRaddgsk/pKFKQ</td>
<td>5</td>
<td>2.8</td>
<td>1210</td>
<td>60</td>
</tr>
<tr>
<td>FADRaddguaC/pKFKQ</td>
<td>5</td>
<td>2.3</td>
<td>650</td>
<td>0</td>
</tr>
<tr>
<td>FADRaddguaCgsk/pKFKQ</td>
<td>5</td>
<td>2.3</td>
<td>850</td>
<td>60</td>
</tr>
<tr>
<td>FDR/pKFKQ</td>
<td>0</td>
<td>16.4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>FDRgsk/pKFKQ</td>
<td>0</td>
<td>15.1</td>
<td>140</td>
<td>120</td>
</tr>
</tbody>
</table>

Fig. 3. Time Course of Cell Growth and Inosine Accumulation. Mutants were cultured at 37°C in flasks with 20 ml of MS medium containing 5 mg of adenine, 50 mg of guanine, and 50 mg of kanamycin per liter. Inosine was estimated by HPLC analysis.

A, FADRaddguaB/pKFKQ strain; B, FADRadd/pKFKQ strain.

○---○, inosine; □---□, growth; △---△, residual sugar.

and about 3.2% without the addition of guanine (Fig. 3).

Next, as shown in Table 1, the inosine accumulation in FADRaddgsk/pKFKQ strain slightly increased to 1210 mg/l, compared with 1100 mg/l of the parent strain FADRadd/pKFKQ. Furthermore, a small amount of 60 mg/l guanosine was accumulated unexpectedly, in spite of the biosynthetic pathway from IMP to GMP not being deregulated by GMP.

Successively, FDRgsk strain was also derived from the non-adenine auxotrophic mutant FDR (purF-, deoD-, purR-) by the same disruption method. A FDRgsk/pKFKQ strain could accumulate 120 mg/l guanosine together with a release of growth restriction (Table 1). This strain completely consumed sugar in the medium for about 2 days, and showed the high cell density. This phenomenon that gsk deficiency caused guanosine accumulation even a little, may indicate that the intracellular guanine compounds pool is usually maintained in a slightly high level concentration, although it is known that GMP biosynthetic pathway is regulated by GMP, for example, there is a competitive inhibition of IMP dehydrogenase activity by GMP in E. coli.20 Therefore, cycling among guanine, guanosine, and GMP, guanosine may have been accumulated by the conversion to guanine from guanosine (deoD-) and to GMP from guanosine (gsk-) being blocked, followed by only the one way conversion to guanosine from GMP by 5'-nucleotidase.

For purpose of further increasing guanosine accumulation, guaC-disrupted mutants in which cannot convert GMP to IMP were derived from FADRadd and FADRaddgsk strains, with the same method described above. The guaC gene was amplified by PCR with the combination of primers No. 5 and No. 6. The sequence for No. 5 was 5'-ctc acg gct ctc gag gcc ag-3' and No. 6 5'-ctc ctc cag ctc tgg gag att aca gg-3'. These sequences were based on the published nucleotide sequences for guaC.21

The amplified DNA fragment of about 2.2 kb was digested with HindIII and PsI, and cloned on pUC19 (pUCguaC). The guaC gene in the pUCguaC
plasmid was disrupted by digesting the \textit{Bg}III site on the 1100th bp of the cloned fragment with \textit{Bg}III, and then filled in by T4 DNA polymerase before being ligated with T4 DNA ligase (Fig. 2). This disrupted gene was cloned on the tsori plasmid pMAN997, using the same \textit{Hind}III and \textit{Pst}I as when it was cloned on the pUC vector. This resulting plasmid was designated pMAN4guaC.

Disruption of the \textit{guaC} gene on the chromosome was done as described above. The candidates for \textit{guaC}-disrupted mutants derived from FADRadd and FADRaddgsk strains, respectively, were selected, and \textit{guaC}-containing fragment was amplified from their chromosomes by PCR using the No. 5 and No. 6 primers described above, and followed by digestion with the restriction enzyme \textit{Bg}II. The clones with a \textit{guaC} fragment that had not been digested by \textit{Bg}II were selected as a strain carrying a disrupted \textit{guaC} gene on its chromosome, and were designated FADRaddguaC and FADRaddguaCgsk, respectively.

However, in a mutant FADRaddguaCgsk/pFKKQ which is deficient in \textit{guaC}, no effect on increasing the guanosine accumulation could be found, as shown in Table 1. Alternatively, the inosine accumulation in strains FADRaddguaC/pFKKQ and FADRaddguaCgsk/pFKKQ decreased clearly from 1100 mg/l to 650 mg/l, and from 1210 mg/l to 850 mg/l, respectively (Table 1).

The analysis of various purine compounds produced in the culture medium was done by HPLC. The other purine compounds than inosine and guanosine was undetectable below 10 mg/l.

These results suggest that the purine nucleotides biosynthesis from R-5-P to IMP was more strongly regulated by the increase of the guanine nucleotides pool, followed by the deficiency of GMP reductase. It can be speculated that the regulating site may be the inhibition of PRPP synthetase (Prs) activity by guanine nucleotides, based on the reason that this mutant strain is \textit{purR} deficient, and desensitized the inhibition of PRPP amidotransferase by GMP. In fact, the inhibition of PRPP synthetase activity by guanine nucleotides has been reported by Petersen, together with the inhibition by adenine nucleotides.

As a further investigation relating to the salvage synthesis system of IMP and GMP, it may be an important point to test the relation between 5'-nucleotidase activity and inosine and guanosine accumulation.

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References


