Note
gsk Disruption Leads to Guanosine Accumulation in Escherichia coli

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We tried some improvement of guanosine production using an guanosine-producing mutant of Escherichia coli
which is deficient in purF (phosphoribosyl-pyrophosphate (PRPP) amidotransferase gene), purA (succinyl-adenosine 5'-monophosphate (AMP) synthetase gene), deoD (purine nucleoside phosphorylase
degeneration 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 guanosine productivity. Alternatively, the gsk (guanosine-inosine kinase) gene disruption caused a considerable amount of guanosine accumulation together with a slight increase in the guanosine 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 guanosine accumulation.

Key words: guaB; gsk; guaC 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 glutamate 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 subtilis1-9 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 guanosine production.5 A mutant strain FADResp/pKFKQ deficient in purF,6 purA,8 deoD,8 purR,9 and add10 was produced, as well as harboring the desensitized PRPP amidotransferase gene (purF) as a plasmid.8 That is, this strain is an adenine auxotroph, deficient in purine nucleotides 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 pKFKQ 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 pKFKQ contains the ribosome binding site and coding region excluding the promoter region, and the transcriptional expression is read with the lac promoter.

The FADResp/pKFKQ 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.8

Thus, further studies were continued to increase the inosine accumulation using the FADResp/pKFKQ 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 in the salvage system.
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 guaB (IMP dehydrogenase gene)\(^{39}\) disruption which blocks the conversion to XMP from IMP, and second, of gsk (guanosine-inosine kinase gene)\(^{39}\) 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 \(B.\ subtilis\)^{39} and \(C.\ ammoniagenes\)^{39} mutants. On the other hand, its effect on inosine kinase deficiency has never been reported.

A guaB or gsk disrupted mutant was derived from strain FADRadd as follows. The chromosomal DNA of \(E.\ coli\) W3110 (ATCC27325)\(^{72}\) was isolated using a Genomic DNA Purification Kit (Edge BioSystems). The guaB 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 tcg gtt acg ctc tg-3' and No. 2 5'-ctc gca tgc tca gga gcc cag acg gta gtt c-3', while for No. 3 was 5'-ctc ggt acc ctc ttg cgt taa gcc atc cca ga-3' and No. 4 5'-ctc gca tgc caa cgt acg gca tta acc ta-3'. These sequences were based on the published nucleotide sequences for guaB\(^{35}\) and gsk,\(^{16,30}\) respectively.

PCR was done with the reaction mixture of about 2.5 \(\mu\)g of chromosomal DNA, 10 pmol of primers, and the LA Taq DNA polymerase system (Takara Shuzo) per 50 \(\mu\)l 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 guaB, 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/pUCguaB strain. As shown in Fig. 2, the guaB gene in the pUCguaB plasmid was disrupted by digesting the \(HincII\) site on the 750th bp and the 800th bp of the cloned fragment with \(HincII\). The small \(HincII\) fragment was then deleted and the plasmid was ligated with BglII linker (8 mer) using T4 DNA ligase.

For gsk, the amplified DNA fragment of about 2.9 kb was digested with \(KpnI\) and \(SphI\), inserted
Disruption of the guaB

Fig. 2. Structure of Genes Constructed for Gene Disruption by Homologous Recombination.

The base pair (bp) designated shows the size of genes amplified by PCR. The open box shows the coding region of each gene.

into the pUC18 plasmid and cloned to produce the JMI109/pUCgsk strain. The gsk gene in the pUCgsk plasmid was disrupted by digesting the Ara51HI and BglII sites on the 900th bp and the 1640th bp of the cloned fragment with Ara51HI 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.

The disrupted genes were recloned into plasmid pMAN997, using the same SacI and SphI, and KpnI and SphI, respectively, as for cloning into the pUC18 vector. The resulting plasmids were designated pMAN4guaB and pMANAgsk, respectively.

The pMAN997 plasmid consists of a temperature-sensitive replication origin (tsori) for homologous recombination, and it was constructed from pMAN031 and pUC19 by replacing the HindIII-VspI fragment.

Disruption of the guaB or gsk gene on the chromosome was done as follows. E. coli FADRad-expressing cells were transformed using pMAN4guaB or pMANAgsk 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 Ara51HI or BglII. The clone with a guaB fragment that had been digested by BgII was selected as a strain carrying a disrupted guaB gene on its chromosome, and was designated FADRadguaB. Alternatively, the clone with a gsk fragment that had not been digested by Ara51HI or BglII was selected as a strain carrying a disrupted gsk gene on its chromosome, and was designated FADRadgsk.

The inosine productivity of guaB- or gsk-disrupted mutants were investigated as follows. E. coli FADRad, FADRadguaB, and FADRadgsk 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 FADRadguaB/pKFKQ strain decreased as compared with that of FADRad/pKFKQ strain. Especially, in FADRadguaB/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 (600 nm)</th>
<th>Productivity</th>
<th>Yield (Inosine /consumed sugar) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADRadd/pFKFQ</td>
<td>5</td>
<td>2.8</td>
<td>Inosine (mg/l) 1100   Guanosine (mg/l) 0</td>
<td>3.2</td>
</tr>
<tr>
<td>FADRaddgsk/pFKFQ</td>
<td>5</td>
<td>2.8</td>
<td>1210         60</td>
<td>3.4</td>
</tr>
<tr>
<td>FADRaddguaC/pFKFQ</td>
<td>5</td>
<td>2.3</td>
<td>650          0</td>
<td>2.7</td>
</tr>
<tr>
<td>FADRaddguaCgsk/pFKFQ</td>
<td>5</td>
<td>2.3</td>
<td>850          60</td>
<td>2.7</td>
</tr>
<tr>
<td>FDR/pFKFQ</td>
<td>0</td>
<td>16.4</td>
<td>100          0</td>
<td>0.25</td>
</tr>
<tr>
<td>FDRgsk/pFKFQ</td>
<td>0</td>
<td>15.1</td>
<td>140          120</td>
<td>0.35</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/pFKFQ strain; B, FADRadd/pFKFQ strain.

M. inosine accumulation per consumed sugar. Namely, the yield of FADRaddguaB/pFKFQ strain was about 3.4%, and that of FADRadd/pFKFQ strain was about 3.0% with the addition of 50 mg/l guanine and about 3.2% without the addition of guanine (Fig. 3).

Next, as shown in Table 1, the inosine accumulation in FADRaddgsk/pFKFQ strain slightly increased to 1210 mg/l, compared with 1100 mg/l of the parent strain FADRadd/pFKFQ. 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/pFKFQ strain was also derived from the non-adenine auxotrophic mutant FDR (purF-, deoD-, purR-)* by the same disruption method. A FDRgsk/pFKFQ 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*.* 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 acc gct ctc gagagg-3' and No. 6 5'-ctc ctc cag gct cgg gat aca gcg-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 *PstI*, and cloned on pUC19 (pUCguaC). The guaC gene in the pUCguaC
plasmid was disrupted by digesting the BgII site on the 1100th bp of the cloned fragment with BgII, and then filled in by T4 DNA polymerase before being ligated with T4 DNA ligase (Fig. 2). This disrupted gene was recloned on the tsori plasmid pMAN997, using the same HindIII and PstI as when it was cloned on the pUC vector. This resulting plasmid was designated pMAN4guaC.

Disruption of the guaC gene on the chromosome was done as described above. The candidates for guaC-disrupted mutants derived from FADRadd and FADRaddgsk strains, respectively, were selected, and 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 BgII. The clones with a guaC fragment that had not been digested by BgII were selected as a strain carrying a disrupted guaC gene on its chromosome, and were designated FADRaddguaC and FADRaddguaGsk, respectively.

However, in a mutant FADRaddguaGsk/pKFKQ which is deficient in guaC, no effect on increasing the guanosine accumulation could be found, as shown in Table 1. Alternatively, the inosine accumulation in strains FADRaddguaC/pKFKQ and FADRaddguaGsk/pKFKQ 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 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,20 together with the inhibition by adenine nucleotides.21

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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