Mutation Research  Short communication

Characterization of mutations induced by  
N-methyl-N’-nitro-N-nitrosoguanidine in an  
industrial Corynebacterium glutamicum strain

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Abstract

Mutations induced by classical whole-cell mutagenesis using \(N\)-methyl-\(N^\prime\)-nitro-\(N\)-nitrosoguanidine (NTG) were determined for all genes of pathways from glucose to L-lysine in an industrial L-lysine producer of Corynebacterium glutamicum. A total of 50 mutations with a genome-wide distribution were identified and characterized for mutational types and mutagenic specificities. Those mutations were all point mutations with single-base substitutions and no deletions, frame shifts, and insertions were found. Among six possible types of base substitutions, the mutations consisted of only two types: 47 G·C \(\rightarrow\) A·T transitions and three A·T \(\rightarrow\) G·C transitions with no transversion. The findings indicate a limited repertoire of amino-acid substitutions by classical NTG mutagenesis and thus raise a new possibility of further improving industrial strains by optimizing key mutations through PCR-mediated site-directed mutagenesis.

Keywords: Mutagenic specificity; \(N\)-methyl-\(N^\prime\)-nitro-\(N\)-nitrosoguanidine; Strain improvement; Corynebacterium glutamicum

1. Introduction

Production strains that are used in industrial amino acid fermentation have been generally constructed by repeating random mutation and selection [1-3]. In this classical approach, \(N\)-methyl-\(N^\prime\)-nitro-\(N\)-nitrosoguanidine (NTG) has been used as the popular mutagen to induce mutants that exhibit improved production [1]. Some of these mutants have been shown to be genetically deregulated with respect to relevant
biosynthetic pathways. However, recent more detailed analysis has revealed that mutations responsible for deregulation, such as the mutations in \textit{thrA} [4], \textit{dapA} [5], \textit{lysC} [6], and \textit{gnd} [7], resulted in only partial desensitization of the enzymes, despite continual efforts of strain improvement. This made us realize again that NTG mutagenesis is not necessarily the best to achieve high desensitization of regulatory enzymes.

NTG induces a relatively wide spectrum of mutations by alkylating purines and pyrimidines, although the mutagen has its own specificity of the types of base substitutions. Such conception is apparently based on previous studies which focused on certain genes to examine the mutagenic specificity of NTG. Gee et al. used six \textit{Salmonella typhimurium} tester strains which carried different missense mutations in the histidine-biosynthetic operon to determine the specificity of reversion via NTG-induced base substitutions [8]. Their conclusion was that the mutagen induced preferentially G\textsuperscript{\textdagger}C \textrightharpoonup A\textsuperscript{\textdagger}T transitions and, to a lesser extent, A\textsuperscript{\textdagger}T \textrightharpoonup G\textsuperscript{\textdagger}C transitions and A\textsuperscript{\textdagger}T \textrightharpoonup C\textsuperscript{\textdagger}G transversions. Wang et al. used both \textit{E. coli recA}-positive and \textit{recA}-negative strains to investigate the types of base substitutions in NTG-induced mutations in the \textit{tonB} gene [9]. Also in this work, the mutagenic specificity observed was similar to that mentioned above, while other types of base substitutions such as A\textsuperscript{\textdagger}T \textrightharpoonup T\textsuperscript{\textdagger}A and G\textsuperscript{\textdagger}C \textrightharpoonup T\textsuperscript{\textdagger}A transversions were found in the \textit{recA} background. However, as far as we know, there has been no report examining the mutagenic specificities of NTG on a genome-wide scale in classically derived industrial production strains.

Some specific mutations induced by chemical or spontaneous mutagenesis have been examined for their types of base substitutions in limited kinds of amino acid-producing mutants of \textit{Corynebacterium glutamicum} and its relatives \textit{Brevibacterium flavum} and \textit{Brevibacterium lactofermentum} [10-16], \textit{Escherichia coli} [17], and \textit{Serratia marcescens} [18]. Results are summarized in Table 1, which includes...
two cases of the NTG-induced mutations. Although both NTG-induced mutations show the same pattern of base substitution (G·C→A·T transition), these are not enough for discussing not only the spectrum of NTG-induced mutations but the mutagenic potential for strain improvement in amino acid-producing organisms, especially in C. glutamicum.

Our laboratories have recently determined the whole genome sequence of the wild-type strain of *C. glutamicum*, ATCC 13032 [19]. Following this, we analyzed mutations introduced at specific locations in the genome of a *C. glutamicum* L-lysine producer derived through multiple rounds of NTG mutagenesis, followed by reconstruction of the producer by assembling only beneficial mutations in a wild-type background [6,20,21]. In this process, we have identified numerous mutations accumulated in the producer's genome as reported previously [6,7,22,23]. This time, we examined the types of base substitutions of those extensive mutations, which disclosed an extreme bias in the patterns of base substitutions beyond our expectation. Here we describe the results and discuss limited usefulness of classical whole-cell mutagenesis using NTG for strain improvement.

2. Materials and methods

2.1. Bacterial strains and plasmid

The L-lysine producer used for characterization of mutations is *C. glutamicum* B-6 [24]. This production strain was derived by multiple rounds of NTG mutagenesis from the wild type *C. glutamicum* ATCC 13032 and has many mutations that lead to resistance to an L-lysine structural analog, S(2-aminoethyl)-L-cysteine, rifampicin, streptomycin, and 6-azauracil. NTG treatment to induce strain B-6 was carried out by incubating cells at 30°C for 30 min in 50 mM Tris-maleate buffer (pH 6.0) containing 400
µg/ml of NTG as described previously [25]. *E. coli* DH5α was used as a host for cloning of the PCR products. Vector pESB30 [6] was used to clone the PCR products.

2.2. Media

Complete medium BY [26] was used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. When required, kanamycin was added at the final concentration of 20 µg/ml. For growth of *E. coli*, LB medium [27] was used.

2.3. Recombinant DNA techniques

Standard protocols [27] were used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli*. Chromosomal DNA was extracted from protoplasts of *C. glutamicum* B-6 by the method of Saito and Miura [28]. The protoplasts were prepared by the method of Katsumata et al. [29]. PCR was performed with a DNA Thermal Cycler GeneAmp 9700 (Perkin Elmer, USA), using *Taq* polymerase (Roche, Germany).

2.4. Characterization of mutations

The sequences of all genes indicated by the gene symbols in Fig. 1 were determined for L-lysine producer B-6 as described previously [6]. Mutations were identified by comparing the sequences with the corresponding wild-type sequences. The whole-genome sequence of the wild-type strain *C. glutamicum* ATCC 13032 is available under the accession numbers, **BA000036** (Kyowa Hakko Kogyo and Kitasato University) and **BX927147** (Degussa AG and Bielefeld University).
3. Results and Discussion

In *C. glutamicum*, there are more than 60 genes for the conversion of glucose to L-lysine (Fig. 1). These include genes for the relevant terminal pathways and transport, the glycolytic pathway, the pentose phosphate pathway, and TCA cycle. We determined the sequences of all the genes in L-lysine producer B-6, revealing a total of 50 mutations with a genome-wide distribution (Fig. 1). Those mutations were all point mutations with single-base substitutions and no deletions, frame shifts, and insertions were found. These base-pair mutations consisted of 34 missense mutations causing amino-acid substitutions, 15 silent mutations causing no amino-acid substitutions, and one nonsense mutation leading to a change to a stop codon.

Among the 34 missense mutations, four specific mutations, *hom59* (a T to C exchange at position 176, leading to V59A), *lysC311* (a C to T exchange at position 932, leading to T311I), *pyc458* (a C to T exchange at position 1372, leading to P458S), and *gnd361* (a C to T exchange at position 1083, leading to S361F), were defined as useful mutations relevant to L-lysine production, as described previously [6, 7, 21]. One nonsense mutation, *mqo224* (a G to A exchange at position 672, leading to W224opal), was also a useful mutation for improved L-lysine production [21, 22]. Some of these useful mutations were characterized for their phenotypic consequences, which were given in the legend of Table 2. The other 30 missense mutations and the 15 silent mutations are assumed to be secondary mutations introduced into the genome concomitantly with the introduction of the useful mutations.

All these mutations were classified based on the types of base substitutions, which were summarized in Table 2. Among six possible base substitutions, 94% (47/50) were G·C → A·T transitions and the remainder (3/50) were A·T → G·C transitions.
Unexpectedly, any other 4 types of transversions were not found. This means that NTG induced only two types of base substitutions out of the six possible types. To verify this mutational spectrum, we extended our analysis over additional 50 point mutations defined on other metabolic pathways in strain B·6. As the result, we confirmed substantially the same specificity of base substitutions (data not shown), revealing a limited repertoire of base substitutions by NTG mutagenesis in C. glutamicum.

The mutagenic preference to the types of base substitutions was basically in agreement with the previous conception that the mutagen induces preferentially G·C → A·T transitions. However, the spectrum of mutations was much narrower than the results reported for certain genes of Gram-negative E. coli [9] and S. typhimurium [8], in both of which transversions have also taken place. The mutagenic spectrum was suggested to be affected by the genetic background used [9], and thus, the extreme bias in the patterns of base substitutions in our study might reflect the differences in DNA replication or DNA repair systems between the Gram-negative bacteria, E. coli and S. typhimurium, and Gram-positive C. glutamicum.

The fact that more than 90% of the NTG-induced mutations were G·C → A·T transitions means a limited variation in amino-acid substitutions occurred by the mutagen. For instance, in case of the gnd361 mutation (Ser361Phe) which was found to be responsible for diminished allosteric regulation of 6-phosphogluconate dehydrogenase [7], there were hardly any other choices of amino-acid substitutions, because the predominant mutational type of the G·C → A·T transition resulted in only the change from TCC codon (Ser) to TTC codon (Phe) or to TCT (Ser). Even if it should happen that the other rare mutational type of the A·T → G·C transition occurs within the same TCC codon (Ser), the resulting amino-acid substitution is limited to only the change from TCC codon (Ser) to CCC codon (Pro). Such a limited variation in amino-acid substitutions by
NTG is not confined to the gnd361 mutation but is true of other cases reported as positive mutations for L-lysine production; e.g. the lysC311 mutation (Thr311Ile) [6] with probable changes from ACC codon (Thr) predominantly to ATC codon (Ile) or ACT codon (Thr), and rarely to GCC codon (Ala); the mqo244 mutation (Trp224stop) [22] with probable changes from TGG codon (Trp) predominantly to TAG stop codon or TGA stop codon, and rarely to CGG (Arg) codon.

The extreme bias in the patterns of amino-acid substitutions by NTG in C. glutamicum raises a question how reliable the mutagen is in order to induce a mutant with the most desirable property. Thus, we should throw doubts on the quality of mutated enzymes of classically derived industrial strains. In fact, the gnd361 and lysC311 mutations mentioned above have been shown to cause only partial deregulation of each gene product from allostERIC inhibition [6,7], which is now reasonably attributed to the use of NTG. In this sense, it is worth attempting to optimize NTG-derived key mutations by site-directly changing amino acid residues to other residues which are scarcely obtained by the mutagen. Through this approach, we have actually succeeded in higher deregulation of several key enzymes and thereby improved amino acid production in C. glutamicum. One such example has already been demonstrated for L-arginine production [30].

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oxidoreductase increases L-lysine production by *Corynebacterium glutamicum*,


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Fig. 1. *C. glutamicum* genome map of the genes for sequence analysis. All predicted genes relevant to L-lysine biosynthesis from glucose were arranged around the genome provided by DDBJ (http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032). The mutated genes identified by comparative genomic analysis between L-lysine producer B-6 and its parental wild-type were underlined.
<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Mutagen</th>
<th>Strain</th>
<th>Gene(s)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Transition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C·G→A·T</td>
<td>NTG</td>
<td><em>C. glutamicum</em> ATCC 21850</td>
<td><em>trp</em> operon</td>
<td>[10]</td>
</tr>
<tr>
<td>G·C→A·T</td>
<td>NTG</td>
<td><em>S. marcescens</em></td>
<td><em>proB</em></td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>Hydroxylamine</td>
<td><em>E. coli</em> W3110</td>
<td><em>aroG</em></td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Spontaneous</td>
<td><em>B. flavum</em> ATCC 14067</td>
<td><em>atp</em></td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>Spontaneous</td>
<td><em>C. glutamicum</em> R</td>
<td><em>bgIF</em></td>
<td>[12]</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C·G→G·C</td>
<td>Nitrous acid</td>
<td><em>C. glutamicum</em> ATCC 21850</td>
<td><em>aroF, aroG, aroH</em></td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>Spontaneous</td>
<td><em>B. flavum</em> ATCC 14067</td>
<td><em>atp</em> operon</td>
<td>[11]</td>
</tr>
<tr>
<td>C·G→A·T</td>
<td>EMS</td>
<td><em>C. glutamicum</em> ATCC 13032</td>
<td><em>lysC</em></td>
<td>[14]</td>
</tr>
<tr>
<td>T·A→G·C</td>
<td>Spontaneous</td>
<td><em>B. lactofermentum</em></td>
<td><em>trpE</em></td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Spontaneous</td>
<td><em>C. glutamicum</em> ATCC 13032</td>
<td><em>malE</em> up</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>Spontaneous</td>
<td><em>C. glutamicum</em> R</td>
<td><em>bgIF</em></td>
<td>[12]</td>
</tr>
</tbody>
</table>

EMS, ethyl methanesulfonate; *malE* up, upstream region of *malE*.
Table 2

Types of base substitutions among NTG-induced mutations in *C. glutamicum* B-6

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Number detected</th>
<th>Mutated genes and base changes (amino-acid changes)</th>
</tr>
</thead>
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<tr>
<td><strong>Transition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G·C→A·T</td>
<td>47</td>
<td>gnd, <strong>ATC</strong>(I)→<strong>ATT</strong>(I), <strong>TCC</strong>(S)→<strong>TTG</strong>(F)*, <strong>CCA</strong>(P)→<strong>TCA</strong>(S); tkt, <strong>GCT</strong>(A)→<strong>ACT</strong>(T)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>zwf, <strong>GCT</strong>(A)→<strong>ACT</strong>(T); opcA, <strong>TCC</strong>(S)→<strong>TTG</strong>(F); ppc, <strong>GCT</strong>(A)→<strong>GTT</strong>(V)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pye, <strong>CCG</strong>(P)→<strong>TGC</strong>(S)*, <strong>AAG</strong>(K)→<strong>AAA</strong>(K), <strong>TAC</strong>(Y)→<strong>TAT</strong>(Y); pgk, <strong>CTT</strong>(L)→<strong>TTT</strong>(F)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gapB, <strong>CGC</strong>(R)→<strong>CGT</strong>(R); gapA, <strong>ATC</strong>(I)→<strong>ATT</strong>(I); eno, <strong>GAG</strong>(E)→<strong>AAG</strong>(K)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pck, <strong>CCA</strong>(P)→<strong>TCA</strong>(S); <strong>ppsA</strong>, <strong>GAC</strong>(D)→<strong>AAC</strong>(N); <strong>pdhA</strong>, <strong>GCG</strong>(A)→<strong>GCA</strong>(A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>pdhB</strong>, <strong>GCT</strong>(R)→<strong>CAT</strong>(H); <strong>pfk</strong>, <strong>GCA</strong>(A)→<strong>ACA</strong>(T), <strong>GAA</strong>(E)→<strong>AAA</strong>(K)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ipd, <strong>CAC</strong>(H)→<strong>CAT</strong>(H), <strong>GCC</strong>(A)→<strong>GTC</strong>(V); <strong>gltA</strong>, <strong>GCC</strong>(A)→<strong>GCT</strong>(A), <strong>AAC</strong>(N)→<strong>AAT</strong>(N)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>odhA, <strong>GCC</strong>(A)→<strong>GTC</strong>(V), <strong>CCA</strong>(P)→<strong>CTA</strong>(L), <strong>GAG</strong>(E)→<strong>GAA</strong>(E); <strong>mdh</strong>, <strong>ACC</strong>(T)→<strong>ATC</strong>(I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>fumH</strong>, <strong>CGC</strong>(R)→<strong>CGT</strong>(R); <strong>acn</strong>, <strong>GAG</strong>(E)→<strong>GAA</strong>(E), <strong>GCC</strong>(G)→<strong>AGC</strong>(S); aceB, <strong>GAA</strong>(E)→<strong>AAA</strong>(K)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aceA, <strong>GCT</strong>(A)→<strong>ACT</strong>(T); <strong>sucC</strong>, <strong>GCA</strong>(A)→<strong>ACA</strong>(T), <strong>GGA</strong>(G)→<strong>GAA</strong>(E), <strong>CCA</strong>(P)→<strong>TCA</strong>(S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tal, <strong>CGC</strong>(R)→<strong>CGT</strong>(R); <strong>mqo</strong>, <strong>TTCC</strong>(F)→<strong>TTT</strong>(F), <strong>TGG</strong>(W)→<strong>TGA</strong>(stop)<em>; <strong>lysC</strong>, <strong>ACC</strong>(T)→<strong>ATC</strong>(I)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>dapE</strong>, <strong>TTC</strong>(F)→<strong>TTT</strong>(F); <strong>dapF</strong>, <strong>AAC</strong>(N)→<strong>AAT</strong>(N); <strong>thrB</strong>, <strong>GCC</strong>(A)→<strong>GTC</strong>(V), <strong>CCA</strong>(P)→<strong>TCA</strong>(S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>thrC</strong>, <strong>ACT</strong>(T)→<strong>ATT</strong>(I), <strong>ACC</strong>(T)→<strong>ACT</strong>(T); <strong>lysA</strong>, <strong>GGG</strong>(G)→<strong>GAG</strong>(E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>A·T→G·C</strong></td>
</tr>
<tr>
<td><strong>Transversion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G·C→T·A</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A·T→C·G</td>
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<tr>
<td>G·C→C·G</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A·T→T·A</td>
<td>0</td>
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</tr>
</tbody>
</table>

Bases that form substitutions are underlined. The five useful mutations relevant to L-lysine production are indicated by asterisks.
Among the useful mutations, the \( \text{GTT(V)} \rightarrow \text{GCA(A)} \) mutation in \( \text{hom} \), the \( \text{ACC(T)} \rightarrow \text{ATC(I)} \) mutation in \( \text{lysC} \), and the \( \text{TGG(W)} \rightarrow \text{TGA(stop)} \) mutation in \( \text{mqo} \) confer on \( \text{C. glutamicum} \) wild-type ATCC 13032 the phenotypes of a partial requirement for \( \text{L-homoserine} \), of resistance to an \( \text{L-lysine} \) structural analog, \( \text{S-(2-aminoethyl)-L-cysteine} \), and of the requirement of \( \text{nicotinamide} \), respectively [6, 22].
C. glutamicum
ATCC 13032

Fig. 1 Ohnishi et al.