Exploration of Genes That Encode a Carbocycle-Forming Enzyme Involved in Biosynthesis of Aminoglycoside Antibiotics from the Environmental DNA

Hideyuki TAMEGAI,† To-ichiro KUKI, Yuri UDAGAWA, Rie AKI, Atsushi NAGAYA, and Shu-ichi TSUKADA

Department of Chemistry, College of Humanities and Sciences, Nihon University, 3-25-40 Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan

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2-Deoxy-scyllo-inosose (DOI) synthase is the enzyme participating in biosynthesis of 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics. The gene which encodes the enzyme can be a marker for screening of DOS-containing aminoglycoside-producer and exploration of its biosynthetic gene. Further, this enzyme is expected to be of use in industry, because it converts sugar into 6-membered carbocycle. In the present study, we identified 21 clones encoding DOI synthase from environmental DNA by degenerate PCR. They were clearly divided into two groups. One appeared to derive from actinomycetes, and the other from non-actinomycetes. The latter group was larger (17 clones) than the former (four clones) despite the fact that only one strain of non-actinomycete was identified for DOS-containing aminoglycoside production. This result indicates that there are still many unidentified non-actinomycetes for DOS-containing aminoglycoside biosynthesis. We showed the possibility of identification of novel aminoglycoside-producing non-actinomycete from soil, and for development of more efficient enzymes for industrial use.

Key words: aminoglycoside; biosynthetic gene; 2-deoxy-scyllo-inosose synthase; industrial use; environmental DNA

2-Deoxy-scyllo-inosose (DOI) synthase is an enzyme involved in the biosynthetic system of aminoglycoside antibiotics, clinically important compounds. Aminoglycosides can be classified into two major groups: those having an aglycone of fully-substituted aminocyclitol biosynthesized from myo-inositol, and those having a 2-deoxystreptamine (DOS) moiety as an aglycone. DOI is a specific intermediate for the biosynthesis of DOS-containing aminoglycoside. DOI synthase and a corresponding gene (btrC) have been identified from the butirosin-producer Bacillus circulans during the study of butirosin biosynthesis.1-4) No corresponding enzyme or gene was found in the biosynthesis system of aminoglycoside which contains myo-inositol-derived aglycone such as streptomycin. The enzyme catalyzes the carbocycle-forming reaction from glucose-6-phosphate to DOI using NAD\(^+\) and Co\(^{2+}\) as cofactors. Its reaction mechanism closely resembles that of 3-dehydroquinate synthase, involved in the shikimate pathway of primary metabolism. Many studies of the reaction mechanism and structure of the enzyme have been performed because of its interesting enzymatic features.5-9) Further, the butirosin biosynthetic gene cluster was identified from B. circulans by DNA fragment coding btrC as a probe.10) In order to explore aminoglycoside-producers and aminoglycoside biosynthetic genes, it has been suggested that specific aminotransferase genes (btrS in the case of B. circulans) can be useful markers.11) Homologous genes have been found in both types of aminoglycoside-producers.12-14) Additionally, we tried to identify homologous genes from soil DNA, and several attracting clones were obtained.15) In contrast to the case of the btrS homologue, genes that encode DOI synthase can be markers for DOS-containing aminoglycoside biosynthetic organisms and genes specifically. DOI synthase is also expected to be available for industrial use. Kakinuma et al. have found that catechol, an important industrial compound, can be prepared in one-pot synthesis from D-glucose with two enzymatic reactions using hexokinase and DOI synthase followed by simple chemical conversion.16) Facing a shortage of fossil resources, novel technology is desirable to prepare chemical compounds from sustainable resources. Especially, carbon-neutral materials synthesized by plants must be developed. D-Glucose is one of the most important compounds, because quite large amounts of it are synthesized by plants by photosynthesis. Frost et al. have found that many aromatics can be prepared from D-glucose by enzymes involved in the shikimate pathway and myo-inositol synthase.17-19) They showed that catechol can be prepared from D-glucose using recombinant Escherichia coli, but the yield of the product was very low because of its toxicity to E. coli.20) Further, the

† To whom correspondence should be addressed. Fax: +81-3-5317-9433; E-mail: htamegai@chs.nihon-u.ac.jp
requirement of D-glucose for cell growth of *E. coli* might lower the yield of the final product. Enzymatic conversion *in vitro*, like the method of Kakinuma *et al.*,16 would solve this problem. One of the difficulties in the application of DOI synthase, however, is that the enzyme from *B. circulans* shows comparatively low activity (k<sub>cat</sub> of 7.3 × 10<sup>-2</sup> s<sup>-1</sup> for glucose 6-phosphate).<sup>1</sup> Although a recombinant DOI synthase showed higher activity than the native enzyme, its k<sub>cat</sub> was only 1.0 s<sup>-1</sup> for glucose 6-phosphate.<sup>2</sup> A efficient enzyme is required for industrial use, but the numbers of identified DOS-containing aminoglycoside-producers are limited. Hence, development of a novel source is desirable for the screening of novel DOI synthase both from a clinical and an industrial viewpoint.

Environmental DNA should be a good source for screening of these genes, because it is thought that the number of microorganisms cultured from soil represents less than 1% of the total communities.21 Further, even in the case of isolated microorganisms, the genes from them are not fully utilized. For example, actinomycetes (a major group for production of antibiotics) have been shown to contain many genes for production of secondary metabolites in their genomes although they produce only a few of them under usual culture conditions.<sup>22,23</sup> Thus such genes are also positioned as non-utilized genetic resources, and an environmental DNA library can cover such genes. Technical advances allow a novel approach for the use of such genes from environmental microorganisms.24 Our recent results<sup>15</sup> strongly suggest that there are many unidentified genes for aminoglycoside biosynthesis, perhaps including DOI synthase genes.

In this study, we succeeded in isolating DOI synthase genes from environmental DNA and phylogenetic analysis. Some attracting genes were found among them. These results should contribute to the preparation of a genetic library for screening of enzymes for industrial use and novel producer of DOS-containing aminoglycoside antibiotics.

**Materials and Methods**

**Organism.** *E. coli* DH5α was cultured in LB medium or on LB-agar containing 50 μg/ml of ampicillin when necessary.

**DNA extraction from soil.** The soil samples were collected from Shinjuku Central Park (Tokyo) and Meiji Jingu Gaien Park (Tokyo). DNA extraction was carried out with UltraClean Soil DNA Kit (MO BIO, Carlsbad, CA) according to manufacture’s protocol.

**Cloning of the genes by PCR.** In order to identify brc homologues, three degenerate primers, kanC1f (GCC-GSCMTGMTGTTTYCGGGGATT), kanC2f (CTKRT-KCACGTTCGCCAGCAGC), and kabs3L (CCNAYNG-TRTGNCCRTAYTC) were designed from the well-conserved regions between brc, tbnA (for tobramycin biosynthesis in *Streptomyces tehrerarius*) and gmiA (for gentamycin biosynthesis in *Micromonospora echinospora*). PCR (one cycle at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 51–60 °C for 1 min, and 72 °C for 1 min, and then 72 °C for 10 min) was carried out with these primers, with soil DNA as template. The first PCR was carried out using kanC1f and kabs3L as primers, and the products were subsequently used as templates for nested PCR using kanC2f and kabs3L as primers. Nested-PCR product was subcloned into pT7blue T vector (Novagen, San Diego, CA), and the resulting plasmid was sequenced.

**Molecular biological procedure.** DNA manipulations were performed as described in the literature.25 DNA sequencing was carried out by Bio Matrix Research (Chiba, Japan). PCR was performed by MyCycler (Bio-Rad, Hercules, CA) using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Oligo DNAs for PCR primer were synthesized by Sigma Genosys (Hokkaido, Japan). Purification of plasmids was carried out with GFX MicroPlasmid Prep Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). Restriction enzymes and modification enzymes were purchased from Takara Bio (Shiga, Japan). Genetic analysis was performed with GENETYX-WIN ver. 5.00 (Software Development, Tokyo), and a database search was carried out by BLAST<sup>26</sup> on Internet (http://www.ddbj.nig.ac.jp/search/blast-j.html). An evolutionary tree was constructed by the neighbor-joining method<sup>27</sup> using the CLUSTAL_W program<sup>28</sup> on the Internet (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). All other reagents were of the highest grade commercially available. Frame analyses of obtained sequences were performed by FramePlot<sup>29</sup> on the Internet (http://www.nih.go.jp/jun/cgi-bin/frameplot.pl).

**Results and Discussion**

According to the protocol described in “Materials and Methods,” we obtained 21 individual clones that were homologous with DOI synthase genes for aminoglycoside biosynthesis, as shown in Table 1. We found all of these genes in the soil from Shinjuku Central Park, from which many kinds of aminoglycoside-biosynthetic aminotransferase genes were obtained,<sup>15</sup> but we found no DOI synthase genes in the soil from Meiji Jingu Gaien Park. These findings support our previous suggestion that there are differences in the diversity of aminoglycoside-biosynthetic genes between these places.

Figure 1 shows a phylogenetic tree constructed from deduced amino acid sequences of obtained clones and known homologues. The tree clearly indicates that DOI synthase genes from soil can be classified into two major groups: group A and group B. The clones in group A and those in group B was not highly similar to each other. For example, the homology of deduced amino
The genes in group A showed comparably high GC contents in third letters of codons (higher than 95%), which is a specific property of ORF in actinomycetes. In contrast, the GC contents of those in group B genes were below 95%, and \textit{btrC} from \textit{B. circulans} was included. \textit{B. circulans} is the organism for production of butirosin, as described above, and it is the sole non-actinomycete as an aminoglycoside-producer. Butirosin has a unique 4-amino-2-hydroxybutyric acid (AHBA)-side chain in its structure. It is well-known that butirosin is resistant to certain enzymes that modify aminoglycosides in resistant bacteria due to the presence of AHBA, and AHBA is important in semisynthetic aminoglycoside antibiotics against resistant strains. It is possible that aminoglycosides produced by non-actinomycetes have a unique structure, like butirosin. Thus it is quite significant to find aminoglycoside-producing non-actinomycetes. The DNA fragment obtained can be useful marker for the screening of such organisms. Especially, clones s233 and s9310 were closely related to \textit{btrC}. These genes can serve as probes for interesting organisms.

We obtained 17 clones which might derived from non-actinomycetes in a total of 21 clones. In the case of aminotransferase genes also involved in aminoglycoside biosynthesis, only five were identified to be in the same branch as the butirosin-biosynthetic gene \textit{btrS} among 33 clones. These results might due to a difference in the enzymes in participation in aminoglycoside biosynthesis. Aminotransferase is involved in both \textit{myo}-inositol-derived aglycone-containing and DOS-containing aminoglycoside biosynthesis, and DOI synthase participates only in DOS-containing aminoglycoside biosynthesis. To date, \textit{B. circulans} is the only non-actinomycete for aminoglycoside biosynthesis. Our results suggest that there is substantially less (or no) non-actinomycete for \textit{myo}-inositol-derived aglycone-containing aminoglycoside production, and that there are still many unidenti-

\begin{table}[h]
\centering
\caption{DNA Fragments Obtained}
\label{tab:fragments}
\begin{tabular}{lcc}
\hline
Clones & (accession no.) & Clones (accession no.) \\
\hline
ki1 & (AB245097) & s323 & (AB245108) \\
ki2 & (AB245098) & s324 & (AB245109) \\
ki3 & (AB245099) & s325 & (AB245110) \\
kuki1 & (AB245100) & s326 & (AB245111) \\
kuki2 & (AB245101) & s327 & (AB245112) \\
s192 & (AB245102) & s931 & (AB245113) \\
s193 & (AB245103) & s933 & (AB246701) \\
s231 & (AB245104) & s9310 & (AB246702) \\
s232 & (AB245105) & sha1 & (AB245114) \\
s233 & (AB245106) & sha2 & (AB245115) \\
s321 & (AB245107) & & \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic Tree Showing the Relationships between DOI Synthase Genes and Homologous Genes from Soil. BtrC (AB019237); \textit{B. circulans} (butirosin-producer), \textit{GntB} (AY524043); \textit{M. echinospora} (gentamycin-producer), NeoC (AB211959); \textit{Streptomyces fradiae} (neomycin-producer), ORF11 (AB164642); \textit{Streptomyces kanamyceticus} (kanamycin-producer), RbmA (AJ748131); \textit{Streptomyces ribosidifus} (ribostamycin-producer), TbmA (AJ579650); \textit{S. tenebrarius} (tobramycin-producer), AroB (M80245); 3-dehydroquinase synthase from \textit{Bacillus subtilis}. The tree was constructed based on deduced amino acid sequences of the genes. The sequence of AroB was used as the outgroup for the phylogenetic tree. Bar indicates 0.1 amino acid residue substitutions per site. Each number in parenthesis indicates the GC contents in the third letters of the codons.}
\end{figure}
fied non-actinomycetes for DOS-containing aminoglycoside biosynthesis.

An alignment of the deduced amino acid sequences of DNA fragment is shown in Fig. 2. This result clearly indicates that DOI synthases showed wide diversity in their amino acid sequences, but some amino acid residues were found to be completely conserved in all clones from soil and genes of producers. Especially, Asp135, Lys141, Asn151, Glu183, and Lys186 (numbered from the BtrC sequence of B. circulans) were completely conserved in all clones. Those residues were thought to be important in substrate recognition and enzymatic activity.2,7) Adjacent regions of these important residues were also conserved. This observation suggests that products of the genes obtained should be functional. The DNA fragments obtained in this study...

Fig. 2. Alignment of DOI Synthase Homologues.
Highlighted residues show well-conserved residues in sequences. Asterisks indicate residues considered to be required for enzymatic activity.
did not contain the full-length of the coding region for each DOI synthase. Thus we couldn’t measure the enzyme activities of these gene products, but they can serve as useful markers for the screening of full genes from the metagenomic library. In the present study, we succeeded in identifying genes encoding DOI synthase involved in aminoglycoside-biosynthesis from environmental DNA. The results provide novel information for future work on aminoglycoside biosynthesis and the use of the enzyme for industry.

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References


ducing the ability of producing secondary metabolites. 


