We obtained 19 individual DNA fragments encoding 2-deoxy-scyllo-inosose synthase involved in the biosynthesis of aminoglycoside antibiotics from deep-sea sediments of the Pacific Ocean. Compared with genes from land-based environmental DNA, they showed low diversity. Combined with our previous study concerning the discovery of other aminoglycoside biosynthetic genes from the same deep-sea samples, we suggest the importance of exploration of multiple biosynthetic genes to determine the diversity of aminoglycoside producers. We found that the deep sea is a useful source for screening of these genes.

Key words: deep sea; aminoglycoside; biosynthetic gene; 2-deoxy-scyllo-inosose synthase; environmental DNA

2-Deoxy-scyllo-inosose (DOI) synthase is an enzyme involved in the biosynthetic pathway leading to the formation of 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics. DOI synthase and a corresponding gene (btrC) were identified in the butirosin-producer Bacillus circulans during a study of butirosin biosynthesis.1–3) The enzyme catalyzes the carbocycle-forming reaction from glucose-6-phosphate to DOI using NAD+ and Co2+ as cofactors. Many studies of the reaction mechanism and structure of the enzyme have been performed due to its interesting enzymatic features.4–10) DOI synthase is thought to have evolved from 3-dehydroquininate synthase which is involved in the shikimate pathway of primary metabolism.1,2) This enzyme is not found in other metabolic system up to date, and is considered to be involved specifically in the aminoglycoside biosynthesis.

DOI synthase is expected to be available for industrial use in green chemistry. A novel technology is required to prepare chemical compounds from sustainable resources such as glucose because of a shortage of fossil resources. In this regard, DOI synthase is worthy of consideration. Kakinuma et al. found that catechol, an important industrial compound, can be prepared by single-pot synthesis from D-glucose with two enzymatic reactions using hexokinase and DOI synthase, followed by simple chemical conversion.11) Furthermore, convenient synthesis of DOI has been demonstrated in genetically-engineered Escherichia coli.12)

Nevertheless, the enzyme from B. circulans shows comparatively low activity (a $k_{cat}$ of $7.3 \times 10^{-2}$ s$^{-1}$ for glucose 6-phosphate).13) Hence a more efficient enzyme is required for industrial use.

For general screening of novel genes, environmental DNA is a good source. We have cloned many genes for aminoglycoside biosynthesis from land-based environmental DNA.13,14) Further, we have identified the genes encoding aminotransferase likely to be involved in the biosynthesis of aminoglycosides from deep-sea environmental DNA.15) These genes were homologous to btrS of B. circulans, which encodes L-glutamine:DOI aminotransferase, which is required for the biosynthesis of DOS.16) This suggests that aminoglycoside producers are widely distributed over Earth. Specifically, the deep sea is a good source of novel genetic resources because it is a relatively unexplored domain and is expected to be a habitat of previously unrecognized microorganisms. In this study, we cloned DOI synthase genes from deep-sea environmental DNA in which aminotransferase genes have been already identified.

Deep-sea environmental DNA was prepared in our previous study.15) Deep-sea sediments were collected by the unmanned submersible Hyper-Dolphin from the Hiruo Valley, off Kushiro in the northwestern Pacific Ocean on cruise NT04-11 on October 3–12, 2004. Samples MGM, ML1, ML2, K2 (42°10.755’N, 144°10.507’E, 1,264 m depth), K2C1 (42°10.850’N, 144°10.501’E, 1,257 m depth), and MY1 (42°10.822’N, 144°10.496’E, 1,255 m depth) were used. MGM (0–1 cm from the sea bottom), ML1 (1–10 cm from the bottom), and ML2 (10–17 cm from the bottom) were recovered from the same site with a single core mud sampler. K2 was recovered from almost the same location with a rake. MC1 and MY1 were samples from the surface of the sea bottom at each site. In order to obtain the target genes from the deep-sea environmental DNA, degenerate PCR was carried out as described previously.14) Primers (nucleotide numbers 340–363 for kanC1f, 367–387 for kanC2f, and 727–746 for kabs3L based on the sequence of btrC) were designed from the well conserved region between btrC, tbrA (for tobramycin biosynthesis in Streptomyces tenebrarius) and gtmA (for gentamicin biosynthesis in Micromonospora echinospora). Using these primers, nested PCR was carried out. PCR products were subcloned into pT7-blue T

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vector (Novagen, San Diego, California, USA), and the resulting plasmids were randomly sequenced. DNA sequencing was carried out by Bio Matrix Research (Nagareyama, Chiba, Japan). PCR was performed with a MyCycler (Bio-Rad, Hercules, California, USA) using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California, USA). Oligo DNAs for PCR primers were synthesized by Sigma Genosys (Ishikari, Hokkaido, Japan). A database search was carried out by BLAST17) using the Internet (http://www.ddbj.nig.ac.jp/search/blast-j.html). An evolutionary tree was constructed by the neighbor-joining method18) using the CLUSTAL W program,19) available on the Internet (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). Sequence analysis was carried out by BioEdit.20) The sequences determined in the present study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession nos. AB510568–AB510587.

Using the PCR technique, we identified 23 individual DNA fragments (338–342 bp) encoding putative DOI synthase in environmental DNA from the deep sea of the northwestern Pacific Ocean, as shown in Figs. 1 and 2. These genes were highly homologous to DOI synthase genes deposited in the DDBJ/EMBL/GenBank databases (Fig. 1), but not to the other genes with high similarities. Clones from the same site with the same nucleotide sequences were eliminated (MGMC-1, -5, -6, -9, ML1C-4, and ML2C-4 were as same as MC1C-3 at the amino acid level. BtrC, B. circulans (butirosin-producer, AB019237); GntB, Micromonospora echinospora (gentamicin-producer, AY524043); NeoC, Streptomyces fradiae (neomycin-producer, AB211959); TbmA, Streptomyces tenuevibrio (tobramycin-producer, AJ579650). The clones whose names begin with the letter “M” were obtained in this study. No specific amplification was observed in the samples K2 and MY1. Like the clones from land environmental DNA we investigated previously,14) the important residues for enzyme activity were almost completely conserved (Fig. 1). A phylogenetic tree (Fig. 2) indicated that the genes obtained in this study showed high similarity with each other. Some of them might be explained by PCR error, but clones from MC1 and those from other soil samples might be originated from individual species, because MC1 and the others were collected from different sites. Thus, the clones obtained from this area showed quite low diversity as compared with similar genes from land environmental DNA samples. The homologies between the clones obtained were above 90%, except for MGMC-12 (60%) and -14 (50%). Our previous results indicated that btrS-homologous genes from the same deep-sea environmental DNA showed as much diversity as similar genes from land-based environmental DNA.15) Two possibilities might explain the difference in diversity of these genes. One is that producers of DOS-containing aminoglycoside are not dominant in these areas. Relative to the variation in chemical structure, aminoglycosides can be classified into two major groups. The first group is made up of compounds possessing a common aglycone of DOS, and the other is composed of those having an aglycone of Fig. 1. Alignment of DOI Synthase Homologs. Highlighted residues show well-conserved residues. Asterisks indicate residues thought to be important for enzyme activity. Arrow indicates the position of frame-shift in ML1C-3, 7, 9, and 10. The sequences of MGMC-1, -5, -6, -7, -9, ML1C-4, and ML2C-4 were as same as MC1C-3 at the amino acid level. BtrC, B. circulans (butirosin-producer, AB019237); GntB, Micromonospora echinospora (gentamicin-producer, AY524043); NeoC, Streptomyces fradiae (neomycin-producer, AB211959); TbmA, Streptomyces tenuevibrio (tobramycin-producer, AJ579650). The clones whose names begin with the letter “M” were obtained in this study.
aminoglycosides. It is possible that the producers of aminotransferase is required for the biosynthesis of all DOI synthase is specifically involved in the biosynthesis of myostreptamine which is synthesized from myo-inositol. DOI synthase is specifically involved in the biosynthesis of myostreptamine.

Fig. 2. Phylogenetic Tree of btrC Homologs.
Genes from producers: aroB (M80245, the gene encoding dehydroquinate synthase from Bacillus subtilis); btrC, B. circulans (butirosin-producer, AB019237); gntB, Micromonospora echinospora (gentamicin-producer, AYS2043); kanA, Streptomyces kanamyceticus (kanamycin-producer, AJ582817); neoC, Streptomyces fradiae (neomycin-producer, AB211959); rbmA, Streptomyces ribosidicus (ribostamycin-producer, AJ748131); tbrC, Streptomyces tenebrarius (tobramycin-producer, AJ579650); DET0400, dehydroquinate synthase from Dehalococcoides ethenoges (CP000027). Genes from land environmental DNA: ki1, ki2, ki3, kuki1, kuki2, s192, s193, s231, s232, s233, s321, s323, s324, s325, s326, s327, s931, s933, sha1, sha2 (AB244097-115). The bar indicates 0.1 nucleotide substitutions per site. The sequence of aroB was defined as an outgroup. The clones whose names begin with the letter “M” were obtained in this study.

streptamine which is synthesized from myo-inositol. DOI synthase is specifically involved in the biosynthesis of the former group. On the other hand, btrS-type aminotransferase is required for the biosynthesis of all aminoglycosides. It is possible that the producers of myo-inositol-derived aminoglycosides are dominant at the sampled areas. This suggestion cannot be obtained by analysis of the btrS-homologous genes, because no significant difference was observed in nucleotide sequences between those from DOS-containing aminoglycoside producers and those from streptamine-containing aminoglycoside producers. At least, this might be true for the K2 and MY1 samples, because genes encoding DOI synthase were not found in these samples, although we found putative aminotransferases in these same samples in a previous study. The other possibility is that some of the btrS-homologous genes isolated from the same deep-sea environmental DNA were not from the aminoglycoside producers as discussed in our previous paper. To investigate the diversity of aminoglycoside producers, additional exploration of both genes is required. Most of the genes showed comparably high GC contents at the third position of each codon (more than 95%). This is a specific property of ORFs in actinomycetes. Only two clones, MGMC-12 and -14, exhibited relatively low contents (82% and 72% respectively). In regard to aminoglycoside producers, actinomycetes are likely to be dominant in these areas. In our previous study, 17 clones out of 21 of the genes from land environmental DNA showed low GC contents at the third codon position. Biodiversity of aminoglycoside producers appears to be different in different environments. Most of the genes were closely related to genes identified in the genomes of aminoglycoside-producers and in land-based environmental DNA. However, MGMC-12 was distinct from known clones from producers and land environmental DNA. It might be from aminoglycoside-producers that are specific to the deep sea. The gene product of the clone might show some novel properties as compared with known DOI synthases.

In the present study, we identified many DNA fragments encoding DOI synthase from deep-sea environmental DNA. Further, we also suggest the importance of exploration of multiple aminoglycoside-biosynthetic genes. On the basis of this study, we can now expand the world of potential aminoglycoside producers.

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References