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Molecular Cloning of the Gene for the Key Carbocycle-forming Enzyme in the Biosynthesis of 2-Deoxystreptamine-containing Aminocyclitol Antibiotics and Its Comparison with Dehydroquinate Synthase

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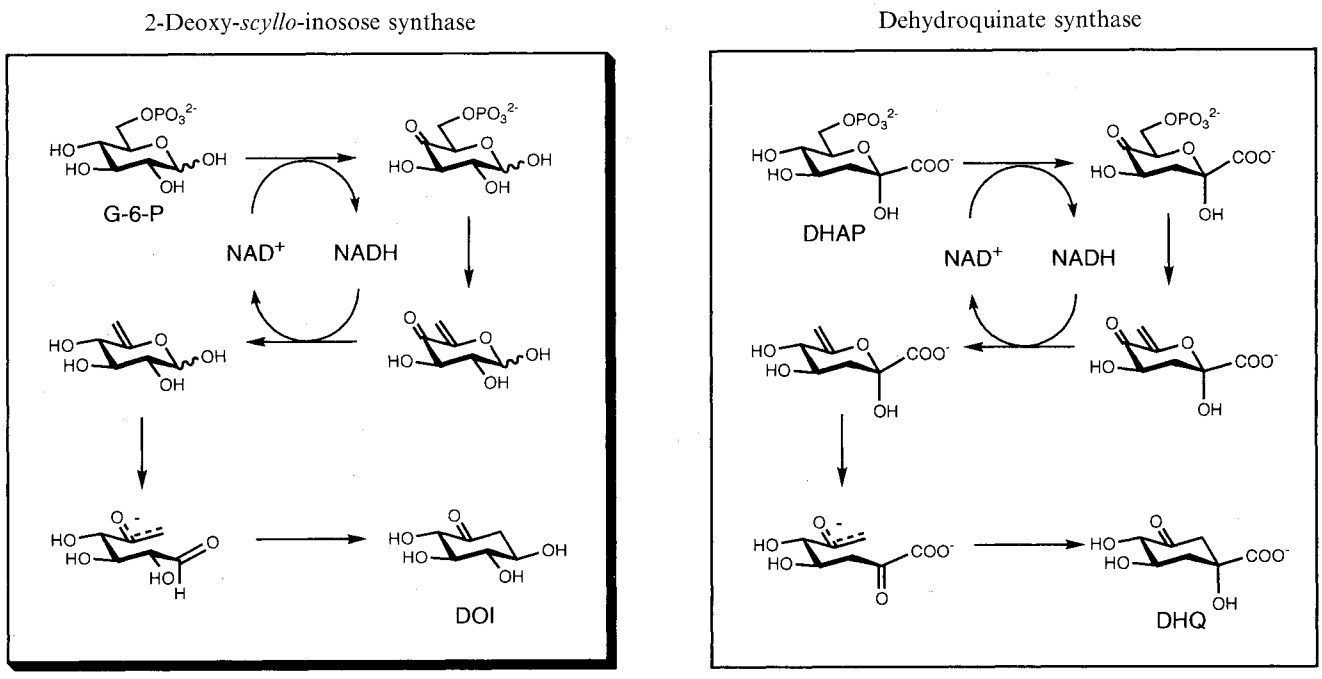
The 2-deoxystreptamine aglycon is a common structural feature found in aminocyclitol antibiotics including neomycin, kanamycin, tobramycin, gentamicin, sisomicin, butirosin and ribostamycin. A key enzyme involved in the biosynthesis of the 2-deoxystreptamine moiety is 2-deoxy-*scyllo*-inosose (DOI) synthase which catalyses the carbocycle formation from D-glucose-6-phosphate to 2-deoxy-*scyllo*-inosose. The recent success of isolating the 2-deoxy-*scyllo*-inosose synthase from *Bacillus circulans* prompted us to clone the gene responsible for this important enzyme by the use of reverse genetics approach. With the aid of DNA probes constructed on the basis of the amino-terminal sequence of the purified 42 kDa subunit of the enzyme, the responsible gene *btrC* was successfully cloned. Subsequently the *btrC* gene was heterologously expressed in *Escherichia coli*, and the 2-deoxy-*scyllo*-inosose synthase activity of the recombinant polypeptide was confirmed by chemical analysis. The *btrC* gene encodes a protein composed of 368 amino acids with a molecular mass of 40.7 kDa. Our previous proposal for the similarity of 2-deoxy-*scyllo*-inosose synthase to dehydroquinate synthase has been confirmed on the basis of their amino acid sequences. Significant differences in the sequences can also be observed however, particularly in the crucial substrate recognition regions. Comparison of the BtrC sequence with those of biosynthetic enzymes for other related microbial products is also discussed.

Over many years, the genetics of the biosynthesis of clinically important classical antibiotics, including β-lactams (penicillins, cephalosporins), non-ribosomal peptides (gramicidins, cyclosporin *etc.*), polyketides (macrolides, tetracyclines, anthracyclines *etc.*), and streptomycin, have been investigated extensively. Surprisingly however, the molecular genetics of the biosynthesis of another major group of classical aminocyclitol antibiotics which contain the 2-deoxystreptamine (DOS) moiety such as neomycin, kanamycin, tobramycin, gentamicin, sisomicin, butirosin, ribostamycin *etc.*,[1,2] has not been reported to date. While many resistance genes for these antibiotics have been identified in the producing organisms and drug-resistant bacteria,[3] those concerning the biosynthesis have evaded discovery.[4] This paper describes the first successful identification of the gene for 2-deoxy-*scyllo*-inosose (DOI) synthase which is a key enzyme required for carbocycle formation during the biosynthesis of the above mentioned DOS-containing aminocyclitol antibiotics.

DOS, which is chemically a pentasubstituted cyclohexane, is a common aminocyclitol aglycon found only in microbial secondary metabolites. The biosynthesis of DOS-containing aminocyclitol antibiotics has been extensively studied, mostly by the use of isotope-tracer

Fig. 1. 2-Deoxy-*scyllo*-inosose synthase and dehydroquinate synthase reaction.



technology with whole-cell systems of producing microorganisms, as well as non-producing blocked (idiotrophic) mutants.[5~8] The biosynthesis of aminocyclitol antibiotics which contain a fully-hexasubstituted cyclohexane moiety biosynthesized from *myo*-inositol including streptomycin and fortimicin have already been studied on the genetic level.[9,10] However, the biosynthetic genes of the DOS-containing aminocyclitol antibiotics have never been identified as explained above. The enzymes involved in the biosynthesis of these antibiotics also remained unclear except for some particular enzymes, *e.g.* transamination enzymes of relevant inososes.[11,12]

For the biosynthetic pathway of DOS-containing aminocyclitol antibiotics, the DOI synthase reaction appears to be a crucial starter step. The DOI synthase catalyses the intramolecular carbocyclization of D-glucose-6-phosphate (G-6-P) into the first non-aminogenous cyclitol DOI.[5,6,13] The reaction mechanism of the enzyme has been elucidated by us as shown in Fig. 1, utilizing cross-over experiments using a doubly isotopically labeled substrate with partially purified enzyme preparations derived from butirosin-producing *Bacillus circulans* SANK 72073 and neomycin-producing *Streptomyces fradiae* IFO 13147.[14~16] The multi-step process includes a first oxidation at the C-4 position of G-6-P, and subsequent elimination of a phosphate group from the activated ulose follows forming an enol or enolate intermediate. Subsequent reduction at C-4, the opening of the hemiacetal ring, and the last aldol-type intramolecular condensation between C-1 and C-6 give rise to DOI. This biosynthetic reaction of DOI synthase in the microbial secondary metabolism appears mechanistically similar to that of dehydroquinate synthase (DHQ synthase) in the shikimate pathway of primary metabolism.[14,15,17,18]

Recently, we reported the first purification of a DOI synthase from *B. circulans*.[19] Some biochemical similarities and differences between DOI synthase and DHQ synthases were observed. DOI synthase from *B. circulans* was isolated as a heterodimeric protein comprised of 23 kDa and 42 kDa subunits. On the basis of similarity to DHQ synthase, the 42 kDa subunit was anticipated to be responsible for the enzyme reaction. In order to prove or disprove the proposed close relationship between DOI synthase and DHQ synthase, which appears to be quite intriguing in terms of molecular and functional evolution as well, we have carried out molecular cloning and functional expression of the gene of DOI synthase from *B. circulans*.

This paper describes the first successful cloning and over-expression of a gene responsible for the biosynthesis of DOS-containing aminocyclitol antibiotics. Also discussed are comparative studies of the amino acid sequences of the gene named *btrC* for the 42 kDa subunit of DOI synthase from *B. circulans*, DHQ synthase, and other mechanistically related enzymes involved in the biosynthesis of microbial secondary metabolites.

Materials and Methods

General Information

Materials from commercial sources include D-glucose-6-phosphate (G-6-P) dipotassium salt (Sigma), NAD^+ (Oriental Yeast Co., Tokyo), reagents for electrophoresis and copper staining (Bio-Rad), and trypsin (Funakoshi, Japan). All other general reagents were of the highest analytical grade available. SDS-removal cartridges were purchased from Michrom BioResources, Inc (Auburn, CA, USA). D-[6,6-$^2H_2$] glucose-6-phosphate dipotassium salt was prepared as described previously.[20,21] Restriction enzymes and T4 DNA ligase were purchased from Takara and used in accordance with the manufacturer's instructions.

High pressure liquid chromatography (HPLC) was performed on a Hitachi L-6000 chromatograph equipped with a Hitachi L-4000 UV-detector and a Hitachi D-2500 integrator, for the enzyme assay. For the enzyme purification, FPLC (Pharmacia Biotech.) was used. ^{1}H-NMR and ^{2}H-NMR spectra were recorded with a JEOL LA-300 or LA-400 spectrometer. Deuteriomethanol (Merck, 99.8 atom% enriched) was used as ^{1}H-NMR solvent.

Bacterial Strains, Plasmids, and Culture Conditions

Butirosin-producing *Bacillus circulans* SANK 72073 was used as the donor of DOI synthase and chromosomal DNA, and was grown in nutrient-glycerol medium at 28°C.[15,19] *Escherichia coli* JM 105 was used for transformation of DOI synthase gene cloning. *E. coli* BL 21 (DE3) was used for the gene expression. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium or on LB plates. Plasmid pUC19 was used as a cloning vector. LB plate containing 50 μg/ml ampicillin was used when pUC19-containing cells were selected. Plasmid pET-30b(+) (Novagen) was used as an expression vector. LB plates containing 30 μg/ml kanamycin were used when pET-30b(+)-containing cells were selected.

Determination of *N*-Terminus Amino Acid Sequence

The 23 kDa and 42 kDa subunits of native DOI synthase purified from *B. circulans* (about 5 μg)[19] were separated on 12.5 % Tricine-SDS-PAGE according to the literature procedure.[22] After the electrophoresis, these proteins were transferred from the gel to a PVDF membrane (Millipore) by electro-blotting. After these proteins were visualized by Coomassie brilliant blue staining, the two bands were excised separately and were subjected to sequence analysis accordingly. Amino acid sequencing was performed on a peptide sequencer (Shimadzu, PPSQ-21, Tokyo) equipped with an LC 10AS liquid chromatograph and an SPD-10A UV-VIS spectrophotometer.

Internal Amino Acid Sequence

For the internal amino acid sequence analysis, the band of the 42 kDa subunit of the *B. circulans* DOI synthase was separated by electrophoresis, which was subsequently visualized by copper staining. SDS-PAGE was performed at 200 V for 40 minutes using a Bio-Rad Mini Protean II Ready Gel Cell and a Ready Gel J 12.5%. The gel was immersed in Milli-Q-purified water for 3 minutes to remove excess SDS, and subsequently stained in a 1 : 10 dilution of Bio-Rad Copper Stain solution for 3 minutes. The gel was used immediately for the preparation of protein samples as described below. The appropriate band and control band were excised from a copper stained polyacrylamide gel with a scalpel, transferred into a polypropylene tube which had been washed by acetonitrile for three times, and destained in a 1 : 10 dilution of Bio-Rad Copper Destain solution. Then, the gel slices were washed twice by shaking in a 100 μl solution of 50% v/v acetonitrile, 0.2 M NH_4HCO_3 for 15 minutes. The buffer was discarded and the gel pieces were dried by vacuum centrifugation. The gel pieces were then reswollen in a 5 μl digestion buffer containing 1 μg of trypsin in 5 μl of 0.2 M NH_4HCO_3 (pH 7.6). When the digest buffer had been absorbed or the gel pieces completely rehydrated, 40 μl of 0.2 M NH_4HCO_3 was added to completely cover the gel and the mixture was incubated overnight at 37°C. Following digestion, the supernatant was transferred to an empty polypropylene tube previously being washed as described above. The gel pieces were extracted twice by 200 μl washes with 60 % v/v acetonitrile containing 0.1% HCOOH for 30 minutes. The combined washes were reduced in volume to approximately 5 μl by vacuum centrifugation, and stored at −80°C until use.

Nano-Liquid Chromatography/Electrospray Ioniza-

tion Ion Trap-Mass Spectrometry/Mass Spectrometry (nano-LC/ESI IT-MS/MS) was performed on a Finnigan LCQ (San Jose, CA, USA). The nano-HPLC system consisted of an ABI 140B dual syringe solvent delivery system (Applied Biosystems, Inc., Foster City, CA) with a Kontron 433 nano detector set (Milano, Italy) to detect 210 nm light. The chromatographic solvents consisted of (A) 0.1% aqueous TFA and (B) acetonitrile/water (9 : 1) with 0.095% TFA. Each sample was injected for 85 minutes with 98% solvent A, using SDS-remove cartridge. The elution was isocratic for 5 minutes with 98% solvent A, and then gradient elutions of 98% A to 85% A (5~15 minutes), 85% A to 40% A (15~55 minutes), and 40% A to 2% A (55~65 minutes) were performed for the isolation of peptides on a Hypersil C18 Nano column (75 μm × 15 cm) packed by LC Packings (San Francisco, CA). The solvents were mixed and split by an Accurate unit (LC Packings) and the flow rate was reduced to *ca.* 200 nl/minute. Proteolytic peptides were injected into the capillary HPLC system and then were introduced into the mass spectrometer after separation.

50% Methanol was used as the sheath liquid at a flow rate 0.5 μl/minute. The trap was run with automatic gain control for all experiments. In this mode, the system automatically selects the trapping parameters to keep the number of ions present in the trap to a constant preset value. The electron multiplier was set to −850 V. The numbers of "microscans" collected were 3 and 2 for the full MS and the MS/MS, respectively. In the CID mode, the trap was filled for up to 50 msec, depending on the number of ions entering the trap per unit of time. In this mode, the threshold to trigger ion selection was 5.0×10^4, and the default collision energy was set to 50%. Nano-LC/ESI IT-MS and Nano-LC/ESI IT-MS/MS were run in an automated nano-LC/MS-MS/MS mode that monitored for a signal threshold and performed MS/MS on the base peak when the threshold criteria were exceed. Molecular weights and partial amino acid sequences of the peptides were attributed to the sequence of the amino acid sequence estimated from the *btrC* gene, using the BioWorks software supplied with the LCQ.

Cloning of the *btrC* Gene from *B. circulans*

Agarose gel electrophoresis and DNA manipulations and transformation in *E. coli* were done as described by SAMBROOK *et al.*[23] On the basis of the partial *N*-terminal sequence of 42 kDa subunit from *B. circulans*, two oligonucleotide, 40 kA (5'-TTYGCNTTYGGNGAR-CAYGT-3') and 40 kB (5'-TCNCCRAANGCRAART-TRAA-3') were designed and customary synthesized by Amersham Pharmacia. The oligonucleotides were 3'-end labeled with DIG (Digoxigenin-11-dUTP, alkali-labile) according to DIG Oligonucleotide 3'-End labeling Kit (Boehringer Mannheim). DIG DNA Labeling Kit (Boehringer Mannheim) was used for the larger DNA fragments obtained through restriction digest.

B. circulans chromosomal DNA was cleaved with several restriction enzymes and the resulting hydrolysates were subjected to agarose gel electrophoresis. DNA fragments were transferred to Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad). Hybridization was performed according to the standard protocol of the kit. Hybridization temperature was 50°C for oligonucleotide (20 mer) and 65°C for the larger DNA restriction fragments. Stringency washes were done with 2 × SSC at 50°C for oligonucleotides or with 1 × SSC at 65°C for the larger DNA fragments. The hybridized bands were visualized using a DIG Luminescent Detection Kit (Boehringer Mannheim).

The fragments of interest which were identified by Southern hybridization were recovered from the agarose gels and ligated to the appropriate dephosphorylated site of pUC19. The ligation mixture was used to transform competent *E. coli* JM 105 prepared using $CaCl_2$. Transformants were selected on LB agar containing ampicillin (50 μg/ml), isopropyl β-D-thiogalactopyranoside (200 μg/ml), and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (40 μg/ml). The transformants were lysed and the DNA was immobilized on a Hybond-N+ membrane (Amersham). The membrane was then screened by hybridization.

DNA Sequencing and Computer Analysis of Protein and DNA Sequences

Various overlapping restriction fragments from the inserted plasmids (pDS1, pDS2 of Fig. 2) were subcloned respectively into pUC19, and sequenced by the dideoxynucleotide chain-termination method using the Thermo Sequenase cycle sequencing kit (Amersham) and M13 Forward primer, M13 Reverse primer (Amersham), and a DNA sequencer (Li-cor, model 4200) according to the protocols of the suppliers. The entire sequences of both strands were determined from double-stranded plasmid DNAs prepared by the alkaline lysis method.[23] The DNA sequences were analyzed using the GENETYX program (Software Development). Homology searches were performed against the EMBL, Genbank or SWISSPROT data libraries using the BLAST[24] and FASTA[25] software on the net.

Overexpression of the *btrC* Gene in *E. coli*

The *btrC* gene was amplified by polymerase chain reaction (PCR) using pDS2 as a template, and the primers employed were 40 kf (5'GGTGGAGGATCCATATGACGACTAA3') and 40 kr (5'AAAGCAAGCTTATCCTGGTTCATCC3') (Amersham Pharmacia). The primer 40kf was so designed to introduce a *Bam*HI and an *Nde*I site (underlined) in place of the natural start codon and for the ability to create a start codon fusion of *btrC* downstream from the ribosome-binding-site of the expression vector pET-30b(+). The primer 40 kr was designed to introduce a *Hind*III site (underlined) for the later ligation of the *btrC* DNA fragment into pUC19 *Bam*HI/*Hind*III or pET-30b(+) *Nde*I/*Hind*III. PCR was performed in a Gene Amp PCR System 9700 (Perkin Elmer, Applied Biosystems) using UlTma DNA polymerase (Roche). The reaction mixtures (100 μl) contained 50 μg of pDS2 DNA, 60 pmol of each primer, 0.25 mM dNTPs, and an incubation buffer. The following conditions were used for the PCR reaction: the enzyme was added after an initial denaturation for 1 minute at 95°C, followed by 30 cycles [95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds] and 72°C for 7 minutes. The PCR-amplified product was digested with *Bam*HI and *Hind*III, and was further cloned into the appropriate site of pUC19, and a resulting plasmid (pDS3) was isolated. After sequencing pDS3, the insert DNA was isolated after digestion with *Nde*I/*Hind*III, and was again ligated into the *Nde*I-*Hind*III site of pET-30b(+). The resulting derivative pDS4 was transformed into *E. coli* BL 21(DE3). The preculture of a transformant containing pDS4 was performed with shaking at 37°C until OD_{600} reached 0.6. The preculture was used to inoculate a growth culture medium (1/100 of volume). The growth culture was shaken at 37°C until the OD_{600} reached 0.6, at which point IPTG was added (final concentration of 0.4 mM) and the incubation was continued for 2~3 hours. The cells were harvested by centrifugation (4,000 × *g* 10 minutes, 4°C), and washed with a 50 mM Tris-HCl buffer containing 0.2 mM $CoCl_2$ (pH 7.7) (4,000 × *g* 10 minutes, 4°C). The cells were stocked at −75°C until use for purification of the recombinant enzyme.

Confirmation of DOI Synthase Activity of BtrC

The cells of *E. coli*/pDS4 were suspended in a 50 mM Tris-HCl buffer containing 0.2 mM $CoCl_2$, pH 7.7 (5 g of wet cells in 150 ml), and were then sonicated by a Branson sonifier Type-250 for 2 minutes × 10 times at 0°C in an iced water bath. The sonicate was centrifuged at 12,000 × *g* for 30 minutes and the resulting supernatant was used as a cell-free extract for enzyme reaction. The reaction was carried out with G-6-P or [6,6-$^2H_2$]-G-6-P as substrate in the presence of 5 ml of the cell-free extract, 5 mM NAD^+, and 5 mM Co^{2+}, at 46°C for 2 hours. The reaction product was derivatized to *O*-(4-nitrobenzyl)oxime and purified by thin-layer chromatography (Merck Kieselgel F_{254}) as described previously.[26] The identity of the *O*-(4-nitrobenzyl)oxime of DOI was unambiguously confirmed by HPLC, ^1H- and ^2H-NMR spectroscopy.[14, 26]

DOI Synthase Assay

The DOI synthase activity was detected by modifying the HPLC method described previously.[19,26] An enzyme preparation (1 μl) was mixed with 10 μl of 50 mM G-6-P, 10 μl of 50 mM NAD^+ and 79 μl of 50 mM Tris-HCl buffer containing 0.2 mM $CoCl_2$ (pH 7.7, final volume was 100 μl, final concentration of G-6-P and NAD^+ was 5 mM). Incubation was carried out at 46°C for 5 minutes. After this, the same protocol was performed as described previously. One unit of the enzyme activity was defined as the production of 1 μmol of DOI per minute.

The *btrC* gene product (BtrC) was biochemically characterized under the standard enzyme assay conditions with a modification of pH or an addition of metal ions. Optimum pH was determined by an enzyme assay in a 50 mM MOPS-KOH buffer between 6.5 to 8.0, and a 50 mM Tris-HCl between 7.0~9.0. For the analysis of a metal ion requirement, $CoCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $CuCl_2 \cdot 2H_2O$, $FeCl_2 \cdot 4H_2O$, $CaCl_2$, $ZnCl_2$, and $NiCl_2 \cdot 6H_2O$ were used at a final concentration of 1 mM. A metal-free condition was made by adding EDTA (final concentration, 1 mM). For the determination of optimum reaction temperature, the enzyme reaction was performed at varying temperatures between 15 to 55°C. The heat stability of the recombinant enzyme was studied by measuring the residual activity after heat-treatment between 20 to 60°C for 5 minutes.

Purification of BtrC

The cell-free extract solution was prepared as described above. The solution was loaded onto a column (2.5 × 15 cm) of DEAE-Sepharose Fast Flow equilibrated with 50 mM Tris-HCl containing 0.2 mM $CoCl_2$ (pH 7.7). The adsorbed proteins were eluted using the same buffer with a linear gradient of NaCl concentration from 0 M NaCl (300 ml) to 0.4 M (300 ml). The DOI synthase activity was observed in the fractions eluting at approximately 0.2 M NaCl. The active fractions were

concentrated into *ca.* 10 ml by centrifugation with Centriprep-10 (Amicon). The concentrate was then loaded onto a Hi Load 26/60 Superdex 200 pg (FPLC) equilibrated with 50 mM Tris-HCl (pH 7.7) containing 0.1 M NaCl and 0.2 mM $CoCl_2$, and the active fraction was obtained similarly.

Physical and Chemical Measurements

The protein content of enzyme preparations was estimated by the Lowry's method with bovine serum albumin as a standard.[27] SDS-polyacrylamide gel (containing 10% or 12.5% polyacrylamide) electrophoresis was performed according to the literature procedure,[28] and proteins were visualized by Coomassie brilliant blue staining. The molecular-weight standards used (Bio-Rad) were lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa). Molecular weight determination under nondenaturing conditions was carried out by TSK-gel G3000SW (glass) gelfiltration chromatography. The column was calibrated with standards (BDH Chemicals Ltd.) including carbonic anhydrase (30 kDa), ovalbumin (45 kDa), ovotransferrin (77 kDa), and lactate dehydrogenase (145.9 kDa).

Deposition of the Nucleotide Sequence

The nucleotide sequence data reported in this paper (*btrC*) will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB019237.

Results

Cloning of the *btrC* Gene from *B. circulans*

The amino acid sequence of the amino-terminus of the 42 kDa subunit of DOI synthase from *B. circulans* SANK 72073 was determined to be M T T K Q I ? F A D R ? F N F A F G E H V L. On the basis of the partial sequence F N F A F G E H V, two oligonucleotide probes designated as 40 kA and 40 kB were synthesized. Both of these were DIG-labeled (40 kA and 40 kB) and were used as probes for Southern hybridization with the chromosomal DNA digests prepared by various restriction enzymes (data not shown). Among the DNA fragments that positively hybridized with both probes, a 4.2 kbp fragment obtained by digestion of chromosomal DNA with *Eco*RI was selected and was subsequently ligated into pUC19. The resulting plasmids were used as a size-fractionated chromosomal DNA library of *B. circulans*. *E. coli* JM105 was transformed with the plasmids of the chromosomal library, and the resulting transformants were screened by colony hybridization with the same DIG probes. As a result, only one positive clone (pDS1) was obtained. Nucleotide-sequencing of pDS1 (Fig. 2) clearly showed that the deduced amino acid sequence (M T T K Q I C F A D R C F N F A F G E H V L E SV E S Y I P R D E F) was in good agreement with the amino-terminal sequence of the 42 kDa subunit of DOI synthase described above. However, the open reading frame (ORF) of pDS1 turned out to unfortunately contain only a small fragment of the expected whole gene for the 42 kDa subunit (Fig. 2). Therefore, a DIG labeled *Bam*HI-*Eco*RI fragment of pDS1 was used as a probe in order to identify the whole gene. Through similar Southern hybridization with this probe, a 4.0 kbp *Bam*HI-*Pst*I fragment of the *B. circulans* chromosomal DNA was isolated. This fragment was inserted into pUC19 as described above, and the resulting transformants were screened by colony hybridization using the same probe. In this way, an open-reading frame (ORF) in the 4.0 kbp *Bam*HI-*Pst*I fragment (pDS2) was identified as the whole *btrC* gene for the 42 kDa subunit of DOI synthase from *B. circulans* on the basis of the molecular size (Fig. 2).

For further confirmation of the deduced amino acid sequence of the *btrC* gene, use was made of nano-LC/ESI IT-MS/MS analysis of the internal oligopeptides obtained by the in gel tryptic digestion of the band of 42 kDa subunit. The observed mass values were assigned to the peptides on the basis of the specificity of the enzyme. In addition, the partial amino acid sequences obtained by the LC/ESI IT-MS/MS analysis were also taken into account. Six peptides; T L I K (358~361), E V I R E G L (362~368), F V F A D T R (161~167), I L S E S P P R (168~175), K T L I K E V I R E G L (357~368), and F Q G G E E Y K T L S T V T N L Q E R (68~86) were identified by mass values. Partial amino acid sequences of four peptides, F V F A D T R (161~167), I L S E S P P R (168~175), K T L I K E V I R E G L (357~368), and F Q G G E E Y K T L S T V T N L Q E R (68~86) were confirmed by partial amino acid sequences obtained from nano-LC/ESI IT-MS/MS analyses. Fig. 3 shows an example of the nano-LC/ESI IT-MS/MS spectrum from $(M+2H)^{2+}$ of K T L I K E V I R E G L (357~368) at m/z 636.2. The y-series ions[29] of y_4 to y_9 clearly demonstrated the partial amino acid sequence of–I K E V I–for the peptide K T L I K E V I R E G L (357~368). Similarly, the y-series

Fig. 2. Restriction map around the *btrC* gene.

The outline arrows indicate the positions and directions of the *btr* genes.

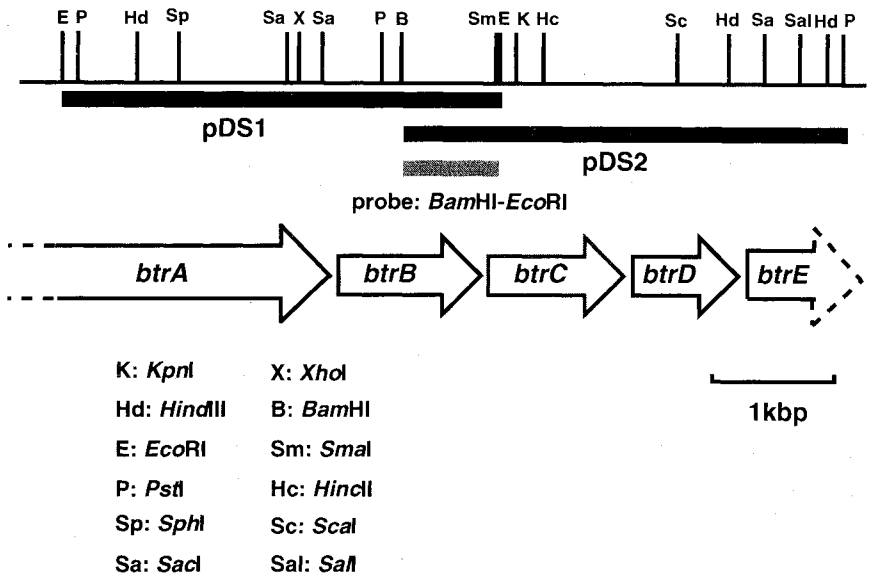
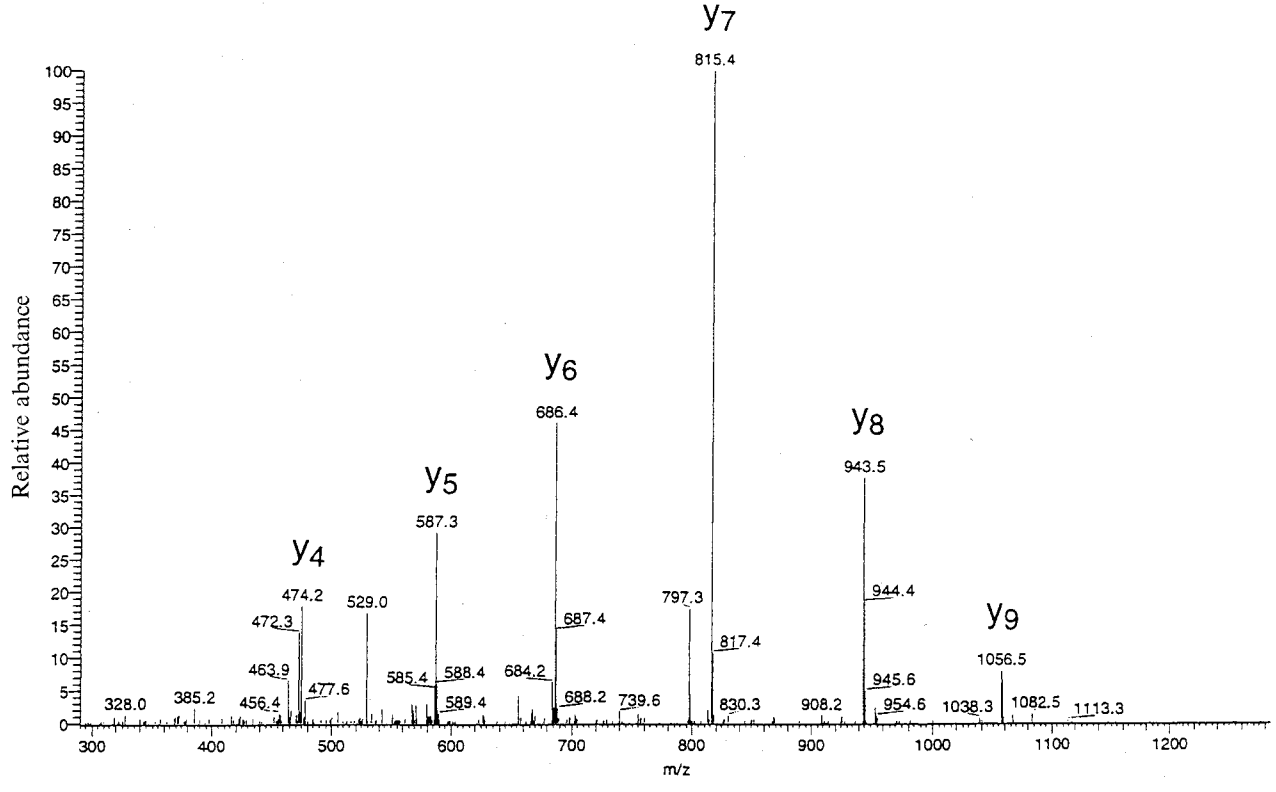


Fig. 3. Nano-LC/ESI IT-MS/MS.

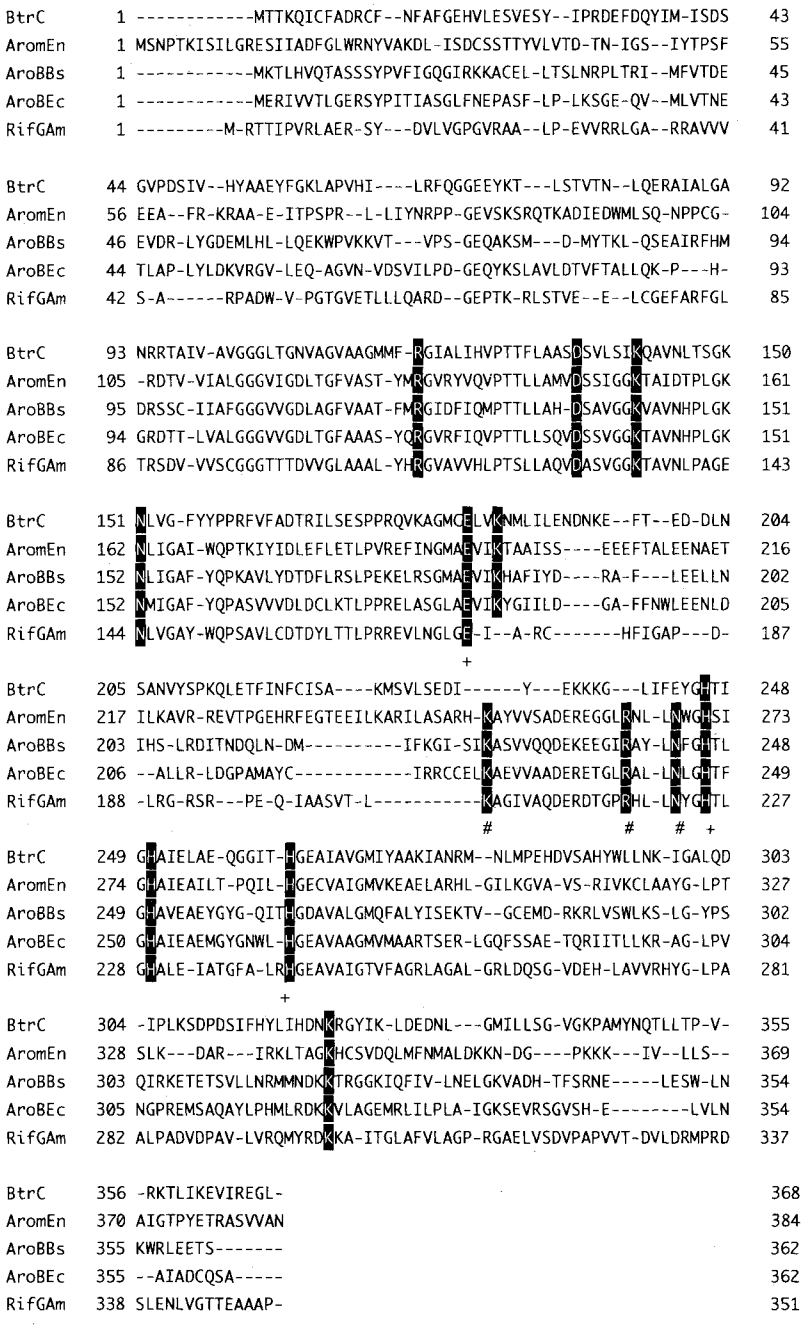


Nano-LC/ESI IT-MS/MS spectrum from $(M+2H)^{2+}$ of KTLIKEVIREGL (357~368) at *m/z* 636.2. The y-series ions of y_4 to y_9 clearly demonstrated the partial amino acid sequence of–IKEVI–of the peptide KTLIKEVIREGL (357~368) which is *C*-terminal regions of the deduced amino acid sequence of the *btrC* gene.

ions of y_6 to $y1_4$ clearly demonstrated the partial amino acid sequence of–E Y K T L S T V–for the peptide F Q G G E E Y K T L S T V T N L Q E R (68～86) (data not shown). Both peptides were predicted to be in the *C*-terminal and internal regions of the deduced amino acid sequence of the *btrC* gene.

The *btrC* gene encodes a polypeptide with a molecular mass of 40,746 Da, comprising 368 amino acid residues. It should be emphasized here that the sequence of the *btrC* gene shows significant sequence similarity to various

Fig. 4. Alignment of BtrC with DHQ synthases sequences.



BtrC, recombinant DOI synthase; AromEn, DHQ synthase of *Emericella nidulans* (DHQ synthase region of AROM protein, SWISS-PROT, P07547); AroBBs, DHQ synthase of *Bacillus subtilis* (SWISS-PROT, P31102); AroBEc, DHQ synthase of *E. coli* (SWISS-PROT, P07639); RifGAm, amino DHQ synthase of *Amycolatopsis mediterranei* (a part of rifamycin biosynthetic gene cluster, AF040570). A functional attribution of the aa residues given reverse face, based on the analysis of the three-dimensional structure of the DHQ synthase domain of *Emericella nidulans* is given below the alignment by the following code: + is metal binding residues. ♯ is not conserved in the BtrC.

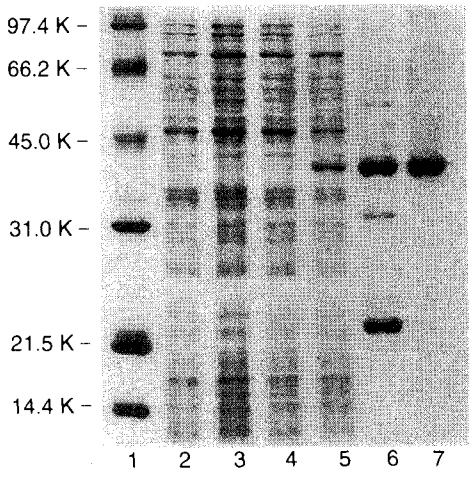
DHQ synthases (AroB) and the catalytic domain of DHQ synthase of *Emericella* (formerly *Aspergillus*) *nidulans* (Fig. 4).[30]

Other ORFs found around the *btrC* gene are also shown in Fig. 2. These ORFs suggest a butirosin biosynthetic gene cluster in the *B. circulans* chromosome. The functions of these genes have yet to be elucidated.

Expression of the *btrC* Gene

It was essential to confirm the function of the *btrC* gene, and an attempt was made to over-express the gene. The corresponding DNA fragment was inserted into an expression vector pET-30b(+), which was then used to transform *E. coli* BL 21 (DE3). A transformant (pDS4) containing the *btrC* gene was thus obtained. The amount of expressed protein BtrC in *E. coli* / pDS4 reached *ca.* 20 % of the total soluble proteins (Fig. 5, lane 5). The DOI synthase activity of BtrC was clearly confirmed by the assay with G-6-P and [6,6-$^2H_2$]-G-6-P using a cell-free extract of the transformed *E. coli* / pDS4. Approximately 3 mg of *O*-(4-nitrobenzyl)oxime derivative of DOI was obtained from 8.4 mg of G-6-P; ^{1}H-NMR (300 MHz, CD_3OD) δ: 1.90 (dd, J=10.9, 13.8 Hz, H-2ax), 3.19 (t, J=8.8 Hz, H-4), 3.51 (dd, J=4.9, 13.8 Hz, H-2eq), 3.3~3.4 (H-3, H-5), 4.03 (d, 9.0 Hz, H-6), 5.25 (s), 7.58 (d, 8.5 Hz), 8.21 (d, 8.5 Hz). The ^{2}H-NMR spectrum was also recorded for the derivative of the deuterated DOI product from [6,6-$^2H_2$]-G-6-P (δ: 1.82, 3.46). These results clearly indicate that BtrC catalyzes the DOI synthase reaction in the absence of the 23 kDa subunit of the native DOI synthase from *B. circulans*, and hence, the *btrC* gene product (BtrC) was verified to be a recombinant DOI synthase.

Fig. 5. Expression of *btrC*.



lane 1; marker, lane 2; cell-free extract (CFE) from *E. coli* / pET30, lane 3; CFE from *E. coli* / pET30 after IPTG induction, lane 4; CFE from *E. coli* / pDS4, lane 5; CFE from *E. coli* / pDS4 after IPTG induction, lane 6; DOI synthase from *B. circulans*, lane 7; purified recombinant DOI synthase (BtrC).

Purification of BtrC

During the purification procedures, recombinant DOI synthase BtrC tended to spontaneously aggregate under the conditions of ammonium sulfate precipitation and dialysis. The enzyme activity was also lost following a short-term storage in a 50 mM Tris-HCl buffer in the absence of Co^{2+} ion (data not shown). These observations indicate that the recombinant DOI synthase is clearly distinct from the native DOI synthase from *B. circulans* from the viewpoint of protein stability. Therefore, the purification protocol for BtrC was modified. Thus, the handling of BtrC was always carried out in the presence of 0.2 mM Co^{2+} in a 50 mM Tris-HCl buffer (Table 1). Approximately 60 % of the purified BtrC were inactivated after 10 days of storage at 4°C or at −75°C even in the presence of 0.2 mM Co^{2+}. The recombinant protein clearly showed a single band of 42 kDa on SDS-PAGE (Fig. 5, lane 7). However, an active enzyme fraction was co-eluted with a standard of

Table 1. Purification table.

Step	Totaol protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Recovery (%)
Extract	360	65	0.18	1	100
DEAE	85	57	0.68	3.8	88
Superdex 200	46	49	1.1	6.0	76

1 U = μmol/minute DOI production.

ovotransferrin (77 kDa) during gel-filtration column chromatography (data not shown). This result suggests that functional BtrC exists as a homodimer under the non-denatured conditions.

An optimum for the enzyme reaction of the recombinant DOI synthase was observed between pH 7.5 and 8.5 (data not shown). This tendency was almost the same as native DOI synthase from *B. circulans* and for DHQ synthases from various sources as well.[19,31] The pH dependence of the rate of enzyme reaction indicates the presence of a catalytically important ionization with an apparent pKa of about 7.0 as has been observed for DHQ synthases.[31]

A temperature optimum of the enzyme reaction was observed at approximately 55°C under the reaction conditions containing NAD^+ and Co^{2+} for a reaction period of 5 minutes (data not shown). However, the recombinant DOI synthase diminished in its enzyme activity, when it was incubated at 50°C for longer periods (data not shown). This suggested that, while the reaction by the recombinant DOI synthase is fast, the protein is rapidly denatured above 50°C. These findings encourged us to perform an enzyme assay at 46°C as in the case of native DOI synthase from *B. circulans*.[19]

The effects of various divalent metal cations on the recombinant DOI synthase are described in Table 2. The presence of Co^{2+} ion was essential for the DOI synthase, because the recombinant enzyme was unable to be purified under the conditions without Co^{2+} as already mentioned. Additional evidence for instability without the presence of Co^{2+} was that the enzyme activity was diminished in the presence of EDTA. Although Mg^{2+}, Ca^{2+}, Mn^{2+}, and Fe^{2+} did not effect the enzyme activity, Cu^{2+} and Zn^{2+} strongly inhibited the enzyme reaction. These results suggest that the recombinant DOI synthase reaction specifically requires Co^{2+} ion.

The time course of DOI synthase reaction under the optimum conditions was examined by using 2 μM of BtrC. The rate of production of DOI was observed to be constant within the first 30 minutes. The kinetic parameters were next analyzed by stopping the enzyme reaction after 5 minutes, and the amount of production of DOI per minute was used as the initial velocity. According to a standard Lineweaver-Burk plot from the initial velocity of the enzyme reaction with the substrate G-6-P, the kinetic constants (k_{cat} and K_m) were determined, k_{cat} being $1.0\,s^{-1}$; K_m (for G-6-P), 2.1×10^{-4}M; k_{cat}/K_m. $4.8 \times 10^3 M^{-1} s^{-1}$, at 46°C and pH 7.7. Similarly, K_m for NAD^+ was determined to be 2.3×10^{-5}M at 46°C and pH 7.7.

Table 2. Effect of divalent metal cations on the enzyme activity.

Additive	Relative V_{max}	Additive	Relative V_{max}
None	1.0	Co^{2+}	1
Mg^{2+}	1.0	Ni^{2+}	0.79
Ca^{2+}	0.87	Cu^{2+}	0.43
Mn^{2+}	0.93	Zn^{2+}	0.050
Fe^{2+}	0.93	EDTA	0

Each metal was added into the enzyme solution in 50 mM Tris-HCl buffer containing 0.2 mM $CoCl_2$.

Discussion

In this study, we were successful, for the first time in cloning the *btrC* gene which corresponds to the functional 42 kDa subunit of DOI synthase from *B. circulans*. This was performed by utilizing a reverse genetics approach using synthetic DNA probes based on the amino-terminal sequence of the purified DOI synthase.[19] This appears to be the first identified example of a gene involved in the biosynthesis of DOS-containing aminocyclitol antibiotics. Previously, AUBERT-PIVERT and DAVIES attempted to analyze the chromosomal DNA regions flanking the butirosin resistance gene encoding an aminoglycoside phosphotransferase (*aphA4*/*butA*) in a butirosin-producing *Bacillus circulans* as an approach to look for the butirosin biosynthetic genes.[4] The putative gene (*butB*) immediately upstream from *aph4* was reported to be unrelated to the biosynthesis of butirosin, but was identified as a gene of the cell-wall-associated protein that eliminates detectable antibiotic accumulation.

The amino acid sequence of the *btrC* gene product (BtrC) shows significant similarity to the sequences of a family of dehydroquinate synthases (DHQ synthases) in the shikimate pathway (Fig. 4), *e.g.*, DHQ synthase of *E. coli* (26.5% in 324 aa overlap), of *Emericella nidulans* (26.4% in 276 aa overlap), and of *B. subtilis* (34.1% in 264 aa overlap), as well as RifG of *Amycolatopsis mediterranei* (28.8% in 302 aa overlap) which is an aminodehydroquinate synthase involved in rifamycin biosynthesis.[32] The BtrC protein clearly belongs to the family of DHQ synthases, and these enzymes may have evolved from a common ancestral enzyme.

The *btrC* gene product, BtrC, was over-expressed and was confirmed to be a recombinant DOI synthase by chemical analysis of the reaction products. The specific

activity of the recombinant enzyme (1.1 μmol/minute/mg) was observed to be much higher than that of the native DOI synthase from *B. circulans* (0.018 μmol/minute/mg). Thus, the kinetic constants of the recombinant enzyme were k_{cat} of $1.0\,s^{-1}$, K_m (for G-6-P) of 2.1×10^{-4} M, K_m (for NAD^+) of 2.3×10^{-5} M. These values were different from those of the native enzyme purified from *B. circulans* (k_{cat} of $7.3\times10^{-2}\,s^{-1}$, K_m (for G-6-P) of 9.0×10^{-4}, K_m (for NAD^+) of 1.7×10^{-4}M).[19] Apparently, the recombinant DOI synthase is more efficient than the corresponding enzyme purified from *B. circulans*. The difference between the DOI synthases in these studies is the presence of the 23 kDa protein. This indicates that the 23 kDa subunit of the *B. circulans* enzyme may regulate the activity and be involved in stabilizing the enzyme complex. The smaller subunit appears not to be directly associated with the reaction catalysis, since both of the native and recombinant enzymes are catalytically active.

The recombinant DOI synthase was found to be rather unstable than the corresponding native enzyme from *B. circulans*. This may be due to an alternative conformation for the recombinant BtrC when compared to that of the native enzyme structure. Based on the chromatographic behaviors of these enzymes in gel-filtration experiment, the DOI synthase from *B. circulans* appears to exist as a heterodimer,[19] whereas the recombinant enzyme is a homodimer. The difference of their stability may indicate that the native 23 kDa subunit is necessary to constitute a stable structure. The function of the 23 kDa protein of DOI synthase from *B. circulans* is thus intriguing in terms of regulation and stability of microbial secondary metabolism. Future cloning and expression of the 23 kDa subunit of DOI synthase from *B. circulans* should shed light on these problems.

The recombinant DOI synthase diminished its activity under the dialysis conditions and during storage in a buffer without Co^{2+}. The activity could not be reconstituted by simple addition of Co^{2+}. These suggest that the recombinant DOI synthase always requires Co^{2+} to conserve the activity. Interestingly, DHQ synthase from *E. nidulans* was reported to also require Co^{2+} or Zn^{2+} for activity, and further, even Eu^{3+} or Sm^{3+} were capable of activating the enzyme.[33] According to the recently published crystal structure of the DHQ synthase of *E. nidulans*, the pentacoordinate Zn^{2+} ion interacts with three amino acid residues.[30] It was considered that the Zn^{2+} ion may facilitate both hydride transfer and proton removal by polarizing an appropriate hydroxy group. The corresponding three amino acid residues, which may interact with Co^{2+}, are conserved in the deduced amino acid sequence of BtrC (designated by + in the sequence of Fig. 4). It appears that the metal binding site is similar in each of these enzymes. However, it should be emphasized that while the Zn^{2+} ion is favorable to DHQ synthase, DOI synthase is totally inhibited by Zn^{2+} ion.

Based on the crystal structure of DHQ synthase of *Emericella nidulans*,[30] the catalytically responsible amino acids, which are in the metal binding and phosphate elimination sites, are almost conserved in DOI synthase (10 out of 13 amino acids). The K197 in the DHQ synthase of *E. nidulans*, which recognizes the hydroxyl at C-4 of the substrate 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP), appears to be conserved in BtrC, and probably functions in recognition of the hydroxyl at C-3 of G-6-P. In contrast, this residue is not conserved in the RifG for the rifamycin biosynthesis.[32] It is considered that the substrate of RifG is 4-amino-DAHP. Thus, this residue may well have an important role in distinguishing between a hydroxyl or an amino group in the substrate. This conserved hydroxy-recognizing residue in BtrC is in good accord with our previous observation that the conversion of 3-deoxy-G-6-P to the corresponding dideoxy-*scyllo*-inosose was inefficient.[16] The most significant difference between DHQ synthase and DOI synthase was found in three residues (K250, R264, N268 in the former). These residues were reported to be responsible for the recognition of the C-1 carboxylate of the DAHP substrate. The corresponding ionic residues are lacking in DOI synthase (Fig. 4), which is reasonable as the G-6-P substrate of the latter enzyme does not contain an ionic carboxylate functionality. Additional differences may be found in the three-dimensional arrangement of relevant functional motifs involved in the phosphate elimination and the aldol cyclization steps (Fig. 1), since the cryptic stereochemistry of the C-6 position of glucose in the DOI synthase reaction of *Streptomyces ribosidificus* was elucidated to be opposite to that of DHQ synthase.[17,18,34,35] More detailed studies will be necessary to obtain further insights into these intriguing questions.

In conclusion, we have cloned the *btrC* gene which encodes for the functional 42 kDa subunit of DOI synthase from *Bacillus circulans* SANK 72073. This is the first successful entry to genetic studies of the biosynthesis of clinically important DOS-containing aminocyclitol antibiotics.

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