Butirosin-biosynthetic Gene Cluster from *Bacillus circulans*

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Butirosin is an interesting 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotic produced by non-actinomycete *Bacilli*. Recently we were successful in purification of 2-deoxy-*scyllo*-inosose synthase from butirosin-producer *Bacillus circulans* as the key enzyme for the biosynthesis of DOS, in cloning of the responsible gene (*btrC*), and in its over-expression in *Escherichia coli*. The present study involved gene-walking approach, which allowed us to find a gene cluster around *btrC*. The function of each gene was further investigated by gene disruption, and the disruptants of *btrB*, *btrC*, *btrD* and *btrM* showed no antibiotic producing activity. Therefore, the gene cluster found so far was determined to be a part of the butirosin biosynthetic gene cluster. Functions of some ORFs are also discussed in terms of butirosin biosynthesis on the basis of database search.

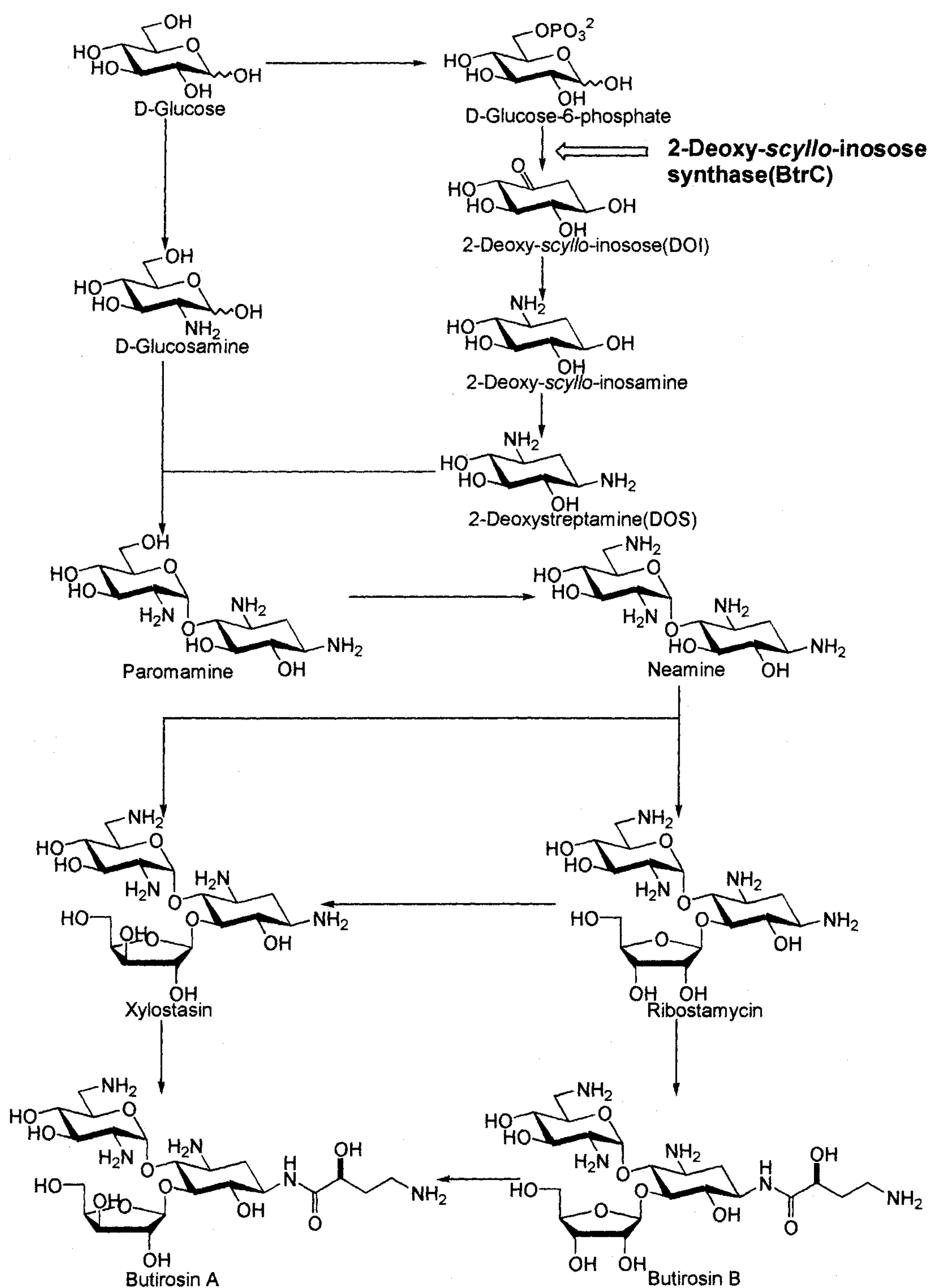
Aminoglycosides are among the most important antibiotics for clinical demands for a long time. Even today, the importance of aminoglycosides is not changed at all, for example, for the treatment of HIV infection.[1,2]

In view of chemical structure, aminoglycosides can be classified into two major groups. One is those having an aglycone of fully-substituted aminocyclitol which is shown or proposed to be biosynthesized from *myo*-inositol. Streptomycin, fortimicin, hygromycin and spectinomycin are among this group, and genetic as well as enzymological studies have been performed for the biosynthesis of the first two.[3~5] The other group of aminoglycosides has a common aglycone of 2-deoxystreptamine (DOS), and clinically important major compounds are included in this category, like neomycin, kanamycin, butirosin, gentamicin, tobramycin, sisomicin, sagamicin *etc.* The biosynthesis of DOS-containing aminoglycosides have been studied mostly by the use of isotope-tracer technology with whole cell systems of producing organisms and blocked mutants.[6~10] The biosynthetic pathway of butirosin, produced by non-actinomycete *Bacilli*, has been proposed as Fig. 1.[8] However, until recently, any biosynthetic genes of DOS-containing aminoglycosides had never been identified. Genetic analysis was carried out for a region around the self-resistance gene (*butA*) on the butirosin-producer *Bacillus circulans*.[11] While a butirosin-transporter gene (*butB*) was found in this particular region, no butirosin biosynthetic gene was identified. The enzymes involved in the biosynthesis of these antibiotics also remained unclear except for some transamination-related enzymes.[12,13] In our laboratory, much study was devoted to 2-deoxy-*scyllo*-inosose (DOI) synthase, the key enzyme for the initial step of DOS biosynthesis.[14~19] The enzyme catalyzes the carbocycle-forming reaction from glucose-6-phosphate to DOI in the presence of NAD^+ and Co^{2+} as cofactors. The multi-step process includes the first oxidation at C-4 of glucose-6-phosphate, and subsequent elimination of a phosphate group from the activated ulose follows to form 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 occurs to form DOI.

Recently, DOI synthase was purified to an electrophoretically homogeneous state from a butirosin-

Dedicated to the memory of Sir EDWARD ABRAHAM.

Fig. 1. Pathway for butirosin biosynthesis.



producer *Bacillus circulans*.[20] Further, the responsible gene (*btrC*) was identified and over-expressed in *Escherichia coli*.[21] These appeared to constitute the first of the biosynthetic enzymes and genes of DOS-containing aminoglycosides. With these results in hand, our attention was next focused on clarifying the over-all biosynthetic system for butirosin at genetic level, because genes for bacterial secondary metabolism usually reside in cluster.

Accordingly, it was anticipated in this case also that certain butirosin-biosynthetic genes could be located near *btrC*.

This paper describes gene walking approach which was undertaken to investigate the butirosin-biosynthesis system around *btrC* on the genome of *B. circulans*. As a result, a series of open-reading frame (ORF) were found. Construction of targeted-gene disruptants and phenotype analyses of them clearly showed that this is a cluster for the biosynthesis of butirosin in *B. circulans*. Functions of some ORFs in butirosin biosynthesis are also discussed on the basis of database search with known enzymes.

Materials and Methods

Organisms and Culture Conditions

B. circulans SANK72073, kindly provided by Sankyo & Co. (Japan), was grown on a medium described previously[16] for the test of antibiotic activity, or on a Pen medium (beef extract 1.5 g, yeast extract 1.5 g, polypeptone 5.0 g, glucose 1.0 g, NaCl 3.5 g, K_2HPO_4 3.68 g and KH_2PO_4 1.32 g in 1 liter of water) for genetic manipulations, both at 28°C. *E. coli* JM105 and JM109 was grown in LB medium as liquid medium or agar plate, containing antibiotics when needed (50 μg/ml of ampicillin for pUC19- or pUC119-derived plasmids, 100 μg/ml of chloramphenicol and 5 μg/ml of erythromycin for pHB201-derived plasmids, 20 μg/ml of tetracycline for tetracycline resistance gene cassette-containing plasmids). All of the plasmid constructions were performed by using *E. coli* JM105 or JM109.

Gene Walking for Cloning of *btr* Genes

DNA manipulations were performed as described in the literature.[22] Construction of pDS5 was carried out as follows. *Eco*R I-*Hind* III fragment (0.7 kb) was recovered from pDS2,[21] and labeled with digoxigenin (DIG) with DIG DNA Labeling Kit (Boehringer Mannheim) to yield a probe of D-2. Genome of *B. circulans* was digested with *Kpn* I and *Sac* I, and subsequently loaded on agarose gel electrophoresis. DNA fragments were transferred to Zeta-Probe GT Genomic Tested Blotting Membrane (BioRad), and was hybridized with a probe at 65°C according to the standard protocol of the manufacturer's instruction. Membrane was washed with 1×SSC at 65°C, and the positively hybridized bands were visualized using a DIG Luminescent Detection Kit (Boehringer Mannheim). The fragments of interest were recovered from a gel and subcloned into the multicloning site of pUC19. The resulting plasmids were transformed into *E. coli* JM105, which was then plated on LB agar containing 50 μg/ml of ampicillin. The colonies were transferred to HyBond-N^+ membrane (Amersham Pharmacia), and screened by hybridization with the same probe, as described above, to yield pDS5. Other clones (pDS10, 11, 12, 13 and 14) were obtained with the same methods using appropriate restriction enzymes and probes summarized in Tables 1 and 2. The nucleotide sequence of each inserted clone was determined by construction of plasmids with digestion of pDS5, 10, 11, 12, 13 and 14 by appropriate restriction enzymes and subcloning into pUC19, and sequencing of them.

Construction of Disruption Vectors

Plasmid pHB2Tc (*btrB* disruption vector) was constructed as follows. Two primers (BD-s, for introduction of *Sac* I site, and BD-a, for introduction of *Eco*R I site) were used for PCR amplification of *btrB* (95°C for 1 minute, and 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds in total 30 cycles, and then 72°C for 7 minutes). The PCR product (1.3 kb) was then 5′-phospholylated by T4 polynucleotide kinase, and further subcloned into *Sma* I-digested pUC119. After confirming the sequence and digestion, *Sac* I-*Eco*R I fragment was subcloned into *Sac* I and *Eco*R I site of pHB201 (Tanaka *et al.*, unpublished). The resulting plasmid was digested with *Bam*H I (the restriction site located on 429 bp from the starting codon of *btrB*), and the tetracycline resistance gene cassette (1.9 kb, recovered from pBEST309[23] with *Bam*H I) was inserted in reverse direction to yield pHB2Tc. Direction of tetracycline resistance gene cassette was confirmed by *Eco*R I digestion using *Eco*R I site located at the end of the gene cassette.

Plasmid pHB3Tc (*btrC* disruption vector) was constructed as follows. A *Bam*H I-*Sca* I fragment (1.8 kb) was recovered from pDS2 and subcloned into *Bam*H I-*Eco*R V site of pHB201. The resulting plasmid was digested with *Nae* I (located on 340 bp from the starting codon of *btrC*), and the aforementioned tetracycline resistance gene cassette (recovered from pBEST309 with *Sma* I) was inserted in reverse direction to yield pHB3Tc.

Plasmid pHB4Tc (*btrD* disruption vector) was constructed as follows. A *Eco*R I-*Sac* I fragment (1.7 kb) was recovered from pDS2 and subcloned into *Eco*R I-*Sac* I site of pHB201. The resulting plasmid was digested with *Sca* I (located on 300 bp from the starting codon of *btrD*), and the tetracycline resistance gene cassette (recovered from pBEST309 with *Sma* I) was inserted in reverse direction to yield pHB4Tc.

Plasmid pHB13Tc (*btrM* disruption vector) was

Table 1. Plasmids and primers using in this study.

Plasmids

pUC19	Ampicillin resistance, *E. coli* clonimg vector
pUC119	Ampicillin resistance, *E. coli* clonimg vector
pHB201	Erythromycin and Chloramphenicol resistance, *E. coli* and *Bacilli* shuttle vector (Tanaka et al., unpublished)
pBEST309	Containing tetracycline resistance gene cassette (ref. 23)
pDS1	pUC19 containing *Eco*R I fragment (4.0 kb) of *B. circulans* genome (ref. 21)
pDS2	pUC19 containing *Bam*H I-*Pst* I fragment (3.6 kb) of *B. circulans* genome (ref. 21)
pDS5	pUC19 containing *Kpn* I-*Sac* I fragment (3.0 kb) of *B. circulans* genome
pDS10	pUC19 containing *Sac* I fragment (4.6 kb) of *B. circulans* genome
pDS11	pUC19 containing *Pst* I-*Sal* I fragment (1.9 kb) of *B. circulans* genome
pDS12	pUC19 containing *Bam*H I-*Eco*R I fragment (2.3 kb) of *B. circulans* genome
pDS13	pUC19 containing *Hin*d III fragment (3.3 kb) of *B. circulans* genome
pDS14	pUC19 containing *Hin*d III-*Sal* I fragment (3.2 kb) of *B. circulans* genome
pHB2Tc	*btrB* disruption vector
pHB3Tc	*btrC* disruption vector
pHB4Tc	*btrD* disruption vector
pHB13Tc	*btrM* disruption vector

Primers

BD-s	TTAGAGCTCAGGAGTGAACGATGAA	For *btrB* disruption
BD-a	TAGAATTCATGTTTAACCTCCACCG	For *btrB* disruption
BC-s	ATATCTGATCGCCGTATCGC	For confirming *btrB* disruption
BC-a	AAAACACCGGTCCGCAAAAC	For confirming *btrB* disruption
CC-s	TTCGTTCTATACGACCGGAACCG	For confirming *btrC* disruption
CC-a	ATATCTTCGTCCTGTTTGTCCGG	For confirming *btrC* disruption
DC-s	GGCGATGTATAACCAAACGC	For confirming *btrD* disruption
DC-a	TTTCCATGGAAAGCACTCCT	For confirming *btrD* disruption
MC-s	GCATATTTCGGATCCCTGGCACAATG	For confirming *btrM* disruption
MC-a	GATCTCCCTTGTCACGGCTGAAGGC	For confirming *btrM* disruption

Table 2. Construction of plasmids for determination of gene cluster.

Plasmid to yield (Size of insert)	Probe	Restriction enzyme for genome digestion
pDS5 (3.0 kb)	D-2 (*Eco*R I-*Hin*d III fragment of pDS2, 0.7 kb)	*Kpn* I, *Sac* I
pDS10 (4.6 kb)	D-3 (*Pst* I-*Sac* I fragment of pDS2, 0.6 kb)	*Sac* I
pDS11 (1.9 kb)	D-5(*Sac* I-*Sal* I fragment of pDS10, 0.9 kb)	*Pst* I, *Sal* I
pDS12 (2.3kb)	D-6 (*Hin*d III-*Kpn* I fragment of pDS5, 0.6kb)	*Bam*H I, *Eco*R I
pDS13 (3.3 kb)	D-4 (*Bam*H I-*Hinc* II fragment of pDS12, 0.7 kb)	*Hin*d III
pDS14 (3.2 kb)	D-7 (*Hin*d III-*Pst* I fragment of pDS11, 0.8kb)	*Hin*d III, *Sal* I

constructed as follows. Plasmid pDS13 was digested with *Sna*B I (located on 369 bp from the starting codon of *btrM*), and the tetracycline resistance gene cassette (recovered from pBEST309 with *Sma* I) was inserted in reverse direction to yield pHB13Tc.

All of the plasmids and primers using in this study were summarized in Table 1.

Double-crossover and Selection

B. circulans SANK72073 was transformed with each disruption vector by electroporation by the method of KUSAOKE *et al.* with slight modifications.[24] The cells of *B. circulans* in early log-phase was harvested by centrifugation (10,000×*g*, 5 minutes), and the cells were washed with 7 mM HEPES-NaOH buffer containing 272 mM of saccharose, 1 mM of $MgCl_2$, pH 7.3 for 4 times. The collected cells were suspended in 0.5 ml of the same buffer, and 1 μl of plasmid solution in TE buffer was added to 100 μl of the cell suspension. The mixture was allowed to stand in an ice bath for 10 minutes, and was subjected to electroporation with EasyjecT Optima (EquiBio, UK). Subsequently, 900 μl of SOC medium was added to the suspension, which was then incubated at 37°C for 1.5 hours and plated on LB agar containing 10 μg/ml of tetracycline, 1 μg/ml of erythromycin and 20 μg/ml of chloramphenicol. Transformation was confirmed in all cases by recovering the plasmid from each transformant by Plasmid Midi Kit (QIAGEN). The resulting transformant was grown on Pen medium containing 1 μg/ml of tetracycline for 24 hours×3 times, and for 72 hours. A 10 μl aliquot of culture was plated on LB plate containing 10 μg/ml of tetracycline. The colonies were screened by using 2 types of LB agar. One contained 10 μg/ml of tetracycline only, and the other contained 10 μg/ml of tetracycline, 1 μg/ml of erythromycin and 20 μg/ml of chloramphenicol. Colonies which showed a phenotype of tetracycline-resistant, erythromycin- and chloramphenicol-sensitive were picked up and cultured. Disruption of the target gene was confirmed by PCR using the genome of disruptant as a template. Primers were designed from the outside of the fragment for using each disruption vector (BC-s and BC-a for *btrB* disruption, CC-s and CC-a for *btrC* disruption, DC-s and DC-a for *btrD* disruption and MC-s and MC-a for *btrM* disruption). The program was; 94°C for 5 minutes, and 94°C for 1 minute, 50°C for 1 minute, 72°C for 3 minutes (for *ΔbtrM*, 4.5 minutes) in total 30 cycles, and then 72°C for 7 minutes. The PCR products were 5′-phospholylated by T4 polynucleotide kinase, and subcloned into *Sma* I-digested pUC119. Insertion of tetracycline resistance gene cassette was confirmed by DNA sequencing.

Analysis of Antibiotic Activity

Wild type and mutants of *B. circulans* were cultured in the medium described previously[16] for 2 days. The culture was then centrifuged (10,000×*g*, 15 minutes), and the supernatant was subjected to antibiotic assay by paper disk diffusion method.[25]

Reagents and Materials

DNA sequencing was carried out with LONG READIR 4200 (Li-Cor) according to the manufacturer's protocol. PCR was performed by GeneAmp PCR System 9700 (Amasham Pharmacia) using ULTma DNA polymerase (Applied Biosystems). Oligo DNAs for PCR primer were synthesized in Amersham Pharmacia. Purification of plasmids was carried out by Plasmid Miniprep Kit (BioRad) or GFX Micro Plasmid Prep Kit (Amersham Pharmacia) unless otherwise stated. Restriction enzymes and modification enzymes were purchased from TaKaRa (Japan). Genome preparations were carried out with Dr. GENTLE (TaKaRa). Genetic analysis was performed with GENETYX-MAC ver. 10 (Software Development, Japan), and database search was carried out by FASTA and BLAST2 on Internet. All other reagents were of the highest grade commercially available.

Accession Number

The sequence determined in the present study have been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB033991.

Results and Discussion

Gene-walking approach allowed us to identify 17 ORFs (2 of them were not fully-cloned) around the *btrC* gene (Fig. 2). All the ORFs were located in same direction, and positioned closely to each other. Further, in the region so-far examined, neither terminator-like nor promoter-like structure was identified. Thus, the region found in the present study appeared to be a part of single operon. The deduced amino-acid sequences of these ORFs were analyzed with BLAST2 and FASTA (Table 3), which suggested comparatively low homology with known proteins. It should be noted that these ORFs showed no significant homology with the streptomycin-biosynthetic genes. Apparently therefore, these *btr* genes are novel and evolutionally-different from those of *myo*-inositol-derived aminoglycoside biosynthetic genes. Previously described

Fig. 2. Genetic map of butirosin-biosynthetic gene cluster in *B. circulans*.

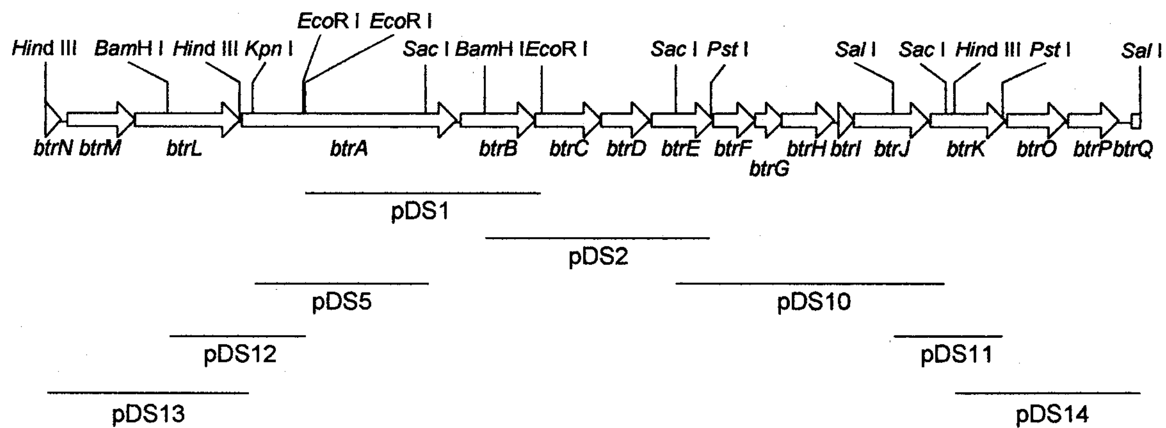


Table 3. Summary of the *btr* genes and comparison with database.

ORF	Size (amino acids)	Proposed function/homology	Typical homology	Accession numbers of selected homologous proteins
btrA	1,209	No typical homology		
btrB	432	glutamate-1-semialdehyde 2,1-aminomutase	25.3%	Z82044
btrC	368	2-deoxy-*scyllo*-inosose synthase (clarified)		
btrD	275	Vancomycin biosynthetic gene (function:unknown)	29.7%	AJ223998
btrE	349	Alcohol dehydrogenase	30.1%	D90769
btrF	232	Putative dehydrogenase	32.9%	AL031307
btrG	156	No typical homology		
btrH	302	No typical homology		
btrI	87	Acyl carrier protein	32.5%	AE001739
btrJ	419	biotin carboxylase	23.7%	AE000742
btrK	428	PLP-dependent decarboxylase	29.1%	AE000897
btrL	604	No typical homology		
btrM	389	Glycosyltransferase	26.2%	AE000693
btrN	-			
btrO	341	FMN-dependent monooxygenase	29.8%	Z99108
btrP	279	No typical homology		
btrQ	-			

PLP: Pyridoxal-5-phosphate
FMN: Flavin mononucleotide

butA (self-resistance gene) and *butB* (extracelluler transporter of butirosin)[11] were not found in the region so far analyzed in the present study.

Proposed Function of BtrM

BtrM was homologous with certain glycosyltransferases (Fig. 3). In the biosynthetic route of butirosin, two glycosyl

Fig. 3. Alignment of BtrM with glycosyltransferases.

Gal: galactocyltransferase from *Pyrococcus abyssi* (AJ248287), GluNac: Lipopolysaccharide *N*-acetylglucosaminyltransferase from *Methanobacterium thermoautotrophicum* (AE000805), Man: mannosyltransferase from *Aquifex aeolicus* (AE000693).

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BtrM    178:SDCIGSNHLSHSIDCPFCSRLKTELLGKKTVLFLGRIAHEKGWSTFVSVAKELADKIGDL 237
Gal     169:NAPI-VE-IPNGVNIE-RFNGRGREWGTRNILYVGRLEPRKGVNYLISAM-KF-VE--G- 220
GluNac  173:RNPDGIHIIPNGVDPE-LIDSVTPA-TGNYIIFVGRLAPHKHVDHLIEVFSKLVIDFPDL 230
Man     168:PKRLMSDYVDEKFK-DF-IELKNK--GRKIVLSIGRLVEYKGYKYLIEAAKYINNNIS-I 222

BtrM    238:QFIVCGDGPQREAMEEQIKAANLQNQFRITGFISHKFVSCYLHHAQLFILPS--HHEEFG 295
Gal     221:KLTIVGDGSMRKVLKMQAKKLGVEDKVEFLGFISQEELILLYKKSEVFVLPS--LSEAFG 278
GluNac  231:RLEIIGDGVERARLKAMVDECGIRDSVTFHHNLSYPEVISRIRGARVLVLPS--TREGFG 288
Man     223:--VIACSGPLFQSLEEKIETLNLKEKVFLFGRI-NN-VSLYMKNCDVFCLPSITRNEAFG 278

BtrM    296:GSLIEAAIAGVPIISTNNGGPADIF-TH-GETAILKDPGDVSGIADEAYKILTNDSVAES 353
Gal     279:IVLLEAMASEVP-VIGTSVGGIPEII---GDAGIIVPPRDSKALANAINAILSNQKTAKR 334
GluNac  289:MVLAEAGACGVP-AVAYRSGGVVEVI-DDGENGFLVEPCDKEALHDKIKLLISDDELRDR 346
Man     279:LVLVEALYFGKPLITTDVEGSGISYVNQNGITGLVVKPKDPKALAEAINKILKNENLYKQ 338

BtrM    354:LRLHSRPEVVSKELPHCVYPNYLNLYSSKEAAVHEG- 389
Gal     335:LGKLGRKRVERLYSWDVVAERTERLYRGELGDSDTNV 371
GluNac  347:MGSQGRKKVEEEFIWDRVVDEVERTYSFIIARKNTP- 382
Man     339:FSENAK-KRFKEFEISNIGDKILNLY---EEVLK--- 368
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Fig. 4. Conserved 4′-phosphopantetheine binding domain in BtrI and other acyl carrier proteins.

Ser is the site for 4′-phosphopantetheine binding. AcmACP: AF134588, ACPA: Q02054, $ACPP^B$: P80643, $ACPP^E$: P02901.

Protein	Size (Amino acid)	Organism/biosynthesis system	Sequence
BtrI	87	*B. circulans* / Butirosin	LIILDSVTAVNLIVSLE
AcmACP	78	*Streptomyces chrysomallus* / Actinomycin	DVVMDSFTLIVLQHGLE
DLTC	81	*Lactobacillus casei* / D-alanyl-lipoteichoic acid	TGILDSMGTVQLLLEIQ
ACPA	86	*S. coelicolor* / Actinorhodin	DIIYDSLALMETAARLE
$ACPP^B$	77	*B. subtilis* / Fatty acid	DLGADSLDVVELVMELE
$ACPP^E$	77	*E. coli* / Fatty acid	DLGADSLQTVELVMALE

transfer reactions are apparently involved (Fig. 1). One is for paromamine synthesis, and the other is for ribostamycin or xylostasin synthesis. Therefore, BtrM may well participate in either one step.

Proposed Biosynthesis of 4-Amino-2-hydroxybutyric Acid (AHBA)

A set of closely-located genes may suggest the biosynthesis of AHBA, the characteristic appendage of butirosin. BtrI is a homologue of acyl carrier protein (ACP), showing an apparent 4′-phosphopantetheine binding site (Fig. 4).[26] BtrK is homologous with pyridoxal 5′-phosphate (PLP)-dependent decarboxylase. Recently, the structure of ornithine decarboxylase (ODC) from mouse was dissolved by X-ray crystallographic study,[27] and all of the residues involved in the PLP-binding site of ODC appered to be conserved in BtrK as shown in Fig. 5. It

Fig. 5. Structure of the active site in ornithine decarboxylase[27] and the conserved residues in BtrK.

Primed residues belong to the other monomer in the dimeric decarboxylase.
Residues in parentheses are those in BtrK.

Fig. 6. Alignment of BtrO with FMN-dependent monooxygenases.

ASMO: Probable alkanesulfonate monooxygenase from *B. subtilis* (Z99108).

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BtrO     1  :MIALDTIQYYGQIPGV-NHYNGKKEFMENAVKIAQLSDAYGIVGSLSFFNHSVLDPWAVS 59
ASMO     1  :MEILWFIPTHGDARYLGSESDGRTADHLYFKQVAQAADRLGYTGVLLPTGRSCEDPWLTA 60

BtrO     60 :SVIMRHTERHVPLIALQPYMYPPYTAAKLIQSFTYLYDRRIDLNMITGAVTGELQQTGGY 119
ASMO     61 :SALAGETKDLKFLVAVRPGLMQPSLAARMTSTLDRISDGRLLINVVAGGDPYELAGDGLF 120

BtrO     120:IDHSSRYKKLHEYVQVLRLLLESDSAVSFKGDYYELNNLEFKPLLPDKRLFPRIFMSGSS 179
ASMO     121:ISHDERYEATDEFLTVWRRLLQGE-TVSYEGKHIKVENSNL-LFPPQQEPHPPIYFGGSS 178

BtrO     180:EEGLETGLKAAD-FVV-TH-PGPL-EHFKRHFSEKV-QGSAVQSAIRIEIIARESAEQAW 234
ASMO     179:QAGIEAAAKHTDVYLTWGEPPEQVKEKIERVKKQAAKEGRSVRFGIRLHVIARETEQEAW 238

BtrO     235:KIA-H--ARYPGNRQGKIQLRMKTNSESSWQRM--LAELALASETYDEVFWMG-GYMNGG 288
ASMO     239:EAAERLISHLDDDTIAKAQAALSRYDSSGQQRMAVLHQGDRTKLEISPNLWAGIGLVRGG 298

BtrO     289:IYSPVLVGDYEQVAAYLNEYYKLGVKA-VLLG-S-M----Y-SEEDF--IHF-S-RV-K- 334
ASMO     299:A-GTALVGDPQTIADRIAEYQALGIESFIFSGYPHLEEAYYFAELVFPLLPFENDRTRKL 357

BtrO     335:EG-ISNPV----------- 341
ASMO     358:QNKRGEAVGNTYFVKEKNA 376
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Fig. 7. Disruption of *btrB* confirmed by PCR.

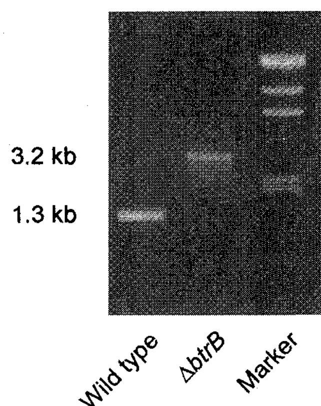
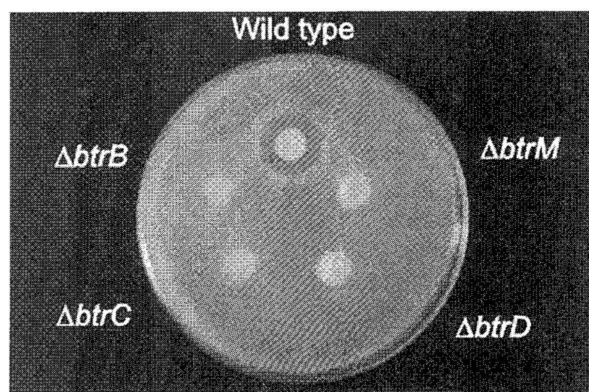


Fig. 8. Antibiotic producing activities of wild type and mutants of *B. circulans*.



seemed most likely, therefore, that BtrK is a PLP-dependent enzyme. BtrO is homologous to flavin mononucleotide (FMN)-dependent monooxygenase as shown in Fig. 6.[28] These findings may suggest the biosynthetic pathway of AHBA, in which a so-far unidentified amino acid is probably decarboxylated, oxygenated and incorporated into the aminoglycoside with an aid of ACP.

The AHBA-side chain is specific for butirosin among the aminoglycosides, and is a clinically important moiety. It is well-known that butirosin is refractory by the existence of AHBA to certain enzymes that modify aminoglycosides in resistant bacteria, and AHBA is important in semisynthetic aminoglycosides active against resistant strains.[29] Thus, the information for the biosynthetic mechanism of AHBA seems to be significant for the development of potential engineered biosynthesis of useful aminoglycosides.

Gene Disruption Studies

To test the dependence of butirosin biosynthesis on the above-mentioned *btr* genes, mutants of targeted-gene disruption were constructed by insertion of tetracycline resistance gene cassette into each gene in reverse direction. Disruption of each gene was confirmed by PCR as described in Materials and Methods. After the tetracycline resistance gene cassette was inserted in *btrB*, the amplified DNA showed a fragment of a size of 1.9 kb larger (3.2 kb) than that of wild type (1.3 kb) (Fig. 7). The same was true for *btrC*, *btrD* and *btrM* disruption (data not shown). In addition, the DNA sequence of the PCR-amplified fragment was analyzed, and reverse-insertion of tetracycline resistance gene cassette was confirmed in each disruptant. Antibiotic production was studied for each disruptants by paper disk diffusion assay, and no antibiotic producing activity was observed for all of the mutants (Fig. 8). These facts clearly demonstrated that BtrB, BtrC, BtrD and BtrM are participated in butirosin biosynthesis. Because all of the ORFs seem to be in a single operon, the gene cluster elucidated in the present study appears to be responsible for a butirosin biosynthesis. More detailed function of each gene should be clarified in due course.

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