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Extended Sequence and Functional Analysis of the Butirosin Biosynthetic Gene Cluster in *Bacillus circulans* SANK 72073

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Abstract Butirosin produced by *Bacillus circulans* is among the clinically important 2-deoxystreptamine containing aminoglycoside antibiotics and its unique structure is found in (*S*)-4-amino-2-hydroxyburyric acid substituted at C-1 of 2-deoxystreptamine. Recently, the key part of the butirosin biosynthetic gene cluster has been identified from *Bacillus circulans* SANK 72073, however the whole gene for the biosynthesis awaited for identification. In the present study, we undertook extended analysis of the butirosin biosynthetic gene cluster and found nine additional open reading flames (ORFs), *btrQ*, *btrR1*, *btrR2*, *btrT*, *btrU*, *btrV*, *btrW*, *btrX* and *orf1* in the cluster. In addition, we constructed disruption mutants of *btrR1* and *btrP-V*, and found that the *btr* genes (*ca.* 24 Kb) between *btrR1* and *btrP-V* are at least required for the butirosin biosynthesis.

Keywords 2-deoxystreptamine, butirosin, biosynthesis, gene cluster, gene disruption, *Bacillus circulans*

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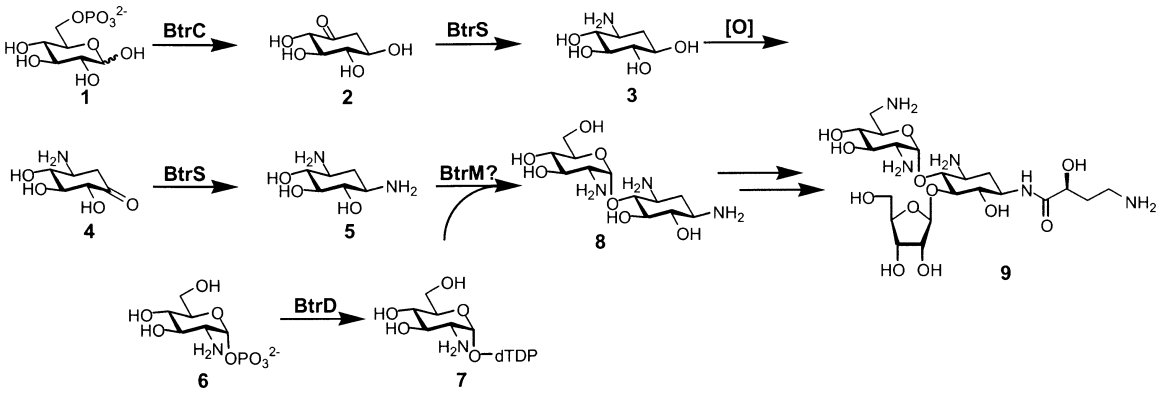
Introduction

Butirosin produced by *Bacillus circulans* is among the clinically important 2-deoxystreptamine (DOS)-containing aminoglycoside antibitoics [1]. However, the rapid emergence of resistant bacteria to this class of antibiotic has urged us to develop structurally novel and more potent antibiotics [2∼4]. In this respect, butirosin is unique in its activity against resistant bacteria due to the substitution with (*S*)-4-amino-2-hydroxybutyryl moiety at C-1 of DOS. This feature allowed to develop amikacin as a commercial semisynthetic antibiotic for the treatment of resistant bacteria [5]. Biosynthetic approach appears to be an alternative to this end [6, 7].

Concerning to the biosynthesis of butirosin, isotope-feeding experiments and cosynthesis methods using blocked mutants allowed to elucidate the gross biosynthetic pathway [8, 9]. More recently, a significant part of the butirosin biosynthetic gene cluster has been identified from *Bacillus circulans* SANK 72073 through the discovery of the protein (BtrC) and of the gene (*btrC*) for 2-deoxy-*scyllo*-inosose synthase, which catalyzes the key carbocyclization of glucose-6-phosphate in the pathway for DOS (Scheme 1) [10∼12]. More recently last year, based on the information of *btrC* and resistance genes, the biosynthetic gene clusters for three other DOS containing aminoglycosides have been identified [13∼18]. Functional analysis of *btrC*, *btrS* and *btrD* has successfully shown that this *btr* gene cluster is involved in the butirosin biosynthesis [10, 12, 19∼21]. BtrS is an unique aminotransferase involved dually in the pathway for DOS [20]. BtrD is a novel enzyme involved in the biosynthesis of dTDP-glucosamine from dTTP and glucosamine-1-



Scheme 1 Biosynthesis of butirosin.

1, D-glucose-6-phosphate; **2**, 2-deoxy-*scyllo*-inosose; **3**, 2-deoxy-*scyllo*-inosamine; **4**, aminodideoxy-*scyllo*-inosose; **5**, 2-deoxystreptamine; **6**, D-glucosamine-1-phosphate; **7**, dTDP-glucosamine; **8**, paromamine; **9**, butirosin B. BtrC, 2-deoxy-*scyllo*-inosose synthase; BtrS, L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase; BtrD, dTDP-glucosamine synthase; BtrM, putative glycosyltransferase

phosphate prior to the glycosylation in the paromamine synthesis [21]. Although seventeen open reading frames (ORFs) have been reported so far [11, 12], all the required genes for the butirosin biosynthesis have not yet been determined. Thus, in order to address this important issue, we have pursued extended analysis of the butirosin biosynthetic gene cluster toward upstream and downstream, and nine additional open reading flames (ORFs), *btrQ*, *btrR1*, *btrR2*, *btrT*, *btrU*, *btrV*, *btrW*, *btrX* and *orf1* have been supplemented to the cluster. The DNA sequence between *btrR1* to *btrT* was independently deposited by Huang *et al.* in different codes names derived from a different type strain of *B. circulans* NRRL B3312 (AJ494863). In the present study, we have further carried out functional analysis of appropriate genes in the *btr* gene cluster by gene disruptions and found that the *btr* genes from *btrR1* through *btrP-V* are at least required for the butirosin biosynthesis.

Materials and Methods

General

Bacillus circulans SANK 72073 was used as the source strain for butirosin biosynthetic genes and was also used for the construction of gene disruption mutants. *Escherichia coli* JM 109 and DH5α were used as the host strain for *btr* gene cloning. *Bacillus subtilis* PCI 219 was used as an antibiotic test strain. The plasmids for gene disruption were constructed with an *E. coli-Bacillus* shuttle vector pHB201 (Tanaka *et al.*, unpublished). Also routinely used was pUC 119 vector for sub-cloning and sequencing. Another vector pT7-blue T (Novagen, USA) was routinely used for sub-cloning after PCR. DNA sequence analysis was carried out with a LONG READER 4200 (Li-Cor) according to the manufacturer's protocol. Other reagents were the highest grade commercially available.

Gene Walking

Standard *in vitro* techniques were used for DNA manipulation [22]. Gene walking toward the upstream of the known region was carried out by inverse PCR. Based on the previously determined sequence [12], two oligonucleotides btrS1: 5′-TTATCGCCCATCTGTTCG-ACTGAAA-3′ and btrS2: 5′-CGCGAGGCAATGGAA-GAACAAATTA-3′ were designed in inverse direction as primers. *Eco*RI-digested chromosomal DNA of *B. circulans* was, after the phenol-chloroform extraction, self-ligated. The resulting circular DNAs were used as PCR template. The PCR conditions were; 1 cycle at 95°C for 10 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 5 minutes, and then 72°C for 7 minutes, using AmpliTaq Gold DNA polymerase (Applied Biosystems, USA). The PCR product was subcloned into pT7-blue T vector, and the resulting plasmid pDS17 was sequenced. Subsequently, pDS18, 19, 20, and 21 were obtained similarly, using *Hinc*II-, *Eco*RI-, *Hinc*II-, and *Stu*I-digested circular DNA fragments as template, respectively and primers pDS18-1: 5′-GCGTACCGTAATGTTTCG-GTTCC-3′ and pDS18-2: 5′-CGGATTGTGCGTAGAA-GCAGAAG-3′ for pDS18, pDS19-1: 5′-GCAAAGC-CTGCTCCCGCCCGATA-3′ and pDS19-2: 5′-CGAGGC-GGGCGGCCTGATCGGGT-3′ for pDS19, pDS20-1: 5′-AACGGACGACTCCACCACCTGAG-3′ and pDS20-2: 5′-CGTTAGTCGTCGTGGCTCATCCG-3′ for pDS20, pDS21-1 5′-CGACATAGACCTGAACAGCCGCC-3′ and

pDS20-2 for pDS21.

Gene walking to the downstream region was carried out with Southern hybridization and inverse PCR. *Sac*I-*Sal*I fragment was prepared from pDS14 [11], and the fragment was digoxigenin (DIG)-labelled with DIG DNA labeling kit (Roche Applied Science, Switzerland) for a probe. *Dra*I-digested chromosomal DNA was subcloned into *Sma*I site of pUC 119 to derive a library. Hybridization was carried out using the probe in a standard manner, and a positive clone containing pDS15 was obtained and then sequenced. In order to analyze the downstream of pDS15, pDS22 were also obtained by inverse PCR as described above, using *Pvu*I-digested and self-ligated chromosomal DNA as a template. The primers, pDS22-1 5′-CGGTTTGGGAA-GACGGATAGCGG-3′ and pDS22-2 5′-CCGGCAGCG-CACCGTTCGGCAGG-3′ were designed from the information of pDS15 sequence.

The sequences determined in the present study have been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers of AB097196.

Disruption of Key *btr* Genes

Disruption of *btrR1*

DNA fragments containing a part of *btrR1* gene, from 107 to 930 (823 bp) and from 1485 to 2327 (842 bp), were amplified by PCR with two sets of primers btrR1-d1: 5′-TGGATCCCCGGCAGGCGGACCCTCGGTTGG-3′ and btrR1-d2: 5′-CGCCGGCCGTTTGCGCTGATTCCGGA-TCGC-3′, btrR1-d3: 5′-AGCCGGCGACTCAGGTGG-TGGAGTCGTCCG-3′ and btrR1-d4: 5′-TGGATCCC-TGTGCTCATGTCCGATTCCTCC-3′ containing *Bam*HI and *Nae*I sites, and subsequently sub-cloned into pT7-blue T vector. After the confirmation of the sequences, the two DNA fragments were so ligated as to construct an in-frame disrupted *btrR1* gene fragment, which was then cloned into a *Bam*HI site of pUC119. After recovery of amplified plasmid, the *Bam*HI fragment containing in-frame disrupted *btrR1* was excised and then inserted into the corresponding restriction site of pHB201. The resulting plasmid pHBbtrR1dlt was introduced into wild type cells of *B. circulans* SANK 72073 by electroporation (Easyjet Optima, EquiBio, UK), and *btrR1* gene disruptant was constructed by homologous recombination according to the previously described method [11]. Disruption of the *btrR1* gene was confirmed by PCR with primers btrR1-f: 5′-GCGATCCGGAATCAGCGCAAACG-3′ and btrR1-d5: 5′-GTCCATGTAATCGCCCGACCGGA-3′ for the amplification of the DNA containing *btrR1*. PCR conditions were 95°C, 5 minutes for denature, 30 cycles of 95°C, 1 minute, 40°C, 1 minute, 72°C, 3 minutes for extension of DNA.

The *btrR1* disruptant was cultured in a glycerol-supplemented nutrient broth. An aliquot of the culture was collected every day for 4 days, and antibiotic activity of each supernatant was tested by a paper disk diffusion method against *B. subtilis* PCI 219.

To complement the *btrR1* mutation, a whole *btrR1* gene was first amplified by PCR with primers R1-sf: 5′-CCTGGATCCGTAACCTAGGGC-3′ and R1-sr: 5′-AATACCTTCTCCGGATCCGCG-3′ and a template *B. circulans* chromosomal DNA. PCR conditions were 95°C, 1 minute for denature, 30 cycles of 95°C, 30 seconds, 60°C, 45 seconds, 72°C, 30 seconds for extension of DNA. The amplified DNA fragment was sub-cloned into pT7-blue T vector. After confirmation of the sequence, the *Bam*HI fragment was inserted into the corresponding restriction site of pHB201 to yield an expression plasmid pHBRs, which was introduced by electroporation into the above-mentioned *btrR1* disruptant for complementation. The complemented strain was cultured in the above-mentioned glycerol-supplemented nutrient broth containing 1 μg/ml of erythromycin. An aliquot of the culture was collected everyday and its supernatant was tested on antibiotic activity by a paper disk diffusion method against *E. coli* JM109. The supernatant of a 5-days-culture was loaded onto a column of Amberite IRC-50 [NH_4^+] resin, which was subsequently washed with water and 0.1 M NH_4OH, and then butirosin-containing fractions were eluted with 1 M NH_4OH. After concentration of the fractions, the residue was separated by TLC (Merck silica gel 60 F_{254}, 0.25 mm thick, 20 cm×20 cm) with a solvent $CHCl_3$: MeOH : NH_4OH : EtOH=4 : 6 : 7 : 1. The antibiotics separated by TLC were directly transferred onto the agar plate containing spore of *B. subtilis* PCI 219. *B. subtilis* was cultured at 37°C overnight and the detected antibiotic was compared with standard butirosin.

Disruption Around *btrP-V*

The *btrP-V* disruptant was constructed by the same manner as above mentioned with the primers btrP-d1: 5′-TGGATCCTGCTTACGGCATAGTCGGGTCCC-3′, btrP-d2: 5′-CAGATCTATATTGCAGCGGGCCtGCGCATG-3′, btrP-d3: 5′-GAGATCTGTGGTCTACGCGATTTCCGG-CGT-3′ and btrP-d4: 5′-AGAATTCCGCTTTGGATGG-CGTTACCGGCC-3′. The mutant was screened by PCR with btrP-d5: 5′-CATGCGCAGGCCCGCTGCAATAT-3′ and btrP-d6: 5′-GTTCCCTACTCCGTCCGGCCACC-3′.

RT-PCR

B. circulans RNA was prepared with RNAprotect™ Bacteria Reagent (Qiagen) and RNeasy Mini Kit (Qiagen). RT-PCR was performed with Qiagen OneStep RT-PCR Kit

(Qiagen) using btrR1-f: 5′-GCGATCCGGAATCAGCG-CAAACG-3′ and btrR1-r: 5′-TTGCCATCCATTCCTG-CTGGGCC-3′ for *btrR1*, btrU-f: 5′-GCAGCAGT-CCCGTGTCAACAAAC-3′ and btrU-r: 5′-GCAATCC-GGGGATCAAGCTCGGG-3′ for *btrU*, btrR1U-f: 5′-GGCCCAGCAGGAATGGATGGCAA-3′ and btrR1U-r: 5′-GTTTGTTGACACGGGACTGCTGC-3′ for the region between *btrR1* and *btrU*, OP-f: 5′-GGCGGAGCAA-GCATGGAAGATCG-3′ and OP-r: 5′-CCCGGAGCTTC-ACGGCCTGGCCG-3′ for the region between *btrO* and *btrP*, PV-f: 5′-CGGCCAGGCCGTGAAGCTCCGGG-3′ and PV-r: 5′-ACGCCGGAAATCGCGTAGACCAC-3′ for the region between *btrP* and *btrV*, VQ-f: 5′-GTGGT-CTACGCGATTTCCGGCGT-3′ and VQ-r: 5′-CCGCC-GCCAACCGCGCTGGCAGA-3′ for the region between *btrV* and *btrQ*, according to the manufacturer's protocol. The PCR conditions were; 1 cycle at 50°C for 30 minutes and 94°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and then 72°C for 10 minutes.

Western Blotting

The lysis of *B. circulans* was carried out with final 2% of SDS and 2% of mercaptoethanol. After standard SDS-PAGE, the proteins were transferred onto PVDF membrane by electro-blotting (horizeblot, ATTO). The membrane was soaked in a solution containing BtrC polyclonal antibody with immunblotting kit (Bio-Rad). The expression of BtrC in *B. circulans* was visualized with horseradish peroxidase detection kit (Bio-Rad).

Results and Discussion

Extended Analysis of *btr* Gene Cluster

Gene walking toward the upstream of the so far established *btr* gene cluster was carried out by inverse PCR in order to figure out the starting region, since all the known ORFs are aligned in normal direction. Four additional hypothetical ORFs, *btrR1*, *btrU*, *btrR2*, *btrT* and a partial ORF *orf1* were found in the upstream of the *btrS* gene (Table 1 and Figure 1). The *orf1* gene at the far end showed homology to a gene encoding a hypothetical protein of *B. halodurans* and was identified in the opposite direction to all other *btr* genes. Therefore, *orf1* appears to be in a different operon. It should be pointed out then that a consensus promoter sequence was found in the upstream of *btrR1* (Figure 1) [23]. Thus, an operon starting the *btrR1* gene was envisioned to be the butirosin biosynthetic gene cluster. In fact, the deduced amino acid sequence of *btrR1* showed significant homology to TetR transcriptional regulator family [24]. In addition, the deduced amino acid sequence of *btrR2* showed high homology to the hypothetical protein of *Burkholderia cepacia* and suppressors of fused (Su(fu)) in human, mouse, and *Drosophila*, which function as a negative regulator of the Hedgehog segment polarity pathway by physically interacting with several proteins in the signaling pathway [25∼29]. 3D-PSSM search [30, 31] for a model structure of BtrR2 also showed a high similarity to the Su(fu) of human with low E value. Thus, BtrR2 may interact with butirosin biosynthetic proteins and suppress the activity in tropophase. Consequently, it may be

Table 1. Summary of the newly identified *btr* genes and comparison with database

ORF	Size bp (aa)	Homological proteins, size of protein (aa), source, tag number	Homology (aa/aa, %)
btrP	642 (214)	fructose-2,6-bisphosphatase, 199 aa, *Lactobacillus gasseri*, ZP 00046611	48/165, 29%
btrQ	1371 (457)	hypothetical protein, 508 aa, *S. tenebrarius*, CAE22474	195/451, 43%
		putative sugar-alcohol dehydrogenase, 507 aa, *Micromonospora echinospora*, AAR98543	194/457, 42%
		oxidoredcutase, 508 aa, *S. kanamyceticus*, BAD20757	191/451, 42%
btrR1	654 (218)	putative tetR-family transcriptional regulatory protein, 233 aa, *S. coelicolor* A3(2), NP 625398	41/99, 41%
btrR2	1143 (381)	hypothetical protein, 362 aa, *Burkholderia cepacia* R18194, ZP 00212925	166/358, 46%
		Suppressor of Fused, 433 aa, *Homo sapiens*, AAF23890	103/301, 34%
btrT	234 (78)	no typical homology	
btrU	576 (192)	putative NAD(P)H oxidoreductase, 197 aa, *Bacillus subtilis*, NP 388411	91/184, 49%
btrV	249 (83)	hypothetical protein, 117 aa, *Photorhabdus luminescens* subsp. *Laumondii* TTO1, NP 931337	26/77, 33%
btrW	1758 (586)	putative ABC transporter, 585 aa, *B. subtilis*, NP 388852	309/576, 53%
btrX	>1375 (>459)	putative ABC transporter, 666 aa, *B.cereus*, ZP 00235844	227/458, 49%
orf1	>514 (>172)	hypothetical protein BH3120, 163 aa, *B. halodurans* C-125, BAB06839	41/150, 27%

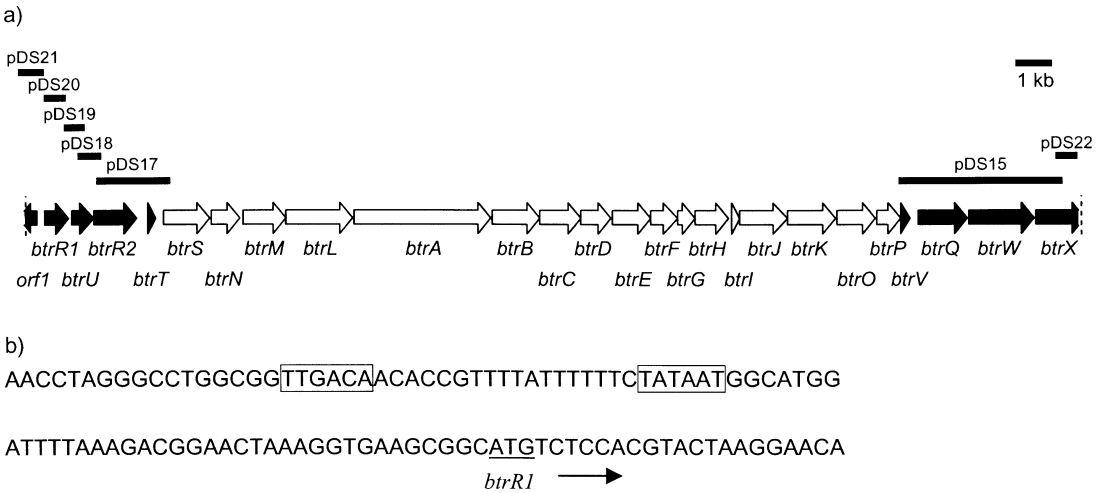


Fig. 1 a) Genetic map of the *btr* gene cluster. Black arrow indicates the ORFs found in the present study. b) Upstream of *btrR1*. Boxes indicate the consensus promoter sequences.

proposed that the combination of BtrR1 and BtrR2 may regulate the butirosin biosynthesis. The deduced *btrU* gene product showed a high homology to NAD(P)H oxidoreductase of various microorganisms. The *btrT* gene was found coding a short protein with no typical homology.

Gene walking to the downstream was carried out with Southern hybridization and inverse PCR. Three additional hypothetical ORFs, *btrV*, *btrQ*, *btrW* and a partial ORF *btrX* were found in this order under the *btrP* gene. It should be pointed out that the previously reported *btrP* gene [11] has to be revised, since an independent ORF *btrV* was newly found between *btrP* and *btrQ* as shown in Figure 1. Interestingly, the deduced BtrV showed quite high homology to hypothetical protein of *Photorhabdus luminescens* subsp. *Laumondii* TTO1 (NC_005126), apparently residing adjacent to a *btrA* homologue in the genome of *Photorhabdus luminescens*. Therefore, BtrA and BtrV may have certain role in combination for the butirosin biosynthesis. The *N*-terminus amino acid of the deduced BtrP showed a similarity to fructose-2,6-bisphosphatases/phosphoglycerate mutases of various microorganisms. Interestingly, the deduced amino acid of *btrQ* gene has high homology to the genes encoding putative dehydrogenase in the DOS-containing aminoglycoside biosynthetic gene cluster, including a tobramycin producer *S. tenebrarius* (AJ579650) [13], a gentamicin producer *Micromonospora echinospora* (AY524043) [15] and a kanamycin producer *Streptomyces kanamyceticus* (AB164142) [17]. BtrQ appears to have close relation to the DOS containing aminoglycoside biosynthesis. The other two genes *btrW* and *btrX* showed significant homology with the genes encoding putative ABC transporters of many species. Aubert-Pivert and Davies previously showed the existence of an bacterial surface protein gene *butB* and a hypothetical protein gene *orfB* adjacent to an aminoglycoside phosphotransferase gene *butA* in *B. circulans* NRRL B3312 [32]. Based on the finding that the insertional mutation of *butB* caused the defection in antibiotic production, they suggested that ButB plays a role in butirosin-export to the outside of the cells. Therefore, the putative transporters *btrW* and *btrX* in the *btr* gene cluster could be related to the exporting system either in combination with the But protein or directly related to the resistance because these genes are obviously in the biosynthetic gene cluster.

Disruption of the *btr* Genes

In order to confirm the relation of the newly identified genes to the butirosin biosynthesis, the *btrR1* gene was first disrupted. The deduced amino acid sequence of *btrR1* showed the significant homology to TetR transcriptional regulator family as mentioned above. Since TetR binds *tetO*, which in turn represses the transcription of *tetA* tetracycline resistance gene, we anticipated that BtrR1 would bind the DNA sequence responsible to the transcription of butirosin biosynthetic genes and regulate the butirosin production. In addition, 3D-PSSM search for the model structure of BtrR1 also showed a high similarity toward TetR transcriptional regulators with low E value. In this hypothetical structure, the *N*-terminus of BtrR1 clearly appeared as a helix-turn-helix motif well conserved as a DNA binding site in the TetR family. Subsequently, we pursued disruption studies of the *btrR1* gene. The *btrR1* disrupted strain did not show any antibiotic production, while the *btrR1* gene complementation recovered the butirosin production. Thus, BtrR1 was found to work as an activator for butirosin biosynthesis in *B. circulans*. Further,

in the *btrR1* disruptant, the transcription of a region *btrV* to *btrQ* was confirmed by RT-PCR. Also, the expression of BtrC was confirmed by Western blotting. Thus, BtrR1 appears to regulate the butirosin biosynthesis, however, the actual target is not clear at the moment. It should be interesting to identify the detailed molecular mechanism of BtrR1 regulation for the butirosin biosynthesis.

In-frame disruption of *btrQ* locating for a downstream of the cluster has been attempted without success. However, the contiguous two genes *btrP-V* just nextmost to *btrQ* was disrupted instead. As a result, the mutant did not show any antibiotic production either. Therefore, *btrP* and/or *btrV* must be required for the butirosin biosynthesis. In addition to the obvious homology of BtrQ to the genes found in the other DOS-containing aminoglycoside producers, the *btr* genes from *btrR1* to *btrQ* would be necessary for the butirosin biosynthesis.

Transcriptional Analysis by RT-PCR

We then investigated the range of transcription of *btr* genes around the upstream and downstream regions. Since there is a promoter like sequence between *btrR1* and *btrU*, RT-PCR was carried out with the mRNA extracted from *B. circulans* at the early stage of growth. As a result, the RNA corresponding to *btrR1*, *btrU* and the sequence between *btrR1* and *btrU* have been already transcribed after 6 hours of fermentation. The RNAs between *btrO* and *btrP*, *btrP* and *btrV*, *btrV* and *btrQ* were similarly analyzed by RT-PCR. As a result, all the sequences were amplified, so that this region should be within a *btr* operon.

In summary, eight additional *btr* genes have been identified in the butirosin biosynthetic cluster in *B. circulans* and the genes between *btrR1-btrP-V* are at least required for the butirosin biosynthesis, although we were not able to conclude the range of *btr* operons. In addition, BtrR1 was clearly found as one of regulatory protein in the biosynthesis of butirosin based on the disruption study. Further analysis of the full *btr* cluster and the regulation mechanism seem necessary to elucidate the whole scenario of the butirosin biosynthesis.

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