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Biosynthesis of aminoglycoside antibiotics: cloning, expression and characterisation of an aminotransferase involved in the pathway to 2-deoxystreptamine†

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The gene *btrR* from *Bacillus circulans* has been cloned and expressed and shown to produce a protein which catalyses the transamination of 2-deoxy-*scyllo*-inosose to give 2-deoxy-*scyllo*-inosamine, an intermediate in the biosynthesis of 2-deoxystreptamine.

Aminoglycosides are a large class of clinically important antibiotics. They are classified into two groups depending on the structure of the aglycone. One class, typified by streptomycin and fortimicin, has the fully substituted aminocyclitol streptamine. The other class has 2-deoxystreptamine as the common aglycone and contains many of the clinically important aminoglycosides like kanamycin, neomycin, gentamicin and butirosin (Fig. 1).[1]

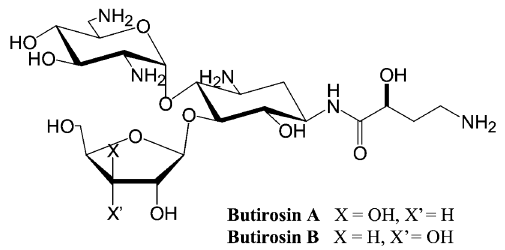
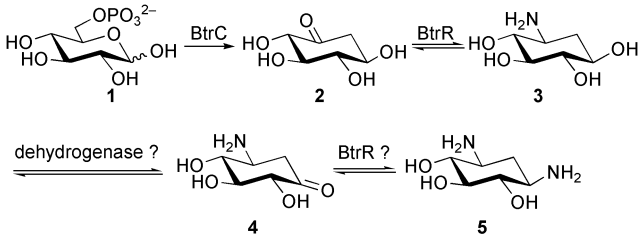


Fig. 1 Structures for butirosin A and butirosin B.

Labelling studies have demonstrated that the biosynthesis of streptamine and 2-deoxystreptamine are quite different. Streptamine is formed from *myo*-inositol[2] whereas 2-deoxystreptamine is biosynthesised from glucose-6-phosphate (**1**) (Scheme 1).[3–7] The initial step in the formation of 2-deoxystreptamine (**5**) has been shown to be catalysed by 2-deoxy-*scyllo*-inosose synthase. This enzyme converts **1** into 2-deoxy-*scyllo*-inosose (**2**) by a mechanism analogous to that of dehydroquinate synthase.[8] It has been purified to homogeneity and the protein sequence was used successfully to locate the gene (*btrC*) in the butirosin producing organism *Bacillus circulans*. Subsequent gene walking from both sides of *btrC* identified additional genes in the butirosin cluster.[9]



Scheme 1 Proposed biosynthetic pathway of 2-deoxystreptamine. **1**. D-glucose-6′-phosphate; **2**. 2-deoxy-*scyllo*-inosose; **3**. 2-deoxy-*scyllo*-inosamine; **4**. 2-deoxy-3-amino-*scyllo*-inosose; **5**. 2-deoxystreptamine.

† Electronic supplementary information (ESI) available: LC-MS, TLC and ESI-Q-TOF MS. See http://www.rsc.org/suppdata/cc/b2/b209799k/

In order to elucidate the complete pathway to 2-deoxystreptamine we have carried out further gene walking experiments using genomic DNA from *B. circulans* NRRL B3312 in an attempt to obtain the whole butirosin gene cluster. During these studies we identified a 1257 bp open reading frame now named *btrR* (accession number AJ494863, EMBL nucleotide sequence database) that has homology to the aminotransferase family of enzymes. The protein sequence of BtrR contains a highly conserved motif that has been observed in a new class of pyridoxal 5′-phosphate (PLP)-dependent transaminase found mainly in the biosynthesis of secondary metabolites. Two of the transaminases involved in biosynthesis of streptamine: StsC, the L-glutamine:*scyllo*-inosose aminotransferase, and StsA, the L-alanine:*N*-amidino-3-keto-*scyllo*-inosamine aminotransferase are both members of this new class of transaminase.[10] Alignment of BtrR with StsC reveals high homology (40.3% sequence identity) between these two proteins, suggesting they may have a similar function.

To determine whether the protein encoded by *btrR* catalyses the transamination of **2** to give 2-deoxy-*scyllo*-inosamine (**3**) or one of the other transamination steps in the biosynthesis of butirosin, the gene was sub-cloned into a pET28 vector and the protein expressed in *E. coli* BL21 (DE3). The over-expressed His_6-tagged protein was purified by Ni^{2+}-NTA affinity column followed by gel filtration and anion-exchange chromatography. Purified BtrR gave a single band on SDS-PAGE gel with a molecular mass around 50 kDa (Fig. 2). The molecular weight of BtrR determined by LC-ESI-MS is 50 714 Da (see supplementary information†) in agreement with the calculated MW of 50 703 Da. Gel filtration chromatography showed an apparent MW of 97 kDa for BtrR determining that it is a homodimer.

Purified BtrR showed two UV-Vis absorptions, one at 335 nm and the other at 415 nm indicating the presence of BtrR-bound pyridoxamine 5′-phosphate (PMP, 335 nm) and PLP (415 nm), respectively. This is similar to that of StsC, one of the transaminases in streptamine biosynthesis pathway from *Streptomyces griseus* and is typical of PLP-containing enzymes.[10] Addition of **2** significantly enhanced the absorption at 415 nm but reduced the absorption at 335 nm suggesting amino transfer from PMP to **2** whilst addition of **3** showed the opposite effect

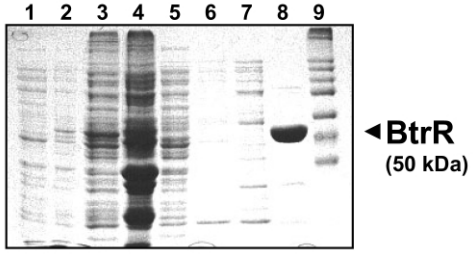


Fig. 2 Expression of BtrR. Lane 1: before induction; lane 2: after induction; lane 3: cell-free extract; lane 4: cell pellet; lane 5: unbound proteins; lane 6 and 7: column-wash fractions; lane 8: purified BtrR; lane 9: protein marker.

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(Fig. 3).[11] This clearly indicates that BtrR binds **2** and **3,** thus suggesting that **2** and **3** are the true substrates for this enzyme.

To test the activity of BtrR we carried out a series of enzymic reactions using **2** and different amino acids as amino donors. Each incubation was carried out for 1 h at 37 °C and contained 0.1 mg ml^{-1} BtrR, 4 mM of **2**, 5 mM of an amino acid in 30 mM Tris·HCl buffer (pH 7.5) containing 0.3 mM PLP. TLC of the reaction mixtures showed that a new compound with the same R_f value as **3**, was formed in the incubation that had L-glutamine as the amino donor (Fig. 4A).

After the completion of the reaction the product was purified and determined to be **3** by ESI-Q-TOF tandem mass spectrometry (identical mass spectra to authentic **3**, see supplementary information†). In the reactions using L-alanine, L-serine, or L-glutamate as amino donors, formation of **3** was also observed but only after overnight incubation at 37 °C indicating that these

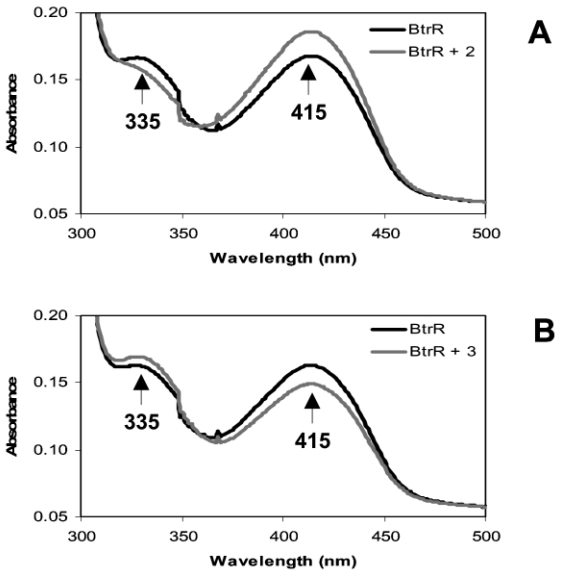

Fig. 3 UV-Vis absorption spectra of BtrR. A: with or without **2** (2-deoxy-*scyllo*-inosose); B: with or without **3** (2-deoxy-*scyllo*-inosamine).

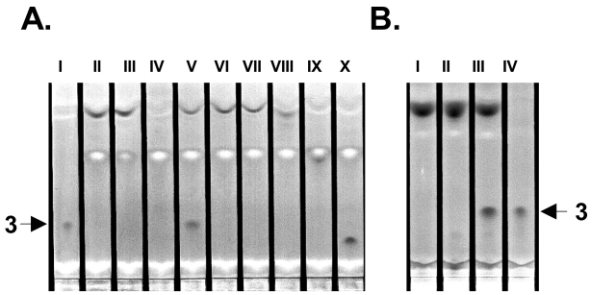

Fig. 4 TLC analysis of BtrR activity: 1 μl of each reaction was applied to a silica TLC gel plate, developed in methanol:ammonia (5:1), and stained using ninhydrin reagent. **A**. BtrR catalysed forward reaction. Lane I: **3** (2-deoxy-*scyllo*-inosamine) standard; lane II: no enzyme; lane III: no **2** (2-deoxy-*scyllo*-inosose); lane IV: no amino donor; from lane VI to lane X: L-glutamine, L-alanine, L-glutamate, L-serine, L-ornithine and L-arginine were used as amino donors, respectively. **B**. BtrC/BtrR-coupled assay. Lane I: no BtrR; lane II: no BtrC; lane III: BtrC + BtrR; lane IV: **3** standard.

amino acids could be taken as poor substrates by BtrR (all of the other common amino acids were not substrates for BtrR).

BtrR was also shown to catalyse the reverse reaction when incubated with **3** and pyruvic acid as an amino acceptor. We did not use α-keto-glutaramate (the transaminated L-glutamine) due to its instability in solution (cyclises spontaneously to form 5-hydroxy-2-pyrrolidone-5-carboxylic acid).[10]

To reconstitute the early part of the 2-deoxystreptamine pathway we also cloned and expressed the first enzyme BtrC. BtrC was cloned by PCR based on the known sequence,[9] inserted into a pET28 vector, expressed in *E. coli* BL 21 (DE3) and purified as described for BtrR except a Co^{2+}-NTA affinity column was used. The BtrC/BtrR coupled assay was carried out using **1** (5 mM) as a substrate in the presence of 5 mM β-NAD, 0.2 mM $CoCl_2$, 20 mM L-glutamine, and 0.1 mg ml^{-1} of each of the enzymes. Analysis by TLC showed that **3** was rapidly formed thus demonstrating that these two enzyme can act concomitantly (Fig. 4B).

The remaining pathway to 2-deoxystreptamine is proposed to involve the oxidation of **3** to **4**, probably catalysed by a dehydrogenase, and finally a transamination to form **5**. Interestingly, BtrR also catalysed amino transfer from **5** to pyruvic acid and therefore it is possible that BtrR also catalyses the transamination from **4** to **5** (the lack of a synthetic standard of **4** has prevented us, at this stage, from confirming this proposal).

In summary, we have cloned, expressed and characterised for the first time L-glutamine:2-deoxy-*scyllo*-inosose aminotransferase, BtrR, involved in the biosynthesis of 2-deoxystreptamine from the butirosin producing strain *Bacillus circulans* NRRL B3312. BtrR has been shown to exist as a homodimer with a subunit molecular weight of 50.7 kDa. L-glutamine has been determined to be the best amino donor for the formation of **3** catalysed by BtrR. We have also shown that the early part of the 2-deoxystreptamine pathway can be readily reconstituted *in vitro* using recombinant BtrC and BtrR.

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