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Biosynthesis of 3′-Deoxy-carbamoylkanamycin C in a *Streptomyces tenebrarius* Mutant Strain by *tacB* Gene Disruption

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Abstract *Streptomyces tenebrarius* H6 mainly produces three kinds of antibiotics: apramycin, carbamoyltobramycin and some carbamoylkanamycin B. In our present study, a dehydrogenase gene *tacB* in the tobramycin biosynthetic gene cluster was disrupted by in-frame deletion. The result of TLC bio-autograph analysis demonstrated the disruption mutant strain produced apramycin and a new antibiotic. The new antibiotic was identified as 3′-deoxy-carbamoylkanamycin C by MS and NMR analysis after isolation and purification. The disruption mutant was restored to produce carbamoyltobramycin in a complementation experiment by the intact *tacB* gene. Our studies suggested that the *tacB* gene encodes a 6′-dehydrogenase, which reduces the 6′-hydroxyl group of paromamine to a keto group, thus facilitating the transfer of an aminogroup to form neamine. This study is the first report on the generation of a tobramycin derivative by gene engineering, and will contribute to clarify the complete biosynthetic pathway of tobramycin.

Keywords *Streptomyces tenebrarius*, in-frame deletion, complementation, 6′-dehydrogenase, 3′-deoxy-carbamoylkanamycin C

Introduction

Aminoglycosides are one of the most important antibiotic families applied for clinical purpose. It have a wide antibacterial spectrum and special antibacterial activity against aerobic gram negative bacilli. However its main side-effects as ototoxicity and nephrotoxicity [1] and bacterial drug resistances compromise its clinical usage. This has necessitated the development of structural novel and more potent antibiotics. Based on chemical structure, aminoglycosides can be classified into two major groups: one has an aglycone fully-substituted aminocyclitol which is synthesized from *myo*-inositol [2]. The other group has a common aglycone of 2-deoxystreptamine (2-DOS). Many important aminoglycosides belong to the second category, such as tobramycin, kanamycin and neomycin.

Compared with other major classes of antibiotics such as macrolides, studies on the biosynthesis of aminoglycosides have fallen behind but in recent years, several gene clusters of 2-deoxystreptamin-containing aminoglycosides have been cloned. Yasumasa Ota *et al.* [3] first reported the gene cluster of Butirosin, identified the function of BtrC which catalyzes the carbocycle formation from D-glucose-6-phosphate to 2-deoxy-*scyllo*-inosose (2-DOI) *in vivo*. And then partial biosynthetic gene clusters of tobramycin, gentamycin, kanamycin, neomycin and ribostamycin were cloned from different strains [4∼9]. All these papers proposed the same biosynthetic pathway of 2-DOS, and most genes encoding for enzymes catalyzing each step of 2-DOS biosynthesis have been discovered [3∼11]. Finally pseudodisaccharide intermediate paromamine is produced through the addition of dTDP-glucosamine to 2-DOS with the help of glycosyltransferase.

There are several dehydrogenases in the gene cluster of tobramycin, but only the function of TbmA (2-DOI synthase) was demonstrated [4]. In this paper, we explored the function of *tacB* gene which located in tobramycin

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biosynthetic gene cluster. Based on gene disruption and complementation experiments, along with the structure analysis of the new antibiotic produced by the *tacB* disruption mutant, our studies suggest that the *tacB* gene encodes a 6′-dehydrogenase, which catalyzed the 6′-hydroxyl group of paromamine to form a keto group. This is the first report on blocking tobramycin derivative biosynthesis due to the ceased expression of a dehydrogenase gene in context with the *tacB* disruption.

Materials and Methods

General

Methods for isolation and manipulation of DNA were used as described by Sambrook *et al.* [13]. Transformation of *Escherichia coli* followed the $CaCl_2$ method [13]. Conjugation transformation of *Streptomyces tenebrarius* H6 was performed according to the method described previously [14]. The reference substance 3′-deoxy-carbamoylkanamycin C (M.W.=511.2) was generated from a mutagenesis strain [16] and purified in our laboratory. Ion-exchange resins were purchased from Shanghai Huazhen Co. Ltd.

Strains, Plasmids and Culture Conditions

Bacterial strains and plasmids used in the study are listed in Table 1. Agar medium (SM) was used for the *S. tenebrarius* H6 sporulation [17] and MS [15] for the conjugation between *S. tenebrarius* H6 and *E. coli* ET12567 (pUZ8002). Liquid medium (SGGP) was used for *S. tenebrarius* H6 or mutant strains mycelium growth. For antibiotics production in *S. tenebrarius* H6 or mutant strains, fermentation was carried out by using seed culture medium and fermentation medium [17]. 2×YT medium used in conjugation was prepared as described by Hopwood *et al.* [15]. If necessary, erythromycin was added at a concentration of 100 μg/ml. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C, supplemented with 50 μg/ml apramycin, 50 μg/ml ampicillin, 25 μg/ml chloromycetin or 25 μg/ml kanamycin when necessary. *Bacillus subtilis* was incubated in bioassay medium [17].

Gene Disruption

The recombinant plasmid was constructed for targeted disruption of *tacB* as follows: A 2.15-kb fragment containing *tacB* gene was amplified from wild-type *S. tenebrarius* H6 genomic DNA with primer 1 (CATAAGCTTCAGCCCGAGGACACGC) and primer 3 (GCGCTCTAGATGACCGTCTCCAAGGG). PCR condition was 95°C, 5 minutes for denaturation, 30 cycles of 94°C, 2 minutes, 59°C, 30 seconds, 72°C, 2.5 minutes for extension of DNA. The amplified PCR product was purified, digested with *Hin*dIII and *Xba*I and cloned into the same site of pIJ2925 to yield pSPU501. The *Sal*I-*Sal*I fragment internal to *tacB* was released and self-ligated as to construct an in-frame disrupted *tacB* gene named *ΔtacB*. And then, the erythromycin-resistant gene (*ermE*) fragment from plasmid pWHM2 was inserted into the *Kpn*I site. The resulting *ΔtacB-ermE* fragment was excised with *Bgl*II and cloned into the *Bam*HI site of pHZ132 [18] to create pHZ-*ΔtacB*, which was transferred into *E. coli* ET12567

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant features*	Source or reference
Strains		
Streptomyces tenebrarius H6	Wild type, carbamoyltobramycin producer	This lab
S. tenebrarius tacB$^-$	Disrupted in *tacB*	This work
S. tenebrarius tacB$^-$*–tacB*	Complement intact *tacB*; Ery^R	This work
Escherichia coli ET12567 (pUZ8002)	Strain used in *E.coli-Streptomyces* conjugation	15
Plasmids		
pHZ132	*E. coli-Streptomyces* shuttle vector used for gene inactivation; Am^R	18
pSPU227	Expressive vector containing *Pmer* promoter+*to* terminator; Am^R	This lab
pWHM2	Erythromycin-resistant gene *ermE* in 1.7-kb *Kpn*I fragment used as selective marker; Am^R	25
pSET152	Integrative (suicide) vector for gene complementation; Ap^R	19
pHZ-*ΔtacB*	pHZ132 derivative containing *ΔtacB*+*ermE*; Am^R	This work
pSET-*tacB*	pSET152 derivative containing *Pmer*+*tacB*+*to*+*ermE*; Ap^R	This work

* Am^R, ampicillin resistant; Ap^R, apramycin resistant; Ery^R, erythromycin resistant.

(pUZ8002).

Plasmid pHZ-*ΔtacB* was introduced into *S. tenebrarius* H6 by conjugation, which was carried out on MS medium at 28°C for 20 hours. After overlaying with erythromycin (100 μg/ml) and nalidixic acid (50 μg/ml), incubation was continued at 28°C for 7~9 days. Single-crossover exconjugants of *S. tenebrarius* H6 were obtained using erythromycin as a selective marker. After three rounds of sporulation in the absence of erythromycin, two types of erythromycin-sensitive strains, namely *tacB*-disruptants and wild-type revertants, were obtained for detection of antibiotic products. To confirm the double-crossover integration of *ΔtacB* into the *tacB* locus, genomic DNA of exconjugant was isolated, and PCR was carried out using primers 1 and 3, the amplification product was subjected to sequencing analysis, and the result was compared with the sequence of *tacB* in the Genbank.

Complementation of *tacB* in the Gene Disruption Mutant

To complement the *tacB* disruption mutant, the intact *tacB* gene was amplified from the genomic DNA of *S. tenebrarius* H6 with primer a (GGGACGG*TCTAGA*-ATGCTGCTGATCTCAG) and primer b (CAT*AAGCTT*-CAGCCCGAGGACACGC). The amplified fragment was cloned between the *Xba*I and *Hin*dIII sites of the expression plasmid pSPU227. The *Bgl*II fragment containing *Pmer*+*tacB*+*to* was inserted into the *Bam*HI site of pIJ2925, and the *ermE* gene was sub-cloned as a *Kpn*I fragment into the same plasmid. The resulting *Pmer*+*tacB*+*to*+*ermE* fragment was excised with *Bgl*II and cloned into the *Bam*HI site of pSET152 [19], resulting in the final plasmid pSET152-*tacB*.

pSET152-*tacB* was introduced into the *tacB* disrupted mutant strain by conjugation from *E. coli* ET12567 (pUZ8002). The erythromycin-resistant (Ery^R) exconjugants were selected for detection of antibiotic products.

Analysis of Antibiotics by TLC and Bio-autography

The fermentation filtrates of the *tacB* disruption mutants, the *tacB* complementary colonies (Ery^R) or *S. tenebrarius* H6 were applied to silica GF_{254} TLC glass plate (Huiyou, China), and developed with a solvent system of PrOH : MeOH : ammonia solution (25%)=25 : 20 : 23 at 37°C for 3.5 hours, then the plate was placed on agar medium seeded with *B. subtilis* for 10 minutes, and the TLC plate was removed finally the agar medium was incubated overnight at 37°C.

Purification and Identification of the New Antibiotic

The antibiotic products of wild-type strain, *tacB* disruption strain and complementary strain were isolated and purified following the protocols described previously [16, 20].

For HPLC-ELSD analysis, the method of Hong Li-ya [21] was used, in which a reverse C_{18} column (4.6 by 200 mm; 5 μm; Diamonsil, USA), mobile phase 0.2 mol/liter TFA : MeOH (95 : 5) at a flow rate of 1.0 ml/minute was used. The drift tube temperature was 105°C, the detector was SofTA-200S (USA). High-resolution MS was performed on a Waters Quattro micro API spectrometer (USA). The NMR data was acquired on an AV-600 (600 MHz) spectrometer.

***In Vitro* Antibacterial Activity**

The *in vitro* antibacterial activities were determined by agar method as described earlier [22]. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth of the organism.

Results and Discussion

Generation and Analysis of *ΔtacB* Mutant *S. tenebrarius tacB*$^-$

In order to avoid polar effects on the downstream gene transcription, an in-frame deletion was introduced into *tacB*. The disruption strategy was described above and illustrated in Fig. 1.

The *E. coli-Streptomyces* shuttle vector pHZ132 containing *ΔtacB* (an internal *Sal*I fragment was deleted) and erythromycin-resistant gene *ermE* was introduced into *S. tenebrarius* H6 by conjugation from *E. coli* ET12567 (pUZ8002). The erythromycin-resistant exconjugants were obtained. Following erythromycin-free cultivation for three rounds of sporulation, two types of double-crossover strains, namely *ΔtacB* strains and wild-type revertant strains were obtained and then identified by PCR analysis. PCR with primer 1 and 3 produced a 2.0 Kb from *ΔtacB* strains but not from wild-type revertant strains. Furthermore, this PCR amplification product from one *ΔtacB* strain was subjected to sequencing analysis with the primer S: 5′-TTCGGCCCCGGGTGCGC-3′ and the result showed that only one *Sal*I site in *ΔtacB* together with the internal 143-bp fragment was deleted. This *ΔtacB* strain was named as *S. tenebrarius tacB*$^-$.

The growth profile of *S. tenebrarius tacB*$^-$ in liquid medium CP was identical to that of the wild-type strain, while the formation of mycelia and spores was indistinguishable from that of the wild-type strain. TLC analysis of the fermentation broth showed that *S.*

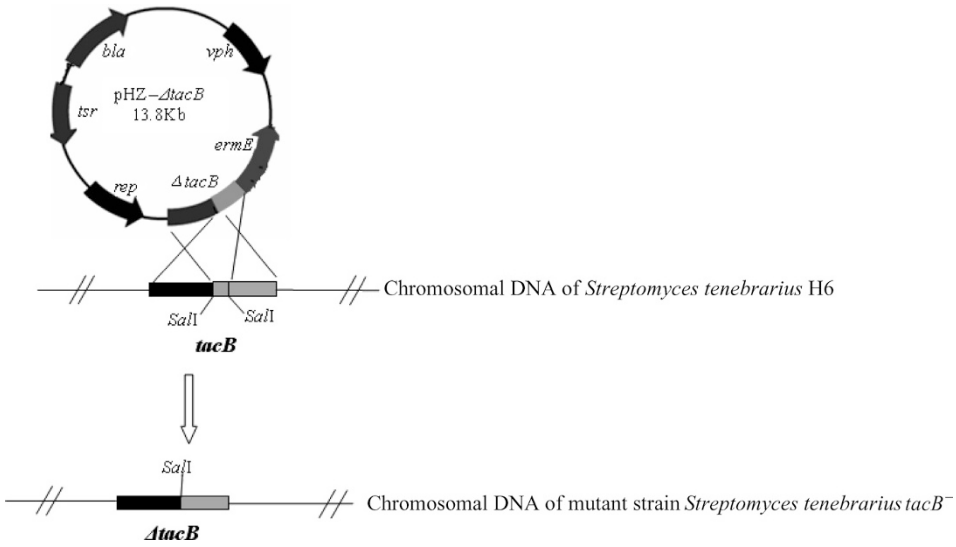

Fig. 1 The plasmid pHZ-*ΔtacB* used in conjugation and the result of in-frame deletion of *tacB via* double crossover (the gray part was deleted).

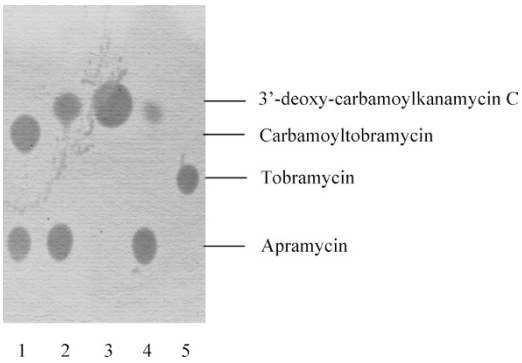

Fig. 2 TLC analysis of the fermentation broths of *S. tenebrarius* H6 and disruption mutants.

1, *S. tenebrarius* H6; 2&4, *S. tenebrarius tacB*$^-$; 3, 3′-deoxy-carbamoylkanamycin C; 5, tobramycin.

tenebrarius tacB$^-$ produced a new component, whose Rf value was approximate to that of 3′-deoxy-carbamoylkanamycin C [16], as shown in Fig. 2.

Purification and Identification of the New Antibiotic

To determine the structure of the new antibiotic produced by *tacB* gene disruption mutant, the antibiotic products of *S. tenebrarius tacB*$^-$ were isolated and purified. Positive ESI-MS spectrometry confirmed the presence of 3′-deoxy-carbamoylkanamycin C in the sample by yielding molecular ions (*m*/*e* for $[M+H]^+$) of 512.2 ($C_{19}H_{37}N_5O_{11}$, calcd 511.2) [16], as show in Fig. 3.

The distinct peak at 367.2 also agrees with the mass of the sum of the DOS and 6′-carbamoyl-kanosamine. The result from NMR analysis further confirmed that this new antibiotic is 3′-deoxy-carbamoylkanamycin C [16, 23]. The ^{13}C-NMR data of the new antibiotic was listed in Table 2 and shown in Fig. 4.

Complementation of the Mutant Strain *S. tenebrarius tacB*$^-$

In order to confirm that the new antibiotic production in *S. tenebrarius tacB*$^-$ was due to the loss function of *tacB* and excluded the possibility of polar effects in the disruption mutant, restoration of the intact *tacB* gene in the *ΔtacB* strain, *S. tenebrarius tacB*$^-$ was carried out.

The complementary plasmid pSET-*tacB* was constructed as described above, in which the expression of the intact *tacB* gene is under the control of the mercury resistant promoter (*P*mer) [24]. pSET-*tacB* was introduced into the disruption mutant *S. tenebrarius tacB*$^-$ by conjugation, and Ery^R exconjugants were selected, named *S. tenebrarius tacB*$^-$*–tacB*. HPLC-ELSD analysis confirmed that carbamoyltobramycin production was partially restored in *S. tenebrarius tacB*$^-$*–tacB* in comparison with the wild-type *S. tenebrarius* H6 as the control, as shown in Fig. 5. This demonstrated that the production of 3′-deoxy-carbamoylkanamycin C in *S. tenebrarius tacB*$^-$ was due to a loss of *tacB* function.

***In Vitro* Activity**

The antibacterial activity of 3′-deoxy-carbamoylkanamycin C against several bacterial is presented in Table 3. The result showed that 3′-deoxy-carbamoylkanamycin C has antibacterial activity to commonly encountered and some

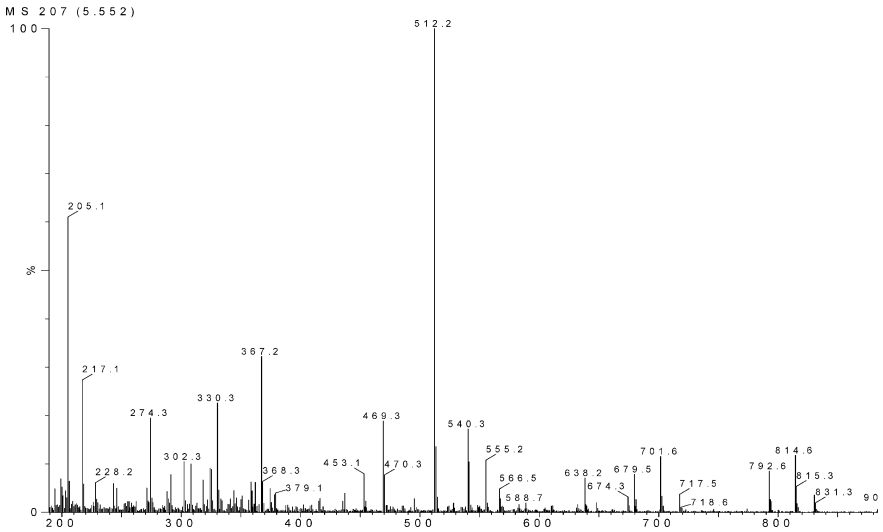

Fig. 3 Mass spectrum of the sample of *S. tenebrarius tacB*$^{-}$.

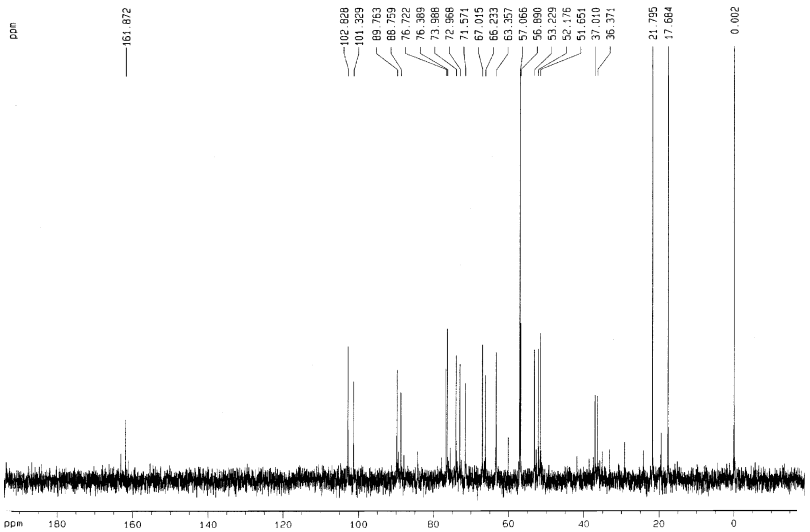

Fig. 4 The ^{13}C-NMR analysis of the new antibiotic.

Table 2 ^{13}C-NMR date of the new antibiotic*

Position	δ (ppm)	Position	δ (ppm)	Position	δ (ppm)
1	53.2	1′	101.3	1″	102.8
2	36.3	2′	51.6	2″	72.9
3	52.1	3′	37.0	3″	56.8
4	88.7	4′	67.0	4″	71.5
5	76.7	5′	76.3	5″	73.9
6	89.7	6′	63.3	6″	66.2
				$CONH_2$	161.8

* Internal standard: DSS.

clinically important bacteria, although it is less active than tobramycin.

Discussion

For studying the role of the putative dehydrogenase TacB, we constructed the *tacB* in-frame deletion mutant *S. tenebrarius tacB*$^{-}$. TLC bioautography showed that the mutant strain produced a new antibiotic. After isolation and purification, the structure of the new antibiotic was further determined as 3′-deoxy-carbamoylkanamycin C by MS and NMR analysis.

The gene downstream of *tacB* is *tacA*. The intergenic region between *tacB* and *tacA* contains two palindromic

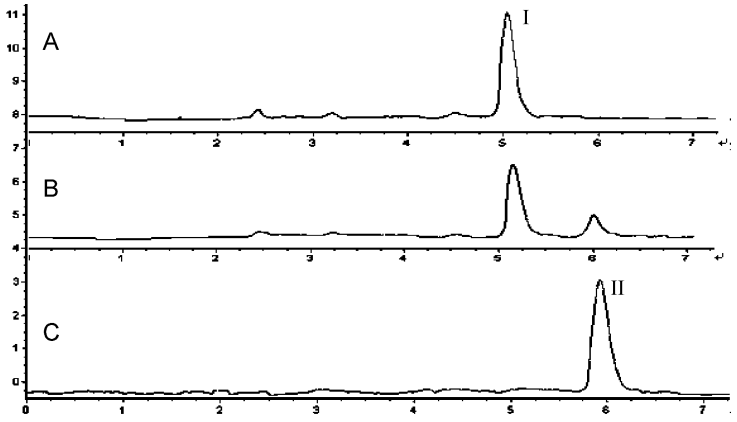

Fig. 5 HPLC-ELSD analysis of the isolated compound from *tacB* disruption strain *S. tenebrarius tacB*$^{-}$ (A), complementary strain *S. tenebrarius tacB*$^{-}$*–tacB* (B), wild-type strain *S. tenebrarius* H6 (C), where I correspond to 3′-deoxy-carbamoylkanamycin C and II correspond to carbamoyltobramycin.

Table 3 Comparison of MICs between 3′-deoxy-carbamoylkanamycin C and tobramycin

Organism	MIC (μg/ml)	
	3′-deoxy-carbamoylkanamycin C	tobramycin
Escherichia coli	16	2
Bacillus subtilis	8	4
Staphylococcus aureus	≧32	4
B. proteus	8	4
Pseudomonas aeruginosa	≧128	16
B. pumilus	16	1

sequences (*GGGCCG*GGCGT*CGGCCC*CG; C*ACCGGT*-TCCTCAC*ACCGGT*T) that may form stem-and-loop structures in the RNA, which might act as transcription terminators of readthrough transcripts from genes upstream *tacB*. This may reduce the chance of polar effects or additional mutations in the disruption strain, which was demonstrated by the integration of an intact *tacB* gene. By performing the restoration of the intact *tacB* gene back into the *ΔtacB* strain, carbamoyltobramycin production was partially restored, this ruled out the possibility of a polar effect and proved that the production of 3′-deoxy-carbamoylkanamycin C in *S. tenebrarius tacB*$^{-}$ was due to the loss of *tacB* function.

TacB was 76% identical to the KanI in the kanamycin biosynthetic gene cluster [7], was similar to the BtrQ (59%) in butirosin biosynthetic gene cluster [26], ParQ (56%) in paromomycin gene cluster, and NeoG (54%) in neomycin biosynthetic gene cluster [11]. After bioinformatic analysis, ^{20}GSGASG^{25} sequence was found in TacB. This dinucleotide binding motif of the Rossmann fold (GXGXXG) was conserved in dehydrogenases from different *Streptomyces*, which suggested the function of TacB as a dehydrogenase. Our result not only supported this hypothesis but also clarified the specificity of this enzyme as 6′-dehydrogenase in tobramycin biosynthesis. The structural difference between 3′-deoxy-carbamoylkanamycin C and carbamoyltobramycin is that the former has a 6-hydroxyl group while the latter has a 6-amino group. The TacB is responsible for reducing the 6′-hydroxyl group of paromamine to a ketone group, then enabling the putative aminotransferase TacC to form neamine. Then, neamine is catalyzed to carbamoyltobramycin [4]. This is the first gene identified responsible for aminoglycoside biosynthesis after the formation of paromamine.

The new antibiotic 3′-deoxy-carbamoylkanamycin C is active against some common bacteria, although it is less active than tobramycin. However, the toxicity of 3′-deoxy-kanamycin C is lower than tobramycim [16, 27].

In summary, two major outcomes have been achieved. Firstly, we identified that TacB is a 6′-dehydrogenase, secondly, we got a new antibiotic, 3′-deoxy-carbamoylkanamycin C, by manipulating the biosynthesis through gene inactivation of *tacB*. The strategy of gene inactivation and generation of new derivatives without chemical synthesis is a promising method and a step forward to the development of new antibiotics in order to overcome problems of infections caused by multi-resistant pathogens.

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