ChemPubSoc Europe

DOI: 10.1002/cbic.201500258

CHEMBIOCHEM Full Papers

Biosynthesis of Epimers C2 and C2a in the Gentamicin C Complex

Yawen Gu,[a] Xianpu Ni,[a] Jun Ren,[a] Huiyuan Gao,[b] Da Wang,[b] and Huanzhang Xia*[a]

Gentamicin is a broad-spectrum aminoglycoside antibiotic widely used to treat life-threatening bacterial infections. The gentamicin C complex consists of gentamicin C1, gentamicin C1a, and epimers gentamicin C2 and gentamicin C2a. At present there is a generally accepted pathway of gentamicin biosynthesis, except for detailed understanding of the epimerization process involving gentamicins C2 and C2a. Here we have investigated the biosynthesis of these epimers. JI-20B—an intermediate in the gentamicin biosynthetic pathway—and its epimer JI-20Ba were generated by in-frame deletion within *gen*P, which encodes a phosphotransferase that catalyzes the first step of 3′,4′-bisdehydroxylation in gentamicin biosynthesis. GenB1 and GenB2 are aminotransferases with different substrate specificities and enantioselectivities. JI-20Ba, containing a 6′*S* chiral amine, a precursor of gentamicin C2a, was synthesized from G418 by GenQ/GenB1 through sequential oxidation/transamination at C-6′. GenQ/GenB2 catalyzed the synthesis of JI-20B, containing a 6′*R* chiral amine, a precursor of gentamicin C2, from G418. GenB2 catalyzed the epimerization of JI-20Ba/JI-20B and of gentamicins C2a/C2.

Introduction

Gentamicin, a broad-spectrum antibiotic produced by *Micromonospora echinospora*,[1] is a complex mixture of four major compounds—gentamicins C1, C1a, C2a, and C2—and numerous minor compounds such as gentamicin C2b. Gentamicin belongs to the group of 4,6-disubstituted 2-deoxystreptamine (DOS) aminoglycoside antibiotics; its chemical structure consists of the core aminocyclitol moiety 2-DOS, together with purpurosamine and garosamine linked to positions C-4 and C-6, respectively. The differences between the four major compounds are to be found in the purpurosamine component (Scheme 1).

Gentamicin is widely used to treat bacterial infections, particularly those caused by Gram-negative bacteria.[2,3] Its bactericidal activity is based on selective binding to the bacterial 16S rRNA A-site (decoding aminoacyl site) and interfering with initiation and translation fidelity during protein synthesis. Gentamicin is also used as a therapeutic agent to treat human genetic disorders caused by nonsense mutations.[4,5]

The emergence of multidrug-resistant bacteria[6,7] caused by antibiotic abuse, together with the lack of new antibiotics, mean that it is likely that aminoglycosides will continue to be in demand because of their low cost and high antibacterial activity. Unfortunately, gentamicin causes serious side effects such as ototoxicity and renal toxicity, and this restricts its wide clinical application. Commercial gentamicin is a mixture of gentamicins C1 and C1a and the epimers C2a and C2. Surprisingly, it has been reported that C2, which has the same bactericidal efficacy as C2a, exhibits no nephrotoxicity and little cellular toxicity.[8] Therefore, the biosynthesis of C2a and C2 as a prerequisite for obtaining gentamicin with a particular composition appears worth investigating. The DNA sequence of the gentamicin biosynthetic gene cluster (GenBank accession numbers AY524043 and AJ628149) in *M. echinospora* has provided the basis for determining the complete biosynthetic pathway.[9]

The established gentamicin biosynthetic pathway (Scheme 1) branches at gentamicin X2, with one branch producing JI-20A and C1a and C2b, and the other producing G418, JI-20B, and C2a, C2, and C1. GenK is a C-6′ methyltransferase that converts gentamicin X2 into G418.[10] Gentamicin X2 and G418 are converted into JI-20A and JI-20B, respectively, through coupled oxidation/transamination reactions. GenQ catalyzes the oxidation of X2 and G418.[11,12] GenP catalyzes the 3′-OH phosphorylation step of JI-20A, which is possibly the first step for C-3′ dehydroxylation.[13]

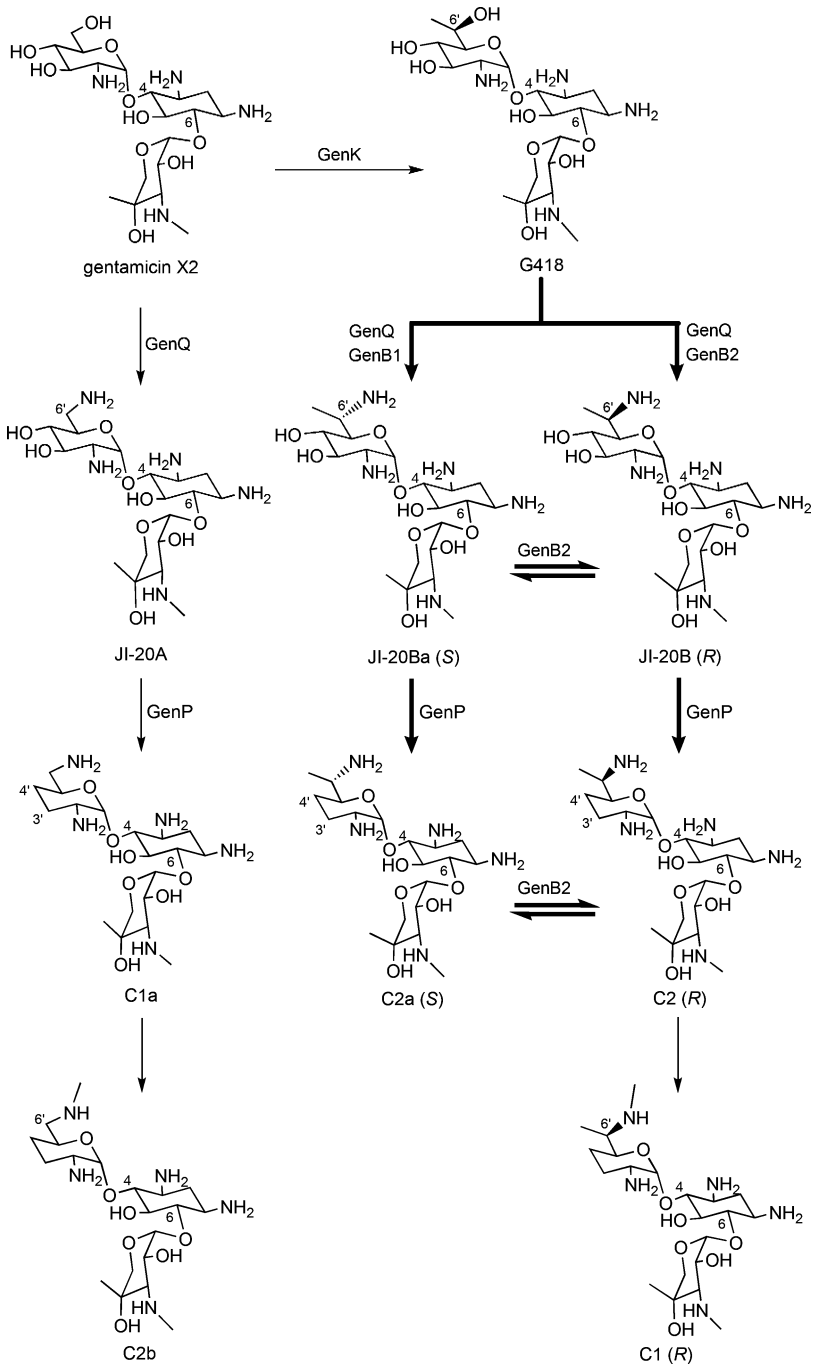
Recently, four potential aminotransferase-encoding genes—*gen*B1, *gen*B2, *gen*B3, and *gen*B4—in the gentamicin gene cluster have been reported.[11] In the formation of JI-20A and JI-20B, transamination is mainly catalyzed by GenB1, whereas GenB2 catalyzes the epimerization between C2a and C2; C2a here is the first product of JI-20B bisdehydroxylation. GenB3 and GenB4 participate in the 3′,4′-bisdehydroxylation of JI-20A and JI-20B.

However, many details in the biosynthetic pathway to gentamicins C2a and C2 are not fully understood. Thus, it is unclear

[a] *Dr. Y. Gu, Dr. X. Ni, Dr. J. Ren, Prof. H. Xia*
School of Life Science and Biopharmaceutics
Shenyang Pharmaceutical University
103 Wenhua Road, Shenyang 110016 (P. R. China)
E-mail: xiahz612@sina.com

[b] *Prof. H. Gao, D. Wang*
Key Laboratory of Structure-Based Drug Design and Discovery of Ministry of Education
School of Traditional Chinese Material Medica
Shenyang Pharmaceutical University
103 Wenhua Road, Shenyang 110016 (P. R. China)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201500258.



Scheme 1. The biosynthetic pathway of the gentamicin complex after gentamicin X2. Biosynthetic enzymes investigated in this study are marked in bold. The new proposed branch point at G418 and the biosynthetic pathway for gentamicins C2a and C2 are shown in bold. The functions of the two pyridoxal-phosphate-dependent (PLP-dependent) enzymes GenB1 and GenB2 are revealed in this study. The phosphotransferase activity of GenP previously demonstrated in vitro was revealed here in vivo. A new pair of epimers JI-20Ba and JI-20B was identified for the first time in this study.

whether other aminotransferases participate in the biosynthesis of C2a and C2, whether C2a is the only C2 precursor, whether the isomerization of C2a to C2 occurs only in the last steps, and whether GenB2 can catalyze the epimerization of JI-20B with the same 6′-chiral carbon as in gentamicin C2 to generate the other conformation.

Compound G418 is first converted into JI-20B, with the same 6′-chiral carbon as is present in gentamicins C2a and C2.[11] In this study, as expected, in-frame deletion within *gen*P, blocking the gentamicin pathway at JI-20A and JI-20B, resulted in the generation of the pair of epimers JI-20Ba and JI-20B (Scheme 1). The analysis of metabolites produced by mutant strains generated by single or double in-frame deletions in the *gen*B1, *gen*B2, and *gen*P genes, together with the results of in vivo biotransformation experiments and in vitro enzymatic reactions, suggest a parallel biosynthetic pathway for the epimer pair C2a/C2, having branched at G418 (Scheme 1). JI-20Ba, containing a 6′*S* chiral amine, is a precursor of C2a and was synthesized from G418 by GenQ/GenB1 through sequential oxidation/transamination at C-6′. JI-20B, containing a 6′*R* chiral amine, is a precursor of C2 and was synthesized from G418 by GenQ/GenB2, whereas GenB2 catalyzed the epimerization of JI-20Ba/JI-20B and of C2a/C2.

Results

In-frame deletion of *gen*P in *M. echinospora* resulted in the synthesis of JI-20A, JI-20B, and epimer JI-20Ba

GenP catalyzes 3′-OH phosphorylation of JI-20A. This is possibly the first step of 3′,4′-bisdehydroxylation in gentamicin biosynthesis.[13] When we introduced an in-frame deletion in *gen*P of the *M. echinospora* wild-type (WT) strain, the phenotypic characteristics of the mutant Δ*gen*P strain, including colony color and shape, mycelium morphology, and growth rates, were not changed. Metabolites in the medium were then purified, concentrated, and subjected to HPLC analysis to assess gentamicin production. Unlike in the case of the WT strain, the gentamicin C complex was undetectable in the culture medium of the Δ*gen*P strain; however, three new peaks that

were eluted earlier than gentamicin C1a were detected (Figure 1 A). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis of the three products indicated that the first peak was JI-20A ($C_{19}H_{40}N_5O_9$, *m/z* 482.2823), and the molecular weights of the other two products were equal to those of JI-20B ($C_{20}H_{42}N_5O_9$, *m/z* 496.2979 and 496.2966; Figure 1 F). The chemical structures of the last two products—designated JI-20Ba and JI-20Bb—were then characterized by ^{1}H, ^{13}C, ^{1}H,^{1}H COSY, HSQC and NOESY NMR analyses (Figure 2, Table 1, and Figures S1–S10 in the Supporting Information).

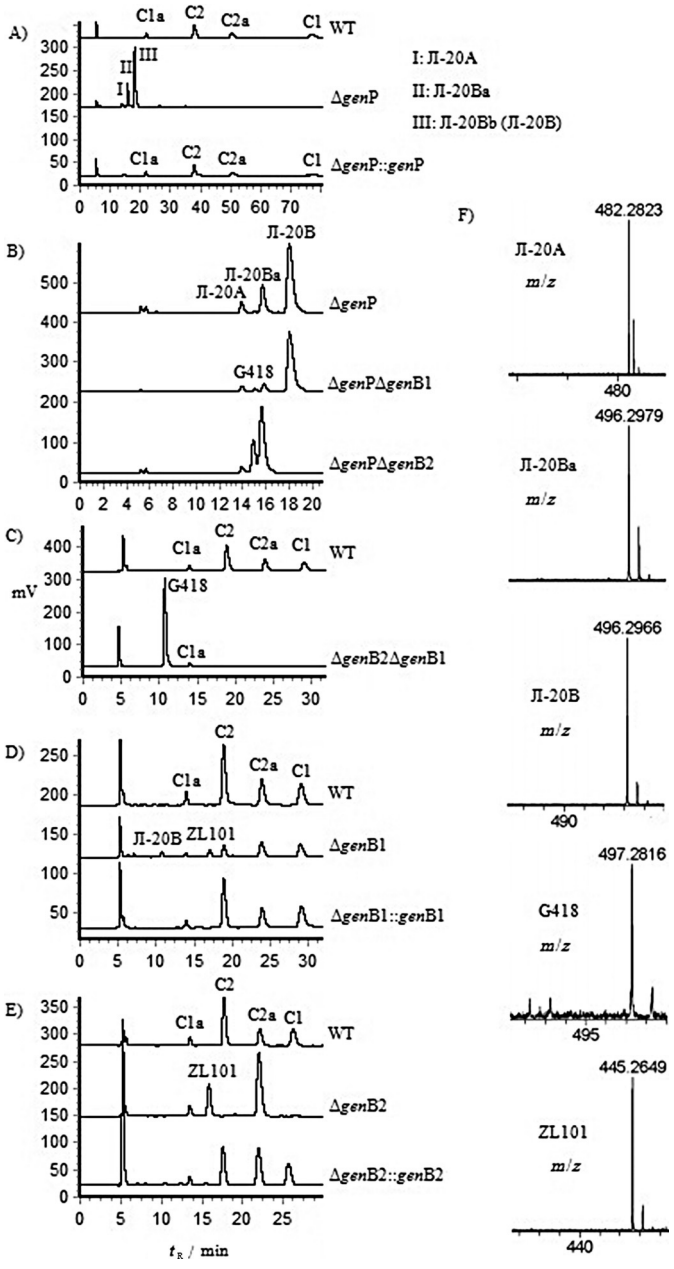


Figure 1. HPLC and HR-ESI-MS analyses of fermentation products generated by *M. echinospora* WT and mutant strains. A) Δ*gen*P and its complementation strain in comparison with the WT strain. B) Δ*gen*PΔ*gen*B1 and Δ*gen*PΔ*gen*B2 in comparison with Δ*gen*P. C) Δ*gen*B2Δ*gen*B1 in comparison with WT. D) Δ*gen*B1 and its complementation strain in comparison with WT. E) Δ*gen*B2 and its complementation strain in comparison with WT. F) Gentamicin-related intermediates produced by mutants Δ*gen*P (JI-20A, JI-20Ba, JI-20B), Δ*gen*B2Δ*gen*B1 (G418), and Δ*gen*B2 (ZL101).

The NMR spectra of compounds JI-20Ba and JI-20Bb were measured in deuterium oxide (D_2O); ^{1}H and ^{13}C NMR spectral data were assigned by analyses of ^{1}H, ^{13}C, ^{1}H,^{1}H COSY, and HSQC NMR spectra (Table 1). The methyl group linked to C-6′ in JI-20Ba demonstrated a chemical shift $\delta_C = 15.11$ ppm; the shift for C-5′ at $\delta_C = 78.43$ ppm was also observed. Meanwhile, spectral data for C6′-CH_3 and C-5′ in JI-20Bb were observed at $\delta_C = 11.31$ and 77.06 ppm.

Comparison of the NMR spectral data for JI-20Ba and JI-20Bb with those reported for C2a and C2,[14] together with analysis of HPLC and HR-ESI-MS data, indicated that compounds JI-20Ba and JI-20Bb were epimers with different configurations at C-6′.

The stereochemistry of JI-20Ba and JI-20Bb was analyzed on the basis of their NOESY spectra (Figure 2, Figures S5 and S10). The presence of key NOE correlations between C6′-CH_3 and H-3′ and H-5′ in the structure of JI-20Ba suggested the α-orientation of the methyl group, whereas NOE correlation between C6′-CH_3 and H-4′ in the structure of JI-20Bb suggested C6′-CH_3 in β-orientation. By the Cahn–Ingold–Prelog rule, we can thus confirm the absolute configurations of the 6′-chiral carbons in JI-20Ba and JI-20Bb as *S* and *R*, respectively.

To determine whether the in-frame deletion in the *gen*P gene influenced the functional activity of other genes and was responsible for metabolic changes in the mutant strain, we constructed the complementary pEAPP plasmid containing *gen*P. When pEAPP was introduced into the Δ*gen*P strain, the gentamicin C complex was synthesized, thus indicating that *gen*P encodes a key enzyme for the 3′,4′-bisdehydroxylation of JI-20A and JI-20B (Figure 1 A).

It was proposed that GenP catalyzes the first step of 3′,4′-bisdehydroxylation in gentamicin biosynthesis, because the deletion of *gen*P inhibited the gentamicin metabolic pathway at JI-20A and JI-20B. A previous study has reported the isolation of JI-20B and its epimer TPJ-B from *M. sagamiensis*.[15] JI-20Bb with the 6′*R* chiral amine corresponds to JI-20B, as was commonly thought. In this study, we have identified JI-20Ba with a 6′*S* chiral amine—the epimer of JI-20B—for the first time. Our findings indicate that epimerization occurs not only for gentamicins C2a and C2, but also for the intermediates.

Pyridoxal-phosphate-dependent aminotransferases GenB1 and GenB2 catalyze the biosynthesis of JI-20Ba and JI-20B, respectively

To examine the synthesis of JI-20Ba and JI-20B, *gen*B1 and *gen*B2 were separately deleted in the *gen*P mutant, and culture media were analyzed for gentamicin secretion. The phenotypic characteristics of the two obtained double mutants—Δ*gen*PΔ*gen*B1 and Δ*gen*PΔ*gen*B2—were not changed. According to HPLC analysis, the JI-20Ba and JI-20B content in the Δ*gen*PΔ*gen*B1 culture was 7.2 and 86.1%, respectively, in comparison with 22.5 and 74.1%, respectively, in the Δ*gen*P culture; JI-20B was not detected in the Δ*gen*PΔ*gen*B2 culture

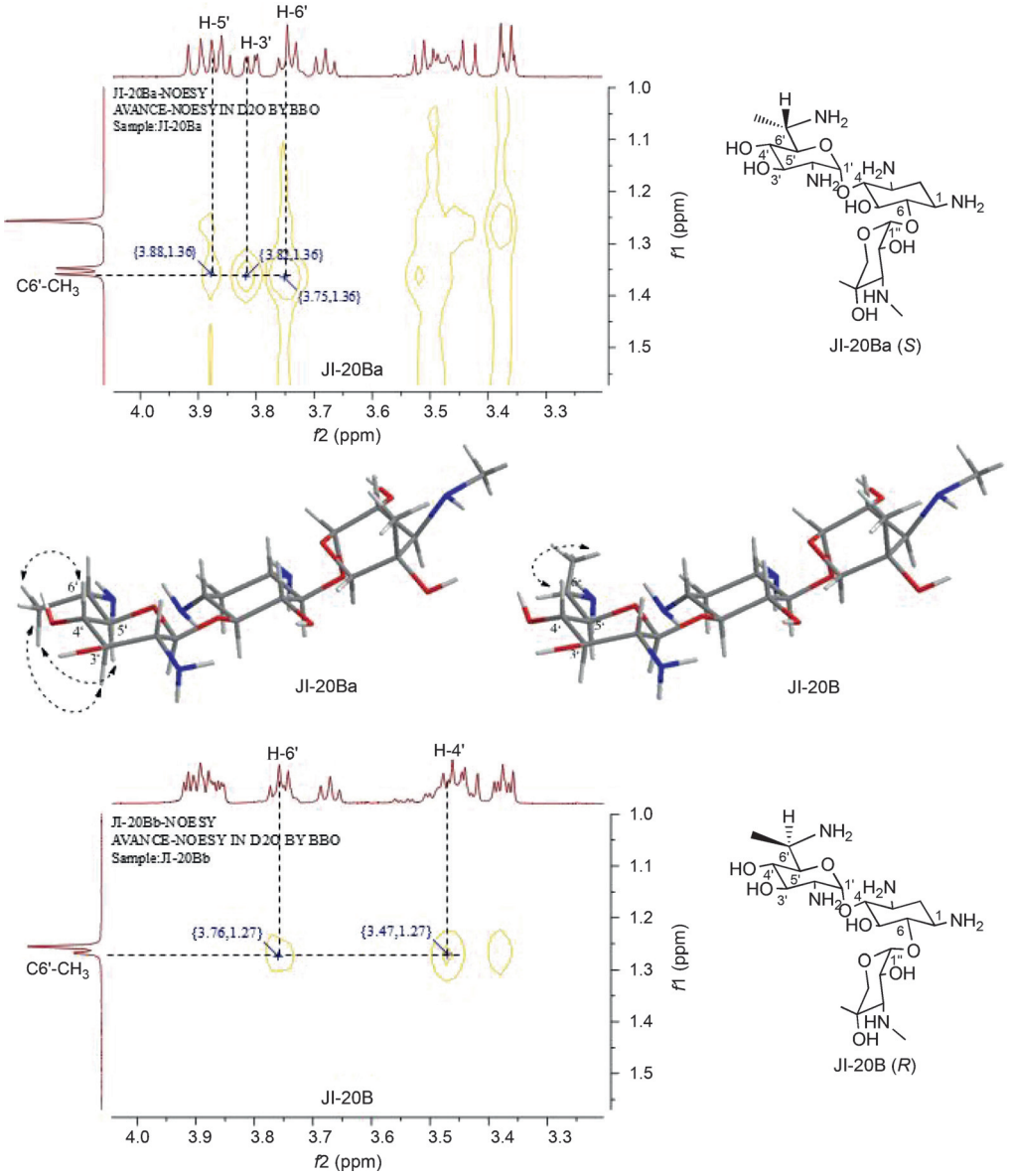


Figure 2. The key NOE correlations and structures of compounds JI-20Ba and JI-20B. NOE correlations of JI-20Ba and JI-20B. The chemical structures of JI-20Ba and JI-20B, also shown in Scheme 1, show α- and β-orientation, respectively, of C6′-CH_3.

medium (Figure 1 A, B and Table 2). A new peak appeared between JI-20A and JI-20Ba, and its identity was confirmed as G418. These results suggest that JI-20Ba was synthesized regardless of GenB1 expression. However, when GenB1 was inactive, the production of JI-20Ba decreased. JI-20B was synthesized from G418 only when GenB2 was expressed.

The effect of double deletion of *gen*B1 and *gen*B2 in the biosynthetic gene cluster was investigated. The phenotypic characteristics of the Δ*gen*B2Δ*gen*B1 mutant were not changed. HPLC analysis of gentamicin secretion in culture medium showed that the double mutant accumulated G418 and gentamicin C1a, but not C2a, C2, and C1 (Figure 1 C and F), thus indicating that the Δ*gen*B2Δ*gen*B1 strain synthesized G418 but not JI-20Ba or JI-20B. These results indicated that GenB1and GenB2 catalyzed the synthesis of JI-20B and its epimer JI-20Ba from G418. No other aminotransferases in *M. echinospora* were able to substitute the activity of GenB1 and GenB2 in this step. G418 is the common precursor of JI-20Ba and JI-20B.

The conversion of G418 to JI-20B (and JI-20Ba) is performed in a two-step oxidation/transamination process. GenQ is a flavin-dependent dehydrogenase that oxidizes G418 to 6′-dehydro-6′-oxo-G418.[11] Purified recombinant GenQ, GenB1, and GenB2 proteins (Figure S11) were used for enzymatic reactions in vitro. GenQ/GenB1 catalyzed the conversion of G418 into JI-20Ba, whereas GenQ/GenB2 catalyzed the conversion of G418 into JI-20B and JI-20Ba (Figure 3 A). Together with the results obtained by in vivo mutagenesis, these data indicate that GenB1 directly catalyzed the synthesis of JI-20Ba from G418. In contrast, the aminotransferase function of GenB2 appeared to be complex. In the oxidation reactions with GenQ, only G418 was detected as the reaction product. The dehydrogenation product of G418 is 6′-dehydro-6′-oxo-G418; it is unstable and

Table 1. ^{13}C NMR and ^{1}H NMR spectral data, proton splitting data, and the key NOE correlations for JI-20Ba and JI-20B in D_2O (600 MHz). No internal standard was used.

Site	JI-20Ba			JI-20B		
	δ_C (ppm)	δ_H (ppm), *J* [Hz]	NOESY	δ_C (ppm)	δ_H (ppm), *J* [Hz]	NOESY
C-1	49.57	3.49 (overlap.)		49.55	3.48 (overlap.)	
2	27.82	1.87 (q, *J* = 12.7 Hz, 1 H) 2.47 (dt, *J* = 4.2, 12.5 Hz, 1 H)		27.79	1.88 (q, *J* = 12.7 Hz, 1 H) 2.47 (dt, *J* = 4.0, 12.2 Hz, 1 H)	
3	48.41	3.49 (overlap.)		48.29	3.48 (overlap.)	
4	83.80	3.70 (t, *J* = 9.6 Hz, 1 H)		83.82	3.68 (t, *J* = 9.6 Hz, 1 H)	
5	68.66	3.86 (t, *J* = 9.6 Hz, 1 H)		68.21	3.91 (t, *J* = 9.6 Hz, 1 H)	
6	74.32	3.76 (t, *J* = 9.0 Hz, 1 H)		74.41	3.76 (t, *J* = 9.0 Hz, 1 H)	
C-1′	96.55	5.86 (d, *J* = 3.9 Hz, 1 H)		95.95	5.97 (d, *J* = 3.7 Hz, 1 H)	
2′	53.41	3.37 (dd, *J* = 3.0, 10.8 Hz, 1 H)		53.39	3.40 (dd, *J* = 4.2, 10.8 Hz, 1 H)	
3′	72.47	3.82 (dd, *J* = 2.4, 10.2 Hz, 1 H)	C-6′-CH_3	72.00	3.87 (dd, *J* = 2.7, 9.9 Hz, 1 H)	
4′	69.93	3.53 (t, *J* = 9.6 Hz, 1 H)		69.87	3.47 (t, *J* = 9.6 Hz, 1 H)	C-6′-CH_3
5′	78.43	3.88 (t, *J* = 8.7 Hz, 1 H)	C-6′-CH_3	77.06	3.88 (t, *J* = 10.2 Hz, 1 H)	
6′	47.21	3.75 (d, *J* = 9.0 Hz, 1 H)		47.43	3.76 (d, *J* = 9.0 Hz, 1 H)	
C-1″	101.45	5.00 (d, *J* = 4.2 Hz, 1 H)		101.42	5.00 (d, *J* = 3.6 Hz, 1 H)	
2″	66.26	4.15 (dd, *J* = 3.7, 10.9 Hz, 1 H)		66.21	4.16 (dd, *J* = 3.7, 10.9 Hz, 1 H)	
3″	63.39	3.38 (d, *J* = 10.8 Hz, 1 H)		63.33	3.38 (d, *J* = 10.8 Hz, 1 H)	
4″	69.84	–		69.82	–	
5″	68.00	3.46 (d, *J* = 12.6 Hz, 1 H) 3.92 (d, *J* = 12.6 Hz, 1 H)		67.87	3.45(d, *J* = 12.6 Hz, 1 H) 3.91 (d, *J* = 12.6 Hz, 1 H)	
C-6′-CH_3	15.11	1.36 (d, *J* = 7.2 Hz, 3 H)	H-3′, H-5′	11.31	1.27 (d, *J* = 7.2 Hz, 3 H)	H-4′
C-3″-*N*-CH_3	34.52	2.83 (s, 3 H)		34.46	2.83 (s, 3 H)	
C-4″-CH_3	20.96	1.26 (s, 3 H)		20.90	1.26 (s, 3 H)	

Table 2. Percentage contents of the gentamicin C complex and its intermediates in cultures of *M. echinospora* wild-type and mutant strains.

Strain	Content [%]								
	JI-20A	G418	JI-20Ba	JI-20B	ZL101	C1a	C2	C2a	C1
WT[a]	n.d.	n.d.	n.d.	n.d.	n.d.	5.8	42.7	24.3	27.2
Δ*gen*P::*gen*P[a]	n.d.	n.d.	n.d.	n.d.	n.d.	6.9	44.8	23.1	25.2
Δ*gen*P[a,b]	3.4	n.d.	22.5	74.1	n.d.	n.d.	n.d.	n.d.	n.d.
Δ*gen*PΔ*gen*B1[b]	4.3	2.4	7.2	86.1	n.d.	n.d.	n.d.	n.d.	n.d.
Δ*gen*PΔ*gen*B2[b]	4.4	24.6	71.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WT[c]	n.d.	n.d.	n.d.	n.d.	n.d.	6.4	45.1	24.0	24.5
Δ*gen*B1[c]	n.d.	n.d.	n.d.	6.0	9.7	7.4	20.9	30.6	25.4
Δ*gen*B1::*gen*B1[c]	n.d.	n.d.	n.d.	n.d.	n.d.	6.9	44.8	24.1	24.2
WT[d]	n.d.	n.d.	n.d.	n.d.	n.d.	6.4	45.3	24.6	23.7
Δ*gen*B2[d]	n.d.	n.d.	n.d.	n.d.	29.4	6.9	n.d.	63.7	n.d.
Δ*gen*B2::*gen*B2[d]	n.d.	n.d.	n.d.	n.d.	n.d.	6.9	33.8	38.8	20.4

[a] The percentage of each composition was calculated from the HPLC-ELSD analysis shown in Figure 1 A by area normalization. [b] The percentage of each composition was calculated from the HPLC-ELSD analysis shown in Figure 1 B by area normalization. [c] The percentage of each composition was calculated from the HPLC-ELSD analysis shown in Figure 1 D by area normalization. [d] The percentage of each composition was calculated from the HPLC-ELSD analysis shown in Figure 1 E by area normalization. n.d.: not detected.

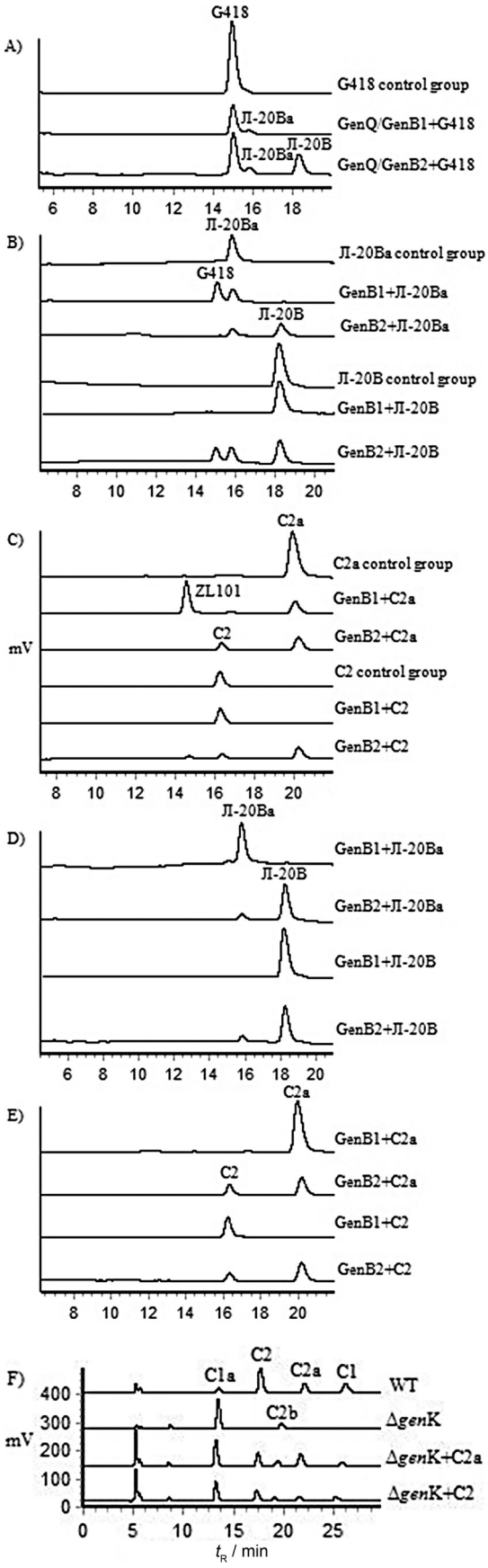
would be reduced to G418. Without coenzyme or amino group donor, the reaction did not occur (data not shown).

Given that Neo18, a 6′-oxoglucosaminyl:L-glutamate aminotransferase encoded in the neomycin gene cluster, catalyzes the amination/deamination of C-6‴ in neomycin,[16] it is reasonable to assume that the C6′-NH_2 groups of JI-20Ba or JI-20B were synthesized and degraded by the same aminotransferase.

We then determined the substrate specificity of GenB2 with regard to JI-20Ba and JI-20B (Figure 3 B), by using α-ketoglutarate, pyruvic acid, and oxaloacetic acid as amino group acceptors, and got similar results. GenB1 catalyzed the deamination of JI-20Ba to afford G418, but JI-20B was not a substrate; however, GenB2 catalyzed the conversion of JI-20Ba into JI-20B but not into G418, and that of JI-20B into both JI-20Ba and G418. The substrates of Neo18 are 6′-dehydro-6′-oxoparomamine and 6‴-deamino-6‴-dehydro-6‴-oxoneomycin.[16] If the deamination product of JI-20B were 6′-dehydro-6′-oxo-G418, it would be unstable and reduced to G418. Without amino group acceptor or coenzyme in the deamination reaction of JI-20Ba/JI-20B, only the substrate was detected (data not shown). These data suggest that GenB2 is the aminotransferase required for JI-20B synthesis and its deamination to afford G418. GenB2 does not act as aminotransferase on JI-20Ba.

GenB2 has epimerase activity and catalyzes the interconversion between JI-20Ba and JI-20B

In previous experiments, JI-20Ba was generated by GenB2 activity both in vitro and in vivo. In vitro, GenB2 catalyzed interconversion between JI-20Ba and JI-20B; this indicates that GenB2 possessed epimerase activity with the equilibrium shifted from JI-20Ba to JI-20B (Figure 3 D). The same experiments were performed with GenB1, but no epimerase activity was detected. Without the enzyme and coenzyme, only the substrate was detected in the attempted epimerization reaction of JI-20Ba/JI-20B (data not shown).



GenB2 also catalyzes the epimerization of gentamicins C2a and C2

To allow further examination of the functions of GenB1 and GenB2 in the biosynthesis of C2a and C2, *gen*B1 and *gen*B2 were separately knocked out by in-frame deletion in the WT strain. The phenotypic characteristics of the mutants Δ*gen*B1 and Δ*gen*B2 were not changed. The Δ*gen*B1 mutant generated C2a and C2 in yields of 30.6 and 20.9%, in comparison with 24.0 and 45.1%, respectively, in the WT strain (Table 2), and also synthesized JI-20B and a new product designated ZL101 (Figure 1D). Δ*gen*B2 produced C1a and C2a along with ZL101, whereas C1 and C2 were undetectable (Figure 1E). Mutant strains Δ*gen*B1, Δ*gen*B2, and Δ*gen*B2Δ*gen*B1 all produced C1a; this was consistent with a recent study on the functions of GenB1 and GenB2 in the gentamicin biosynthetic pathway,[11] but we did not delve into the functions of GenB1 and GenB2 in gentamicin C1a biosynthesis.

Complementation experiments were also performed. The introduction of pEAPB1 into the Δ*gen*B1 mutant restored the production nearly to the WT levels, whereas the introduction of pEAPB2 into the Δ*gen*B2 mutant led to the production of gentamicins C1 and C2; this strongly supports a critical role for GenB2 in the biosynthesis of C1 and C2 (Figure 1D and E). The introduction of pEAPB1 or pEAPB2 into the WT strain did not affect gentamicin production (data not shown). In the complementation experiments, the production was not restored to the WT levels, because the genes were expressed under the control of the *PermE** promoter, which was likely to result in reduced expression.

These data indicate that GenB2 was essential for gentamicin C2 biosynthesis and that C2 and C2a were generated when GenB2 was expressed. Gentamicin C1 was produced from gentamicin C2 by methylation of C6′-NH_2, which is consistent with the results of a previous study.[14]

Figure 3. HPLC analyses of enzymatic products in vitro and biotransformation products in vivo. A) Aminotransferase activity of GenB1 and GenB2 in vitro. JI-20Ba was formed from G418 by the coupled reaction of GenQ/GenB1, whereas JI-20Ba and JI-20B were both formed by the coupled reaction of GenQ/GenB2. JI-20Ba in the second reaction was formed from JI-20B by GenB2. B) Deamination activity of GenB1 and GenB2 with regard to JI-20Ba and JI-20B; G418 is the deaminated product. GenB1 deaminated JI-20Ba but not JI-20B; GenB2 deaminated JI-20B but not JI-20Ba. JI-20B in the GenB2 + JI-20Ba reaction mixture and JI-20Ba in the GenB2 + JI-20B reaction mixture were epimerization products of GenB2. C) Deamination activity of GenB1 and GenB2 with regard to C2a and C2; ZL101 is the deaminated product. GenB1 deaminated C2a but not C2, and GenB2 deaminated C2 but not C2a. Gentamicin C2 in the GenB2 + C2a reaction mixture and C2a in the GenB2 + C2 reaction mixture were epimerization products of GenB2. D) Epimerization activity of GenB1 and GenB2 with regard to JI-20Ba and JI-20B. GenB2 catalyzed the epimerization of both substrates and formed a mixture of JI-20Ba and JI-20B with prevalence of JI-20B. GenB1 did not demonstrate any epimerase activity. E) Epimerization activity of GenB1 and GenB2 with regard to C2a and C2. GenB2 catalyzed the epimerization of both substrates and formed a mixture of C2a and C2 with prevalence of C2a. GenB1 did not demonstrate any epimerase activity. In control groups, enzyme(s) were not added to the reaction mixture. F) Biotransformation of C2a by the Δ*gen*K strain (C1a 35.67%, C2 22.52%, C2b 9.52%, C2a 24.23%, and C1 8.06%) and of C2 by the Δ*gen*K strain (C1a 36.37%, C2 31.22%, C2b 9.24%, C2a 13.86%, and C1 9.31%). The relative amount of each peak product was calculated by the area normalization method.

Biotransformation experiments were performed with a Δ*gen*K mutant characterized by high levels of gentamicin C1a and low levels of gentamicin C2b[17] to validate the interconversion between C2a and C2 in vivo. When GenB2 enzymatic activity was assessed in the Δ*gen*K mutant, we observed that C2a was converted into C2 and C1, whereas C2 was converted into C2a and C1 (Figure 3F), thus indicating that the epimerization of C2a to afford C2 is a reversible reaction.

In the enzymatic reactions in vitro, GenB2 catalyzed interconversion between C2a and C2; this indicated epimerase activity (Figure 3E).[11] The same experiments were performed with GenB1, but no epimerase activity was detected. Without the enzyme and coenzyme, only the substrate was detected in the attempted epimerization reaction of C2a/C2 (data not shown).

ZL101 is the deaminated product of gentamicins C2a and C2

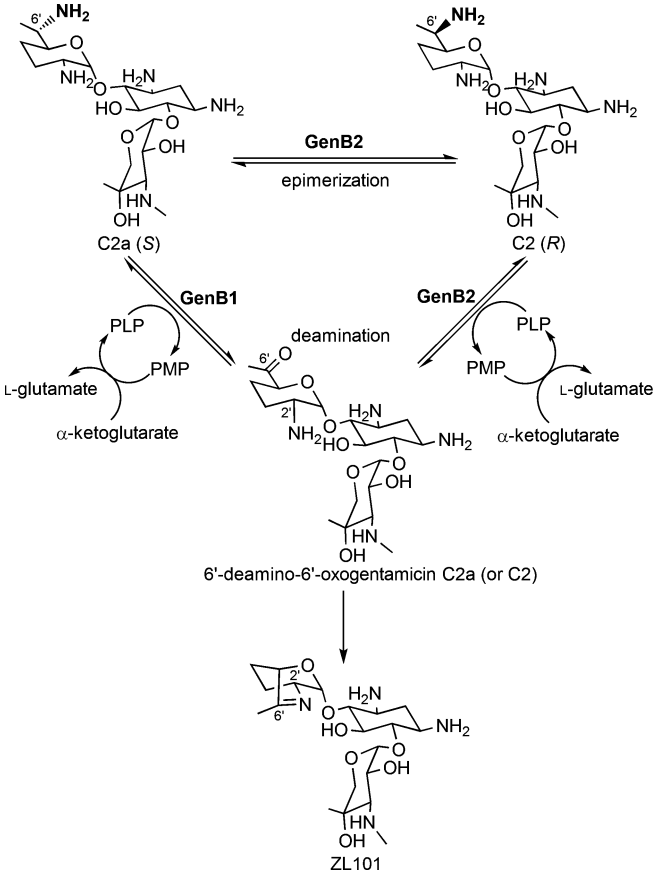
We tested deamination of C2a and C2 by GenB1 and GenB2 (when α-ketoglutarate, pyruvic acid, and oxaloacetic acid were used as amino-acceptors, identical results were obtained). GenB1 converted C2a into ZL101 but did not demonstrate any detectable reaction with C2. GenB2 catalyzed the conversion of C2a into C2 but not to ZL101, and that of C2 into both C2a and ZL101 (Figure 3C). Thus, GenB2 catalyzes the epimerization between gentamicins C2a and C2. GenB1 and GenB2 can only catalyze the deamination of C2a and C2, respectively, thus suggesting that ZL101 is the product of deamination of C2a and C2. Without amino acceptor, or coenzyme, only the substrate was detected in the attempted deamination reaction of gentamicins C2a/C2 (data not shown).

The molecular weight of ZL101 was 444, according to the results of low-resolution (LR) ESI-MS analysis (Figure S12). The peak at *m/z* 322, which indicated the loss of the purpurosamine ring, further indicated that ZL101 was a gentamicin analogue.[18,19] A minor component of the gentamicin complex with the same molecular weight ($C_{20}H_{36}N_4O_7$) had been discovered by Bérdy et al. (1977).[20] In the present study, HR-ESI-MS analysis of ZL101 (Figure 1F) indicated that the *m/z* value of the protonated molecule (445.2649) is consistent with the molecular formula.

In the ^{1}H NMR spectrum of ZL101 (Figure S13), resonances at $\delta = 5.13$ (m, 1H), 4.94 (d, $J = 3.6$ Hz, 1H), 4.39 (d, $J = 2.4$ Hz, 1H), 4.23 (m, 1H), 2.67 (s, 3H), and 1.16 ppm (s, 3H) were characteristic of protons 1′ (anomeric proton of the purpurosamine part), 1″ (anomeric proton of the garosamine part), 5′-, 2″-, 3″-*N*-CH_3, and 4″-*C*-CH_3 groups of the garosamine part. A multiplet at $\delta = 1.53$ ppm was characteristic of H-2_{eq} in the 2-deoxystreptamine ring. Because there is no proton at 6′-C, a 6′-*C*-CH_3 singlet at δ 1.75 ppm was not split as in JI-20Ba and JI-20B. The results of HR-ESI-MS together with the data on fragment peaks obtained by LR-ESI-MS confirmed the identity of ZL101 as compound I-1 discovered by Bérdy et al.[20]

We therefore hypothesized that the direct deamination product of gentamicins C2a and C2 was 6′-deamino-6′-oxogentamicin C2a (or C2), which is unstable and may readily undergo reaction between the 6′-carbonyl group and 2′-amino group, resulting in the loss of one water molecule and the generation of ZL101. Thus, ZL101 is a stable Schiff base, which was not metabolized (Scheme 2).

On the basis of these findings, we propose a new parallel biosynthetic pathway for the epimer pair gentamicins C2a/C2, which is branched at G418 and regulated by GenB1 and GenB2, enzymes with differential activity, substrate specificity, and enantioselectivity. The PLP-dependent aminotransferases GenB1 and GenB2 catalyze C-6′-amination in JI-20Ba and JI-20B, respectively, and these are then converted into C2a and C2, respectively, after 3′,4′-bisdehydroxylation. GenB2 also acts as an epimerase for the pairs JI-20Ba/JI-20B and gentamicins C2a/C2.



Scheme 2. Proposed synthesis of ZL101. The direct deamination product of gentamicins C2a and C2 was 6′-deamino-6′-oxogentamicin C2a (or C2). ZL101 is a stable Schiff base, a product of intramolecular condensation of 6′-deamino-6′-oxogentamicin C2a (or C2).

Discussion

Although the gentamicin biosynthesis gene cluster in *M. echinospora* ATCC15835 was defined in 2004, the detailed synthetic mechanism for gentamicin epimers C2a and C2 remained to be determined. Here, we have revealed a new biosynthetic pathway for these epimers that branches off at G418; it involves the modification of the 6′-chiral carbon and is mediated by GenB1 and GenB2 with differential functional activities

(Schemes 1 and 2), thus suggesting that homologous enzymes in the same pathway can possess significantly different catalytic properties.

This biosynthetic pathway for gentamicins C2a, C2, and C1, containing 6′-chiral carbon, branches at G418. JI-20Ba and JI-20B are synthesized simultaneously from 6′-dehydro-6′-oxo-G418. GenB1 is an *S*-selective transaminase that generates JI-20Ba, containing a 6′*S* chiral amine.[21–24] This is then converted into gentamicin C2a after 3′,4′-bisdehydroxylation. GenB2 is an *R*-selective transaminase generating JI-20B, containing the 6′*R* chiral amine, and this is then converted into gentamicin C2 after 3′,4′-bisdehydroxylation (Scheme 1). Furthermore, GenB2 catalyzes the epimerization of JI-20Ba/JI-20B and gentamicins C2a/C2, which favors the formation of JI-20B and gentamicin C2a with the 6′*R* or 6′*S* conformation, respectively.

The double mutant Δ*gen*B2Δ*gen*B1 accumulates G418, and this indicates that only the GenB1 and GenB2 aminotransferases can catalyze G418 transamination. In the double mutant Δ*gen*PΔ*gen*B1, G418 was first converted into JI-20B by GenQ and GenB2, and then a small amount of JI-20Ba was generated from JI-20B by the epimerase GenB2. In the double mutant Δ*gen*PΔ*gen*B2, G418 was converted only into JI-20Ba by GenQ and GenB1. The presence of G418 in Δ*gen*PΔ*gen*B1 and Δ*gen*PΔ*gen*B2 cultures can be explained by the deficiency in GenB1 and GenB2 activity, leading to incomplete conversion of G418 into JI-20Ba or JI-20B.

In the single mutant Δ*gen*B1, small amounts of JI-20Ba and large amounts of JI-20B were converted into C2a and C2, respectively, after 3′,4′-bisdehydroxylation. The epimerase GenB2 then performed interconversion between C2a and C2, favoring C2a. Gentamicin C2 was deaminated to produce ZL101 by GenB2. JI-20B was not completely converted into C2 and accumulated in the reaction mixture. In the single mutant Δ*gen*B2, JI-20Ba produced by GenB1 was converted into C2a after 3′,4′-bisdehydroxylation, and this was then deaminated by GenB1 to afford ZL101.

Although GenB2 is a bifunctional enzyme, it might not catalyze both transamination and epimerization simultaneously in vitro. Figure 3B and C shows that when the substrate can be deaminated and isomerized by GenB2 (e.g., JI-20B and C2), both reactions occur at the same time; however, when the substrate can only be isomerized by GenB2 (e.g., JI-20Ba and C2a), the enzyme solely displays isomerase activity. The explanation might be that GenB2 participation in the isomerization reaction shifts the dynamic balance, causing conformation changes in the active site responsible for deamination, so this cannot then take place even if JI-20B and C2 are generated.

Our findings differ somewhat from another recent study on GenB1 and GenB2 functions in the gentamicin biosynthetic pathway.[11] In the previous study, the results were conflicting. On the one hand, gentamicin C2a formed from JI-20B was found in the double mutant Δ*gen*B2Δ*gen*B1, whereas G418 could not be converted into JI-20B by GenB3 and GenB4 in vitro, thus suggesting that an aminotransferase other than GenB1–GenB4 participated in JI-20B generation. On the other hand, the quadruple mutant Δ*gen*B4Δ*gen*B1Δ*gen*B3Δ*gen*B2 was unable to produce JI-20B, thus suggesting that no other aminotransferases could catalyze this step. In our study, the Δ*gen*B2Δ*gen*B1 mutant accumulated G418 but not JI-20B, thus confirming that in *M. echinospora*, no aminotransferases other than GenB1 and GenB2 could catalyze this step. Although JI-20Ba and JI-20B are epimers and show the same peak in HPLC, we separated the two compounds by changing the mobile phase, to prolong the retention times. GenB1 catalyzed the transamination of JI-20Ba but not JI-20B. JI-20B was generated only when GenB2 was expressed, and GenB2 could also catalyze the epimerization of JI-20Ba and JI-20B. JI-20B is probably another precursor of gentamicin C2.

Conclusion

This study has described a new parallel branch of the gentamicin C2/C2a biosynthetic pathway starting at G418, and has characterized two aminotransferases—GenB1 and GenB2—with different activity, substrate specificity, and enantioselectivity involved in this pathway. We have also identified JI-20Ba, the epimer of JI-20B, for the first time. G418 is the common precursor of JI-20Ba and JI-20B. JI-20Ba is a precursor for gentamicin C2a biosynthesis, and JI-20B is a precursor for gentamicin C2 biosynthesis. The C6′-amino groups of JI-20Ba and JI-20B are formed by aminotransferases GenB1 and GenB2 respectively. GenB2 is a bifunctional enzyme, also possessing epimerase activity, and catalyzes the epimerization of JI-20Ba/JI-20B and C2a/C2.

Experimental Section

Bacterial strains and culture conditions: Bacterial strains used in the study are listed in Table S2. Wild-type and mutant *M. echinospora* ATCC15835 strains were maintained on solid slanting medium (SSM): soluble starch (1%), $MgSO_4{\cdot}7H_2O$ (0.05%), KNO_3 (0.1%), NaCl (0.05%), asparagine (0.002%), $CaCO_3$ (0.1%), wheat bran (1.0%), $K_2HPO_4{\cdot}3H_2O$ (0.03%), agar (1.5%), pH 7.5 at 37 °C. To isolate genomic DNA, the strains were cultivated in ATCC172 liquid medium for three days. *Escherichia coli* strains were cultured at 37 °C in lysogeny broth (LB) liquid or agar medium supplemented with apramycin (50 μg mL^{-1}), ampicillin (100 μg mL^{-1}), erythromycin (100 μg mL^{-1}), chloromycetin (25 μg mL^{-1}), or kanamycin (25 μg mL^{-1}), as required. *E. coli* DH5α served as the host for molecular cloning. Intergeneric conjugal transfer on MS agar was conducted with *E. coli* ET 12567 (pUZ8002) as the donor, and 2×YT medium was used for conjugation.

Gentamicin was produced by *M. echinospora* strains by a two-stage fermentation. The seed culture was maintained in a 250 mL flask containing seed medium (30 mL): soluble starch (1.0%), soybean powder (1.5%), glucose (0.1%), KNO_3 (0.05%), $CaCO_3$ (0.3%), pH 6.8 at 34 °C, 220 rpm, for 36 h. The culture was diluted 1:10 (*v*/*v*) in fermentation medium (30 mL): soluble starch (5.0%), soy bean powder (3.5%), $(NH_4)_2SO_4$ (0.05%), peptone (0.2%), glucose (1.5%), KNO_3 (0.05%), $CaCO_3$ (0.6%), $CoCl_2{\cdot}6H_2O$ (0.001%), corn starch (1.5%), pH 7.5 and incubated in a 250 mL flask at 34 °C, 220 rpm, for five days.

Construction of gene disruption plasmids: Primers and plasmids used in this study are listed in Table S1. The pD2925Δ*gen*B1plasmid containing *gen*B1 with in-frame deletion was constructed as follows: B11 and B12 fragments were amplified by PCR from WT

chromosomal DNA by use of primer pairs P11/P12 and P13/P14, respectively, and sequentially cloned into the pIJ2925 plasmid; the B11–B12 fragment was then inserted into the suicide shuttle vector pD2925. The pKC1139Δ*gen*B2 vector containing *gen*B2 with in-frame deletion was constructed by inserting two *gen*B2-flanking DNA fragments into the pKC1139 temperature-sensitive shuttle vector. B21 and B22 fragments were amplified by PCR with use of primer pairs P21/P22 and P23/P24 and sequentially cloned into the pKC1139 plasmid. The pKC1139Δ*gen*P vector containing *gen*P with in-frame deletion was constructed as follows: I1 and I2 fragments were amplified by use of primer pairs P1/P2 and P3/P4, respectively, and sequentially cloned into the pIJ2925 vector; the I1–I2 fragment was then inserted into the pKC1139 plasmid. All plasmids were verified by sequencing.

Construction of gene deletion mutants: *E. coli* ET12567 (pUZ8002) was transformed with the pD2925Δ*gen*B1, pKC1139Δ*gen*B2, and pKC1139Δ*gen*P plasmids. The disruption plasmids were introduced into *M. echinospora* through conjugal transfer from *E. coli* on MS agar at 28 °C for 24 h, as described previously.[25] After overlaying of agar with apramycin (50 μg mL^{-1}) and pipemidic acid (PPA, 50 μg mL^{-1}), incubation was continued for eight days at 28 °C. Single-crossover recombinant colonies were first selected for apramycin resistance and were then cultivated on SSM plates without antibiotic at 37 °C. The apramycin-sensitive strains were confirmed by PCR with appropriate primers (Figure S14) and by sequencing.

The disruption plasmid pD2925Δ*gen*B1 was introduced into the Δ*gen*B2 and Δ*gen*P strains through conjugal transfer to obtain double mutants Δ*gen*B2Δ*gen*B1 and Δ*gen*PΔ*gen*B1. The disruption plasmid pKC1139Δ*gen*B2 was introduced into the Δ*gen*P strain through conjugal transfer to obtain the double mutant Δ*gen*PΔ*gen*B2 strain. The genomic DNA of the double mutants was analyzed by PCR with use of two primer pairs (Figure S14).

Complementation of single mutants: The pSPU241 and pEAP1 plasmids were used to construct gene complementation vectors. The *gen*B1, *gen*B2, and *gen*P genes were amplified from WT chromosomal DNA by use of primer pairs CB1–1/exB1–2, CB2–1/exB2–2, and CP-1/CP-2, respectively, and PCR products were inserted into the BamHI and HindIII sites of pSPU241. The cloned genes containing the *PermE** promoter and *To* terminator were digested with BglII and inserted into pEAP1 to generate pEAPB1, pEAPB2, and pEAPP. The complementation plasmids were verified by sequencing and then introduced individually into Δ*gen*B1, Δ*gen*B2, Δ*gen*P by conjugation. The plasmids were integrated into *M. echinospora* chromosomal DNA by site-specific recombination. Complemented exconjugants were identified by erythromycin resistance and confirmed by PCR (Figure S15).

Extraction, purification, separation, and analysis of metabolites from *M. echinospora* strains: Fermentation cultures were adjusted to pH 2.0 with H_2SO_4 (2 M) and agitated for 30 min. After centrifugation at 1600 *g* for 10 min at 4 °C, the supernatant was neutralized with NaOH (2 M). Antibiotics were isolated from the fermentation broth by ion-exchange chromatography with 732 NH_4^+ ion-exchange resin. The anion-exchange column was washed with double-distilled (dd) H_2O until the effluent was colorless, and bound antibiotics were then sequentially eluted with eight column volumes of HCl (0.4 M), NH_4Cl (0.4 M), a mixture of HCl (0.2 M) and NH_4Cl (0.62 M), and again with ddH_2O. The eluates were then passed through 711 H^+ ion-exchange resin for discoloration, and antibiotics were eluted with six column volumes of NH_4Cl (5%). The fractions with the activity against *B. subtilis* were concentrated by reduced pressure distillation, lyophilized, resuspended in ddH_2O, filtered through a 0.22 μm filter, and analyzed by HPLC performed with an Ultimate LP-C18 column (4.6×250 mm; Welch Materials, Inc., Maryland, USA) connected to an evaporative light scattering detector. The mobile phase was trifluoroacetic acid (0.2 M)/methanol (92:8 or 98:2, *v/v*) used at a flow rate of 0.6 mL min^{-1}. Production yields for all strains were calculated with the aid of an external standard. The contents of the components were determined by area normalization with gentamicin C1a as the standard. Separation of the components was performed by dissolving lyophilized gentamicin samples in ddH_2O and loading them onto a D152 NH_4^+ ion-exchange column; elution with 0.01–2 M gradient NH_4Cl was monitored by assaying the eluted fractions for anti-*B. subtilis* activity. Fractions containing single components were combined, concentrated by reduced pressure distillation, lyophilized, and analyzed by MS and NMR.

NMR analysis: All experiments were performed with a Bruker Avance 600 MHz NMR spectrometer operating at 600.13 MHz (^{1}H) and 150.90 MHz (^{13}C) and equipped with the XWIN-NMR software, version 3.0 (Bruker Analytik, Rheinstetten, Germany). For ^{1}H, ^{13}C, ^{1}H,^{1}H COSY, HSQC, and NOESY NMR analyses, JI-20Ba (20 mg) or JI-20B (20 mg) was dissolved in D_2O (500 μL); for ^{1}H NMR analysis, ZL101 (5 mg) was dissolved in D_2O (500 μL).

Biotransformation experiments: The Δ*gen*K mutant was cultured in fermentation medium for 96 h, intermediates of the gentamicin pathway were then added at the final concentration of 1 mg mL^{-1}, and the culture was further incubated for 144 h at 34 °C. Each experiment was repeated three times. Product extraction, purification, and analysis were performed as described above.

Cloning, overexpression, and purification of GenQ, GenB1, and GenB2: The *gen*B1, *gen*B2, and *gen*Q genes were amplified from chromosomal DNA of the WT strain by PCR with use of primer pairs exB1–1/exB1–2, exB2–1/exB2–2, and exQ-1/exQ-2, respectively. PCR products inserted into the NcoI and HindIII sites of the pET32a (+) vector were verified by nucleotide sequencing, and the recombinant and empty plasmids were used to transform *E. coli* BL21 (DE3). The recombinant strain BL21-pET32a was used for blank control.

Transformed bacteria were cultured at 37 °C in LB liquid medium supplemented with ampicillin (100 μg mL^{-1}) until the optical density at 600 nm was 0.4–0.6. Protein expression was induced with IPTG (0.2 mM) at 28 °C for 6 h. The bacteria were harvested by centrifugation, resuspended in binding buffer [20 mL, Tris·HCl (20 mM), NaCl (0.5 M), imidazole (5 mM), pH 8.0], and disrupted by sonication for 10 min with use of a 3 s-on/5 s-off cycle. After centrifugation at 15480 *g* at 4 °C for 10 min, the supernatant was loaded into Ni-Sepharose 6 Fast Flow (GE Healthcare), the column was washed with washing buffer [Tris·HCl (20 mM), NaCl (0.5 M), imidazole (10–50 mM), pH 8.0], and His-tagged recombinant proteins were eluted with elution buffer [Tris·HCl (20 mM), NaCl (0.5 M), imidazole (200 mM), pH 8.0].

The purified enzymes were concentrated and dialyzed against Tris·HCl (pH 8.0, 20 mM) containing KCl (50 mM) and glycerol (10%) and stored at −20 °C. Purified proteins demonstrated single bands in SDS-PAGE gels, and these were in agreement with the calculated molecular weight (MWs) of 63.5, 63.2, and 73.4 KDa (Figure S11). The blank control strain did not produce target proteins.

Enzymatic assays: The GenQ/GenB1 and GenQ/GenB2 coupled assay was performed in Tris·HCl (pH 8.0, 20 mM) containing purified GenQ (30–50 μg), GenB1 (30–50 μg) or GenB2 (30–50 μg),

G418 (0.4 mM), L-glutamate (2 mM) as a donor amino acid, FAD (0.8 mM), and PLP (0.4 mM) at 37 °C overnight.

GenB1 and GenB2 deamination was performed in Tris·HCl (pH 8.0, 20 mM) containing gentamicins C2 (0.4 mM) and C2a (0.4 mM), JI-20Ba (0.4 mM) or JI-20B (0.4 mM), purified GenB1 (30–50 μg) or GenB2 (30–50 μg), PLP (0.4 mM), and α-ketoglutarate (2 mM), pyruvic acid (2 mM), or oxaloacetic acid (2 mM) as amino group acceptors, at 37 °C overnight.

The epimerization assay was performed in Tris·HCl (pH 8.0, 20 mM) containing gentamicins C2 (0.4 mM) and C2a (0.4 mM), JI-20Ba (0.4 mM) or JI-20B (0.4 mM), purified GenB1 (30–50 μg) or GenB2 (30–50 μg), and PLP (0.4 mM), at 37 °C overnight.

In all assays, chloroform was added to the reaction mixtures to quench enzymatic reactions followed by centrifugation. The supernatants were analyzed by HPLC.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81273410) and the Specialized Research Fund for the Doctoral Program of Higher Education (20122134130001).

The authors declare no conflict of interest.

Keywords: aminoglycosides · antibiotics · biosynthesis · epimers · stereoisomerization · transamination

[1] M. A. Weinstein, G. M. Luedemann, E. M. Oden, G. H. Wagman, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog, J. Black, *J. Med. Chem.* **1964**, *7*, 122, 463 – 464.
[2] M. Hainrichson, I. Nudelman, T. Baasov, *Org. Biomol. Chem.* **2008**, *6*, 227 – 239.
[3] G. H. Wagman, M. J. Weinstein, *Annu. Rev. Microbiol.* **1980**, *34*, 537 – 557.
[4] H. Marybeth, A. F. Raymond, M. B. David, *Nat. Med.* **1996**, *2*, 467 – 469.
[5] M. Wilschanski, Y. Yahav, Y. Yaacov, H. Blau, L. Bentur, J. Rivlin, M. Aviram, T. Bdolah-Abram, Z. Bebok, L. Shushi, B. Kerem, E. Kerem, *N. Engl. J. Med.* **2003**, *349*, 1433 – 1441.
[6] R. G. Ridley, *Nat. Med.* **2004**, *10*, S137 – S140.
[7] S. B. Levy, B. Marshall, *Nat. Med.* **2004**, *10*, S122 – S129.
[8] R. M. Sandoval, J. P. Reilly, W. Running, S. B. Campos, J. R. Santos, C. L. Phillips, B. A. Molitoris, *J. Am. Soc. Nephrol.* **2006**, *17*, 2697 – 2705.
[9] J. Unwin, S. Standage, D. Alexander, T. J. Hosted, A. C. Horan, E. M. Wellington, *J. Antibiot.* **2004**, *57*, 436 – 445.
[10] S. Karki, J. Y. Kim, S. H. Park, H. J. Kwon, *J. Korean Soc. Appl. Biol. Chem.* **2012**, *55*, 439 – 442.
[11] J. H. Guo, F. L. Huang, C. Huang, X. B. Duan, X. Y. Jian, F. Leeper, Z. X. Deng, P. F. Leadlay, Y. H. Sun, *Chem. Biol.* **2014**, *21*, 608 – 618.
[12] X. P. Ni, Z. P. Sun, H. Y. Zhang, H. He, Z. Ji, H. Z. Xia, *J. Ind. Microbiol. Biotechnol.* **2014**, *41*, 1383 – 1390.
[13] L. Shao, J. S. Chen, C. X. Wang, J. A. Li, Y. Tang, D. Chen, W. Liu, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1438 – 1441.
[14] D. Ralph, S. Curd, W. Frank, H. Ulrike, *Magn. Reson. Chem.* **2003**, *41*, 589 – 598.
[15] H. Kase, Y. Odakura, K. Nakayama, *J. Antibiot.* **1982**, *35*, 1 – 9.
[16] F. L. Huang, D. Spiteller, N. A. Koorbanally, Y. Y. Li, N. M. Llewellyn, J. B. Spencer, *ChemBioChem* **2007**, *8*, 283 – 288.
[17] D. Li, H. Li, X. P. Ni, H. Y. Zhang, H. Z. Xia, *Microbiol. Res.* **2013**, *168*, 263 – 267.
[18] R. Grahek, L. Zupancic-Kralj, *J. Pharmaceut. Biomed.* **2009**, *50*, 1037 – 1043.
[19] J. W. Park, J. S. Hong, N. Parajuli, H. S. Koh, S. R. Park, M. O. Lee, S. K. Lim, Y. J. Yoon, *Anal. Chem.* **2007**, *79*, 4860 – 4869.
[20] J. Bérdy, J. K. Pauncz, Z. M. Vajna, G. Horváth, J. Gyimesi, I. Koczka, *J. Antibiot.* **1977**, *30*, 945 – 954.
[21] P. K. Mehta, T. I. Hale, P. Christen, *Eur. J. Biochem.* **1993**, *214*, 549 – 561.
[22] H. Hayashi, *J. Biochem.* **1995**, *118*, 463 – 473.
[23] R. A. John, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1995**, *1248*, 81 – 89.
[24] J. N. Jansonius, *Curr. Opin. Struct. Biol.* **1998**, *8*, 759 – 769.
[25] H. Z. Xia, S. Wu, *Wei Sheng Wu Xue Bao* **2000**, *42*, 181 – 185.

Manuscript received: May 20, 2015
Accepted article published: June 17, 2015
Final article published: July 14, 2015