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SHORT COMMUNICATION

Gene Inactivation Study on *gntK*, a Putative *C*-methyltransferase Gene in Gentamicin Biosynthesis from *Micromonospora echinospora*

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Abstract GntK harbors methyltransferase-related cobalamin-binding domain and radical *S*-adenosylmethionine domain. The *gntK*-inactivation mutant of *Micromonospora echinospora* accumulated higher levels of gentamicin Cla and lower levels of gentamicin C1 and C2 isomers compared to the wild-type strain. Based on these results, we propose that GntK is involved in *C*-methylation on C-6' in gentamicin X2 but is dispensable in gentamicin biosynthesis.

Keywords gentamicin biosynthesis · *gntK* · *Micromonospora echinospora* · radical *S*-adenosylmethionine domain *C*-methyltransferase

Gentamicins of *Micromonospora echinospora* origin are aminoglycoside antibiotics composed of 2-deoxystreptamine and two sugar moieties. The gentamicin C complex has been widely used as a clinical antimicrobial agent and is composed primarily of C1, C1a, and C2 isomers (Wagman and Weinstein, 1980). The gentamicin biosynthetic gene cluster has been cloned and sequenced, thereby providing an excellent opportunity to define catalysts involved in gentamicin biogenesis (GenBank accession no. AY524043 and AJ628149) (Unwin et al., 2004). Gentamicin A2 (**A2**), the first pseudotrisaccharide intermediate in gentamicin biosynthesis, is envisioned to undergo biochemical modifications to form gentamicin A (**A**) and subsequently, gentamicin X2 (**X2**) (Fig. 1). Ectopic gene expression was successfully employed to elucidate the biochemical pathway to the first pseudotrisaccharide intermediate, **A2** (Park et al., 2008). However, the genes involved in later modification steps remain unknown with the exception of the *gntE* mutant that accumulates **A2** (Kim et al., 2008). The biosynthetic pathway after the formation of **X2** diverges to form JI-20A and JI-20B. The pathway to JI-20B route begins with *C*-methylation at the C-6 position to produce G-418. Reductive deoxygenation reactions at C-3 and C-4, which are mediated by an unknown chemistry, eventually yield the gentamicin C complex (Testa and Tilley, 1976).

The biosynthesis of gentamicin involves two methyltransfer reactions on non-activated carbons in the steps leading to the formation of **X2** and G-418 (Fig. 1). The product of *gntK* is predicted to mediate *C*-methylation at the C-6 position. GntK is a radical *S*-adenosylmethionine (SAM) methyltransferase and Fom3 of fosfomycin biosynthesis is a representative member of the GntK-like proteins. Fom3 and GntK share limited similarity with 24% sequence identity. The radical SAM superfamily performs diverse biochemical functions (Layer et al., 2004). Recent reports have highlighted the radical SAM methyltransferase modifying adenosine nucleotide (Boal et al., 2011). Foms3 has been proposed to be a *C*-methyltransferase using a SAM-derived 5'-deoxyadenosine radical and methylcobalamin as a catalyst for homolytic C-H cleavage on non-activated carbon and a methyl source, respectively (Woodyer et al., 2007). The proposal of this novel methyltransfer mechanism is guided by the domain organization of Fom3, which has a cobalamin binding domain and radical SAM domain with the characteristic Cys-XXX-Cys-XX-Cys motif. This domain organization is also preserved in GntK. Furthermore, GntK is highly homologous to Fok7 (59% sequence identity), which installs a methyl group at the C-6' position of fortimicin KL1 (Kuzuyama et al., 1995). The chemistry shared between the biosynthesis of gentamicin and fortimicin is C-6′ methylation, supporting the idea that GntK catalyzes methyltransfer reaction at the C-6' position.

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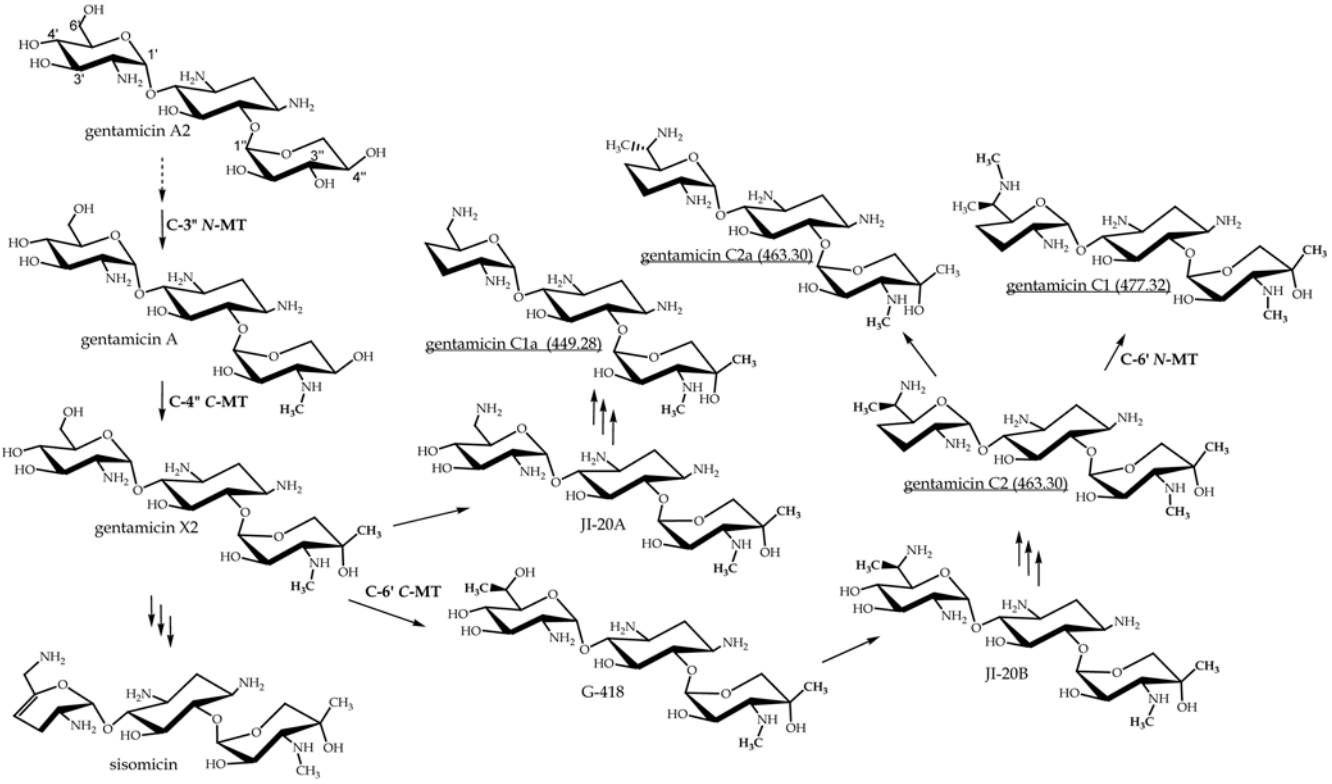

Fig. 1 Proposed gentamicin biosynthetic pathway with the final modification rout of sisomicin biosynthesis. *C*-MT and *N*-MT represent *C*-methylation and *N*-methylation, respectively.

The *gntE* mutant has been previously been shown to accumulate **A2**, supporting that GntE is involved in the *N*-methyltransfer in the bioconversion of **A2** to **A** (Fig. 1) (Kim et al., 2008). In addition, GntE has been suggested to catalyze *C*-methylation on the xylose moiety, because it is a member of the radical SAM methyltransferase (Fig. 1). GntE harbors cobalamin binding and radical SAM domains. However, GntE exhibits limited similarity to GntK. The sisomicin biosynthetic gene cluster was cloned from *Micromonospora inyoensis* (Hong et al., 2009). Sisomicin biosynthesis is closely related to that of gentamicin (Fig. 1). The biosynthesis of sisomicin diverges from the gentamicin pathway at the step modifying **X2**. The structure of sisomicin differs from that of gentamicin in three discrete modifications, C-6' methylation, *N*-methylation at the C-6', and the saturation of the 4'-double bond. The sisomicin biosynthetic gene cluster shares three methyltransferase genes, including *gntE*, with that of gentamicin, which share no less than 90% sequence identity (GenBank accession nos. AJ628149 and FJ160413). Other two additional methyltransferase genes are *genT* (*sis 28*) and *genN* (*sis30*). The roles played by these genes in gentamicin biosynthesis are questionable. Notably, the sisomicin biosynthetic gene cluster lacks a *gntK* homologue. This finding suggests that GntK mediates C-6' methylation and is also possibly involved in *N*-methylation at the C-6' (Fig. 1). The present study demonstrates that a *gntK* knockout mutant retains the ability to produce the gentamicin C complex. However, the component composition differs significantly from that of WT. Compared to WT, the *gntK* knockout mutant accumulates higher levels of gentamicin C1a (**C1a**) and lower levels of gentamicin C2 isomers (**C2**) and gentamicin C1 (**C1**), indicating that *gntK* is not essential in gentamicin biosynthesis but does play a role in the production of **C1** and **C2**.

The *gntK* inactivation plasmid pMJ-HJK-1-35b was prepared by replacing the central region of *gntK* with a thiostrepton-resistance gene (*tsr*) followed by the introduction of the plasmid into *M. echinospora* ATCC 15835 through intergeneric conjugation (Supplementary material). The primary exconjugants with thiostrepton-resistant/apramycin-resistant phenotypes were selected, thereby giving rise to the single cross-over mutants, which were grown in a series of liquid cultures without any antibiotics. Screening the resulting progeny for a thiostrepton-resistant/apramycin-sensitive phenotype gave rise to the *gntK* mutant (Δ*gntK*). The chromosomal structure of the mutant was confirmed by polymeratse chain reaction (PCR) amplification with the primer sequences specific to *gntK* (Fig. 2A). PCR amplification of WT chromosome generated a 1.7-kb fragment, which was replaced with a 1.1-kb fragment in Δ*gntK* (*gntK*::*tsr*) (Fig. 2B). Restriction digestion analysis supported the identity of the resulting PCR fragments (Fig. 2C). A 1.7-kb fragment from WT was digested into 1.25- and 0.45-kb fragments upon treatment

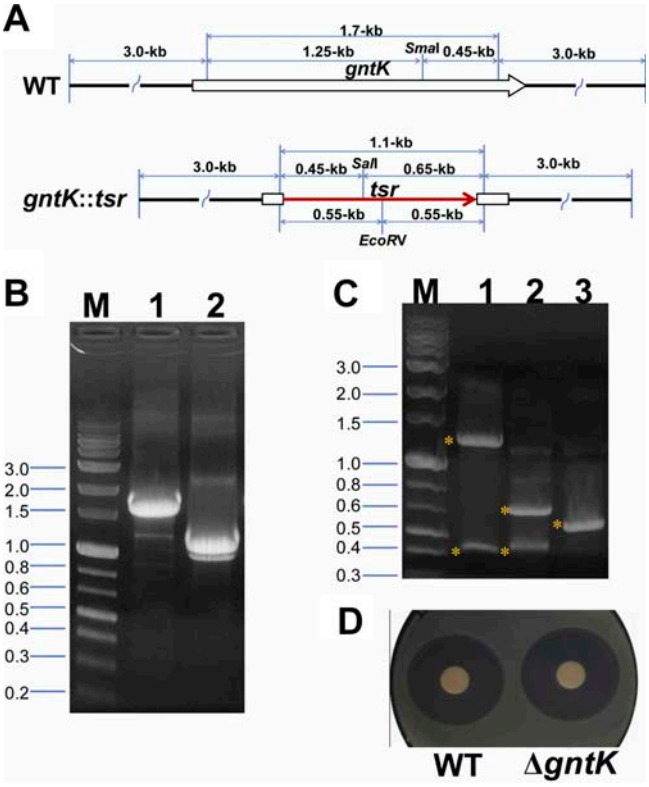

Fig. 2 Gene inactivation of *gntK* by double crossover-mediated gene replacement. (**A**) Restriction maps of *M. echinospora* ATCC 15835 wild-type (WT) and Δ*gntK* (*gntK*::*tsr*) in the *gntK* region showing the predicted sizes of PCR products with primer pair of 5'-TCCGCG GATGCATGGTGGAA-3' and 5'-TGACCGCTGTACAGCGCGAT-3'. The predicted sizes of the restriction digestion products are provided. (**B**) Electrophoretic analysis of the PCR product obtained with the chromosomes of WT (**1**) and Δ*gntK* (**2**). (**C**) Electrophoretic analysis of the PCR product of WT treated with *Sma*I (**1**) and the product of *gntK* with *Sal*I (**2**) or *Eco*RV (**3**) with the relevant fragments indicated with asterisks. Lane **M** refers to the DNA molecular weight marker with the size indication on the left in kb. (**D**) Antimicrobial assays with *Bacillus subtilis* ATCC 6633. The assay organisms were cultivated in a nutrient medium and overlaid onto nutrient agar after suspended in 0.4% soft agar. The assay samples were 200 μL of the supernatants of the GSS medium cultures.

with *Sma*I (Fig 2C, lane 1). A 1.1-kb fragment from Δ*gntK* was cleaved into 0.65- and 0.45-kb fragments by *Sal*I-digestion (Fig 2C, lane 2). *Eco*RV was used to cleave the 1.1-kb fragment into two overlapping 0.55-kb fragments (Fig 2C, lane 3). The genotype of Δ*gntK* was further confirmed by PCR analysis with primers flanking *gntK* (Supplementary material). These results verified that *gntK* was successfully deleted in Δ*gntK*. When the antibacterial activity of the Δ*gntK* and WT culture extracts against *Bacillus subtilis* was tested, comparable levels of activity were exhibited by both strains (Fig. 2D).

The culture extract was partially purified by IRC-50 cation exchange chromatography and analyzed by high performance liquid chromatography (HPLC)-mass spectrometry (MS). Both cultures of WT and Δ*gntK* were processed under the same condition. The extract obtained from 50 mL culture of each strain was finally dissolved in 1 mL of water. In the WT extract, **C1** was determined to be the main component with a lower amount of **C2** (Fig. 3A-2 and -3). **C2** and **C1** were identified by the detection of *m/z* $[M+H]^+$ 464 and 478, respectively (Fig. 3B-1 and -3). The presence of these gentamicin components was validated by the detection of *m/z* 322 using fragmentation mass analysis (Fig. 3B-2 and -4) (Park et al., 2007). HPLC-MS analysis failed to detect **C1a** in the WT extract under the condition employed. This type of the component composition was previously observed in ^{14}C-methyl labeling experiment (Lee et al., 1976) and bioconversion experiment with **A** (Testa and Tilley, 1976).

We hypothesized that Δ*gntK* lost the ability to produce **C1** and **C2** due to lack of C-6' methylation. However, HPLC-MS analysis indicated that Δ*gntK* is capable of producing **C1**, **C2**, and **C1a** (Fig. 3C-1, -2, and -3). **C1a** and **C1** were identified by detecting molecular ions of *m/z* $[M+H]^+$ 450 and 478, respectively (Fig. 3D-1 and -3), and by the detection of *m/z* 322 using fragmentation mass analysis (Fig. 2D-2 and -4) (Park et al., 2007). In this experiment, no intermediate of gentamicin C complex was detected in both extracts of WT and Δ*gntK*. WT accumulated **C1** with a trace amount of **C1a**, whereas Δ*gntK* produced comparable levels of **C1** and **C1a**. These observations indicate that a high portion of **X2** was shuttled into the JI-20A pathway in Δ*gntK*, whereas the G-418 pathway was predominant in WT (Fig. 1). These results demonstrate that *gntK* is dispensable in gentamicin biosynthesis and has a role in C'-6 methylation. Other methyltransferase genes including *gntE* were suggested to partially complement the loss of *gntK* to render the production of **C1** and **C2** in Δ*gntK*. Furthermore, the broad substrate specificity of GntE may compensate for the loss of *gntK* in gentamicin biosynthesis.

In conclusion, *gntK* was characterized as participating in C'-6 methylation. We hypothesize that GntE and GntK are unusual radical SAM methyltransferases that mediate both *C*- and *N*-methyltransfers. These results will guide the exploration of the radical SAM methyltransferases in engineered biosynthesis of unnatural aminoglycosides.

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Supplementary Material

The supplementary material section contains the materials and methods used in this study and the detailed genetic map of the *gntK*-inactivation plasmid, including the PCR analysis results with primers flanking *gntK*.

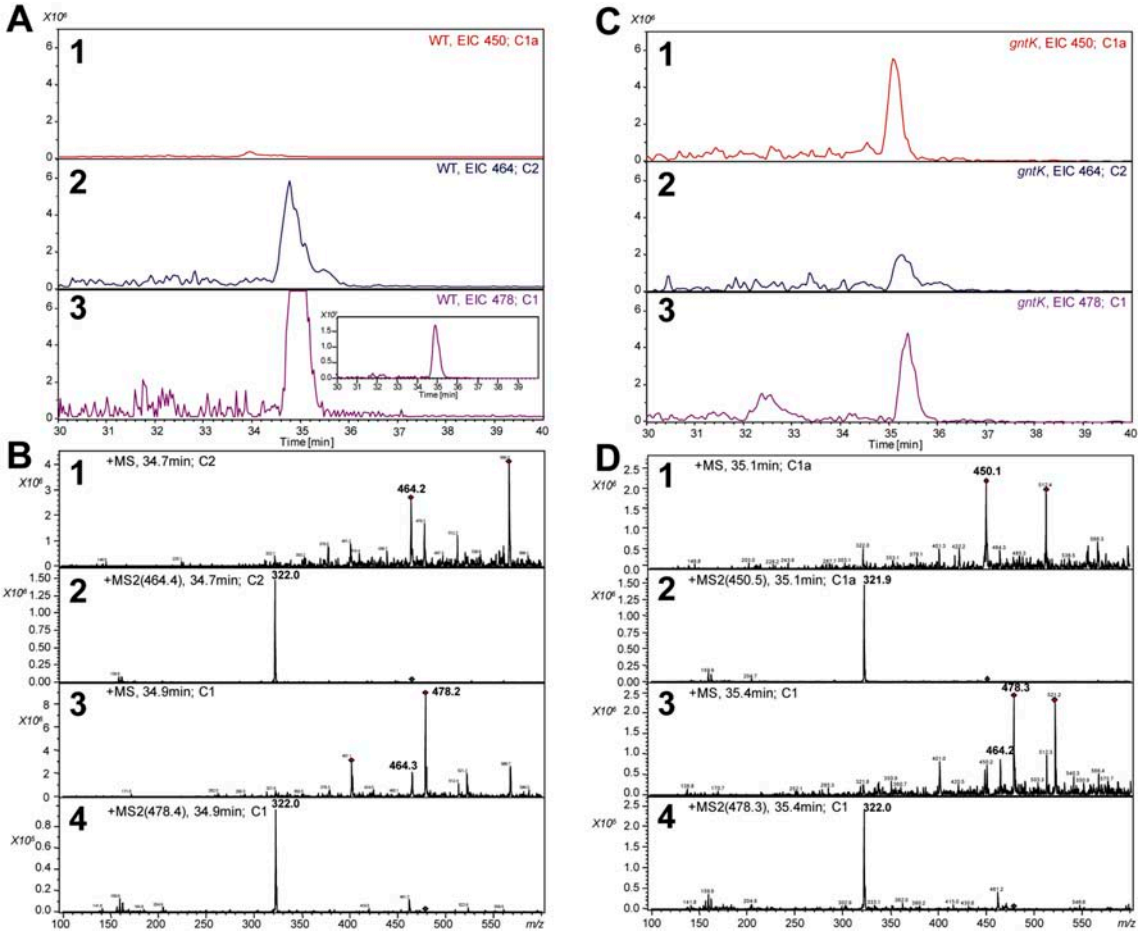

Fig. 3 HPLC-MS chromatograms of the extracts from *M. echinospora* ATCC 15835 WT (**A**, **B**) and Δ*gntK* (**C**, **D**). Selected ion chromatograms of *m/z* 450 for **Cla** (**1**), *m/z* 464 for **C2** (**2**), and *m/z* 478 for **C1** (**3**) of WT (**A**) or Δ*gntK* (**C**). Mass spectra (**1**, **3**) fragmentation mass spectra (**2**, **4**) of **C2** (**1**, **2**) and **C1** (**3**, **4**) from WT (**B**) and those of **C1a** (**1**, **2**) and **C1** (**3**, **4**) from Δ*gntK* (**D**).

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