J Ind Microbiol Biotechnol (2014) 41:1383–1390
DOI 10.1007/s10295-014-1479-3

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Genetic engineering combined with random mutagenesis to enhance G418 production in *Micromonospora echinospora*

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Received: 16 April 2014 / Accepted: 18 June 2014 / Published online: 17 July 2014

Abstract G418, produced by fermentation of *Micromonospora echinospora*, is an aminoglycoside antibiotic commonly used in genetic selection and maintenance of eukaryotic cells. Besides G418, *M. echinospora* produces many G418 analogs. As a result, the G418 product always contains impurities such as gentamicin C1, C1a, C2, C2a, gentamicin A and gentamicin X2. These impurities are less potent but more toxic than G418, but the purification of G418 is difficult because it has similar properties to its impurities. G418 is an intermediate in the gentamicin biosynthesis pathway. From G418 the pathway proceeds via successive dehydrogenation and aminotransferation at the C-6′ position to generate the gentamicin C complex, but genes responsible for these steps are still obscure. Through disruption of *gac*J, which is deduced to encode a C-6′ dehydrogenase, the biosynthetic impurities gentamicin C1, C1a, C2 and C2a were all removed, and G418 became the main product of the *gac*J disruption strain. These results demonstrated that *gac*J is in charge of conversion of the 6′-OH of G418 into 6′-NH_2. Disruption of *gac*J not only eliminates the impurities seen in the original strain but also improves G418 titers by 15-fold. G418 production was further improved by 26.6 % through traditional random mutagenesis. Through the use of combined traditional and recombinant genetic techniques, we produced a strain from which most impurities were removed and G418 production was improved by 19 fold.

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Keywords Strain improvement · Gene disruption · *Micromonospora echinospora* · Genetic engineering · Traditional random mutagenesis · G418

Introduction

Micromonospora echinospora produces a group of aminoglycoside antibiotics, including the gentamicin A, B, and C complexes, Micronomicin and G418. G418, also known as Geneticin, blocks protein synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. G418 is widely used in molecular biology research to select genetically engineered cells. In general, for bacteria and algae concentrations of 5 mg/L are used, and for mammalian cells concentrations of approximately 400 mg/L are used for selection and 200 mg/L for maintenance. G418 is produced by *M. echinospora*, but *M. echinospora* also produces many other aminoglycosides in addition to G418. The principal impurities of G418 are gentamicin C1, C1a, C2, C2a, gentamicin A and gentamicin X2. However, removal of these impurities from G418 is very difficult because of their similar properties. Even more serious, these impurities are much more toxic to commonly used cell lines (as shown in Table 1), and the toxicity of the impurities reduces the quality of G418 by decreasing its selectivity [14].

G418 is an intermediate in gentamicin biosynthesis. Several groups have cloned the gentamicin biosynthetic gene cluster from *M. echinospora* [7, 21]. As shown in Fig. 1, the whole gentamicin biosynthesis pathway has been proposed. Moreover, biosynthesis of gentamicin A2, which is the first pseudo-trisaccharide intermediate of the gentamicin biosynthetic pathway, has been investigated by heterologous expression [15]. However,

gentamicin A2 is just an intermediate of gentamicin and there are still several steps of methylation, amination and deoxygenation needed to form the final gentamicin C complex.

Gentamicin A2 is methylated to form G418, which is then converted to JI-20B by transamination. However, genes responsible for introduction of the gentamicin C-6′ amino group have not been reported. Because the 2-deoxystreptamine-containing aminoglycoside antibiotics gentamicin, neomycin, butirosin and tobramycin share a similar C-6′ amino modification, and introduction of the 6′-NH_2 of neomycin and butirosin is known to be catalyzed and formed by the sequential action of a 6′-dehydrogenase and an aminotransferase, we predicted that the same mechanism may be employed in the biosynthesis of gentamicin [3, 6, 23]. Therefore, if the C-6′ dehydrogenase step is blocked, biosynthesis of most of the impurities found in G418 preparations will be disrupted and the metabolic flux should flow to G418 biosynthesis (Fig. 1). To improve G418 production and eliminate impurities production, the deduced C-6′ dehydrogenase-encoding gene, *gac*J, was disrupted. The resulting disruption strain did not biosynthesize products with C-6′ amination, such as gentamicin C1a, gentamicin C2, gentamicin C2a and C1. This result suggests that GacJ participates in C-6′ dehydrogenation in gentamicin biosynthesis. Furthermore, G418 production was improved by 15 fold in this engineered strain. The metabolically engineered strain was further improved by mutagenesis and selection. This study demonstrates that combined genetic engineering and random mutagenesis is a feasible way to develop strains for industrial use.

Table 1 LD_{10} concentration for different components and different cell lines [14]

	NIH373	CHO	HeLa	GH3	U87
G418	7,200	5,210	6,100	4,300	6,200
Gentamicin X2	527	1,240	321	940	689
Gentamicin A	831	1,310	937	739	492
Gentamicin C1a	110	216	139	153	138
Gentamicin C1b	53	172	281	156	271
Gentamicin C2	128	93	215	173	96

Materials and methods

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this work are listed in Table 2. *Escherichia coli* DH5α was used as a cloning host, growing on Luria–Bertani (LB) liquid or solid medium. Liquid ATCC172 was employed for *M. echinospora* vegetative growth [8]. Solid medium containing soluble starch 1 %, $MgSO_4{\cdot}7H_2O$ 0.05 %, KNO_3 0.1 %, NaCl 0.05 %, asparagine 0.02 %, $CaCO_3$ 0.1 %, wheat bran 1 %, $K_2HPO_4{\cdot}3H_2O$ 0.03 %, and agar 1.5 % was used for *M. echinospora* sporulation, and conjugal transfer was performed on MS agar medium. The wild-type strain *M. echinospora* and its derivative mutants were cultured by a two-stage fermentation at 34 °C using a seed medium that contained 1.0 % soluble starch, 1.5 % soya bean meal, 0.1 % glucose, 0.05 % KNO_3 and 0.3 % $CaCO_3$. After

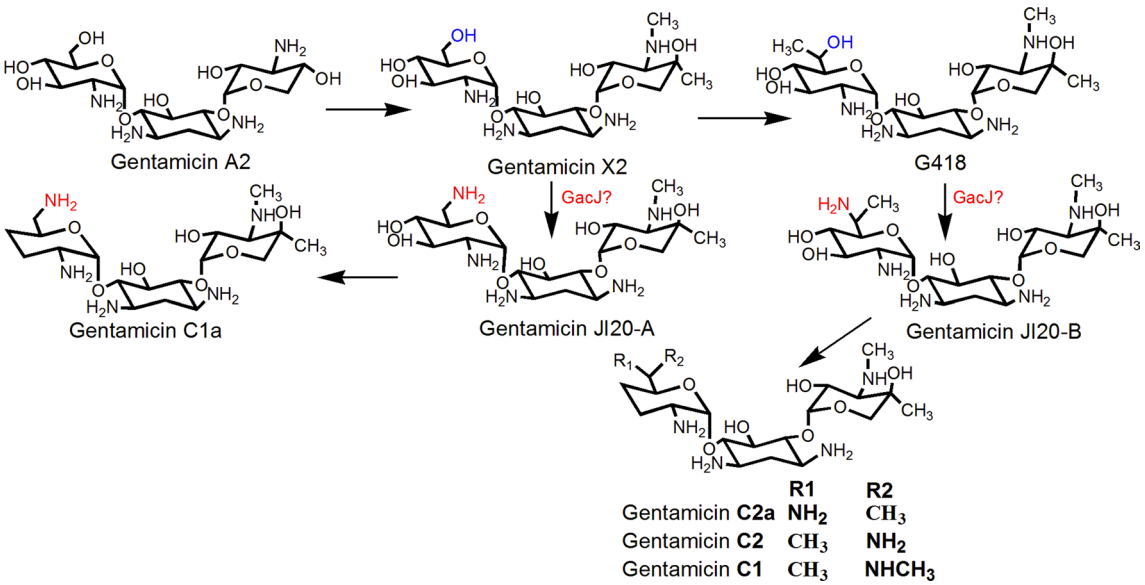

Fig. 1 Proposed biosynthetic pathway of gentamicin

Table 2 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Reference or source
Strains		
E. coli		
DH5α	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*) U169 *hsdR17*(r_k^- m_k^+) *thi-1 gyrA96 supE44 endA1 relA1* φ80d*lacZ* Δ*M15 phoA deoR*	Invitrogen
ET12567	Methylation defective, strain used in *E. coli-Micromonospora* intergeneric conjugation	[12]
M. echinospora		
Wild type	Wild-type strain, gentamicin and G418 producer	ATCC 15835
SPU336	Wild-type strain with *gac*J disrupted	This study
SPU337	High-producing strain, SPU336 through NTG mutagenesis and selection	This study
SPU338	High-producing strain, SPU337 through NTG mutagenesis and selection	This study
Plasmids		
pIJ2925	Cloning vector for *E. coli*, Amp^R	[5]
pKC1139	*E. coli-streptomyces* shuttle vector, Am^R	[2]
pSPU341	Site-specific integrative vector pSET152 *derivative, E. coli-streptomyces* shuttle vector, Amp^R, Erm^R	This study
pSPU335	pMD18-T containing *gac*J upstream and downstream fragments, Amp^R	This study
pSPU336	pKC1139 containing *gac*J upstream and downstream fragments, Am^R	This study
pSPU339	pSPU341 containing intact *gac*J, Amp^R, Erm^R	This study

Amp^R ampicillin resistance, *Am^R* ampramycin resistance, *Erm^R* erythromycin resistance

incubation at 34 °C for 36 h, 3 mL [10 % (vol/vol)] seed culture was used to incubate the fermentation medium and then it was maintained at 34 °C with shaking speed of 220 r/min for 5 days. The fermentation culture medium consisted of soluble starch 5.0 %, soya bean meal 3.5 %, glucose 1.5 %, peptone 0.2 %, KNO_3 0.05 %, $(NH_4)_2SO_4$ 0.05 %, NH_4Cl 0.1 %, corn powder 1.5 %, $CoCl_2$ 0.001 % and $CaCO_3$ 0.6 %.

Construction of *gac*J disruption plasmid

General procedures for DNA manipulation were used [18]. Primers were designed using the biosynthetic gene cluster sequence for gentamicin (GenBank accession number: AJ575934). P1 (AAGCTTTCGCCACGATCC AGACGG) and P2 (GGACAGCGGCCTCTGGGAGGGG CCTGTGCTTCAAGATGA) were used to amplify a 1,392 bp fragment containing the downstream sequence and the last 667 bp of *gac*J. A second 1,184 bp PCR product amplified with primers P3 (TCATCTTGAAGCA CAGGCCCCTCCCAGAGGCCGCTGTCC) and P4 (GAA TTCCGGCGAGCGGGAAGTGAC) contained the first 152 bp of the open reading frame and upstream sequence. The two fragments were fused by overlap extension PCR. The PCR product was ligated with pMD18T (Takara) to generate pSPU335, and then the fused fragment was digested with *Hin*dIII and *Eco*RI, and the resulting fragment was cloned into the same sites of pKC1139 to yield pSPU336.

Construction of *gac*J disruption strain

Disruption plasmid pSPU336 was transferred into *M. echinospora* by conjugation. Since pSPU336 contains an apramycin resistance gene, exconjugants were selected first for the apramycin resistance (the first crossover event) and then for the apramycin sensitivity (the second crossover event) to isolate SPU336, resulting from the desired double-crossover homologous recombination event. The Δ*gac*J::aacVI(III) allele was first exchanged onto the chromosome and then replaced by unmarked Δ*gac*J, using apramycin sensitivity as a screen.

N-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced mutagenesis and mutants screening

A mature *M. echinospora* slant was washed with 20 mL sterile deionized water and the resulting mixture was vortexed. 9 mL of the spore suspension was added to 1 mL of a sterile solution of NTG (4 mg/mL NTG in phosphate buffer). The samples were shaken at 37 °C for 30 min and immediately centrifuged for 10 min at 3,000 r/min. The cells were washed three times with sterile water and the experimental samples were serially diluted with sterile water and plated on solid slanting medium. The plates were incubated at 37 °C until colonies were just visible to the naked eye (about 2 days). Colonies were removed from the plates using a sterile cork borer of 4 mm diameter. Incubation was continued in a humid environment for 5 more

days. The plugs were then placed on the surface of antibiotic assay agar (Difco) seeded with *Bacillus pumilus*. After overnight incubation at 37 °C, parental colonies produced inhibition zones. High-producing strains resulting from the agar plug method were screened in shake-flask cultures. The final mutant was separated and propagated for five successive generations to test its genetic stability.

Antibiotic isolation and analysis

The pH of the culture broth was adjusted to 2.0 with H_2SO_4. The acidified broth was agitated for 30 min and then centrifuged (10,000 r/min; 10 min). The pH of the supernatant was readjusted to 6.8 with NH_4OH. This pre-treated supernatant was centrifuged (10,000 r/min; 10 min) again. The supernatant was applied onto strongly acidic resin 001 × 7 (Shandong Lukang Record Pharmaceutical Co., Lit), and bound substances were eluted with 2 mol/L NH_4OH. A second cation-exchange chromatography step was performed on the weakly acidic resin D152 (Shandong Lukang Record Pharmaceutical Co., Lit). Bound substances were removed by gradient elution with NH_4OH (from 0.1 to 1.0 mol/L).

Culture supernatants were analyzed by bioassay and by thin-layer chromatography (TLC). The solvent system employed for TLC was chloroform–methanol–25 % ammonium hydroxide (1:1:1). Microbiological assay (bioassay) was used to determine broth titers. The bioassay was performed with *B. pumilus* by agar diffusion.

Elutant from the strongly acidic resin 001 × 7 was used as the sample for reversed-phase high-performance liquid chromatography (RP-HPLC) with an evaporative light scattering detector (ELSD). The production of G418 was determined based on HPLC-ELSD, using a reverse phase C18 column with an evaporation temperature of 45 °C and nitrogen pressure of 3.5 bar, with a mobile phase of 0.2 mol/L trifluoroacetic acid–methanol (93:7) at 0.6 mL/min flow rate. Authentic standard gentamicin was purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products); reference standard G418 was purchased from Sangon Biotech.

Complementation of *gac*J mutants

The complementary *gac*J plasmid was prepared by cloning *gac*J in pSPU341 (a plasmid derived from pSET152 with the apramycin resistance gene replaced by ampicillin resistance gene and erythromycin resistance gene) under the control of the P*erm*E*. *gac*J was amplified with primers P5 (GTAGGATCCAGCGGTGATCGGTAGCGGCGC) and P6 (AATTAGATCTCCGACAGGTCACAGGGAG AAAT) from genomic DNA of *M. echinospora* ATCC 15835. The *gac*J gene was fused by overlap extension PCR with P*ermE**, which was amplified with primers P7 (AATTAGATCTTCGCAGGTGCACGCGGTCG) and P8 (GCGCCGCTACCGATCACCGCTGGATCCTACCAACC GGCAC). The PCR products were cloned into *Bgl*II site of pSPU341 site to yield pSPU339. After sequence confirmation, the plasmid was introduced into SPU336 by conjugation. Complemented exconjugants were confirmed by PCR.

Results

Protein phylogeny of *gac*J

The protein phylogeny was constructed by the maximum likelihood (ML) method in MEGA version 5.05 using default settings. Bootstrap method was used to test the phylogeny with 1,000 replications [20].

We collected sequences of C-6′ dehydrogenases of aminoglycosides based on annotations in the GenBank database. *gac*J lies within the branch that contains characterized dehydrogenase from other aminoglycoside antibiotic biosynthetic pathway (Fig. 2). Overall, *gac*J shows 46 and 43 % protein sequence identity to Neo11 and BtrQ, which were clarified as 6′-dehydrogenase in neomycin and butirosin biosynthetic pathway, respectively [3, 6]. On this basis, it is likely that *gac*J is a 6′-dehydrogenase involved in the first step of C-6′ amination in gentamicin biosynthesis.

Disruption of *gac*J in wild-type strain

To block the formation of gentamicin C1a, C2, C2a and C1, *gac*J was inactivated using an in-frame deletion strategy to eliminate possible polar effect on other genes. The *gac*J in-frame deletion plasmid pSPU336, in which an internal

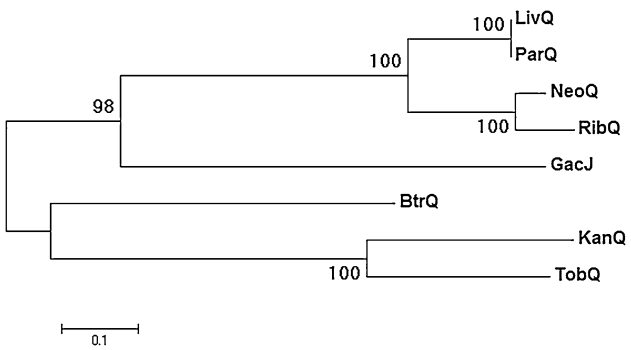

Fig. 2 Phylogenetic analysis of *gac*J. The systems shown (GenBank accession numbers in parentheses) are aminoglycoside dehydrogenases: LivQ, *Streptomyces lividus* (CAG38701); ParQ, *Streptomyces rimosus subsp. paromomycinus* (CAF32378); RibQ, *Streptomyces ribosidificus* (CAG34033); NeoQ, *Streptomyces fradiae* (BAD95834); BtrQ, *Bacillus circulans* (CAF31587); KanQ, *Streptomyces kanamyceticus* (CAF60534); TobQ, *Streptoalloteichus tenebrarius* (CAH18553)

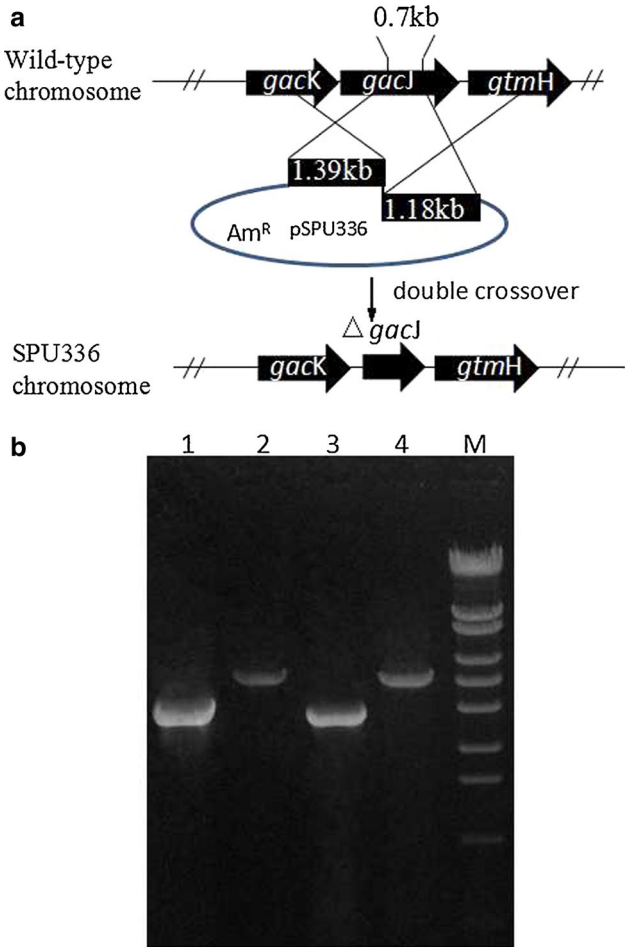

Fig. 3 Targeted gene disruption of *Micromonospora echinospora*. **a** Schematic description of *gac*J gene deletion. **b** PCR analysis with genomic DNA from *M. echinospora* and its mutant strain, using primer P1 and P4. 2.6 kb band was caused by deletion of 705 bp internal fragment in double-crossover mutant strains SPU336 no. 9 (*lane 1*) and no. 11 (*lane 3*), 3.3 kb corresponding to apramycin-sensitive strain no. 10 (*lane 2*) and intact *gac*J gene in wild-type strain (*lane 4*), *lane M* indicates the DNA molecular weight marker (λ-*Eco*T14 digest)

705 bp fragment of *gac*J gene was deleted, was introduced into *M. echinospora* by conjugation. Since pSPU336 contains the *aac*(3)IV gene, exconjugants were first selected for an apramycin resistant phenotype (the first crossover event) and then for an apramycin-sensitive phenotype (the second crossover event) to isolate SPU336 via the desired double-crossover homologous recombination event (Fig. 3a). This desired genotype was confirmed by PCR analysis of genomic DNA (Fig. 3b). PCR products of strain no. 9 and no. 11 were 0.7 kb smaller than the wild-type strain, which was caused by deletion of a 705 bp *gac*J internal fragment through double-crossover recombination. Disruption strains were further verified by DNA sequencing of those PCR products, and the *gac*J disruption strain was designated as SPU336.

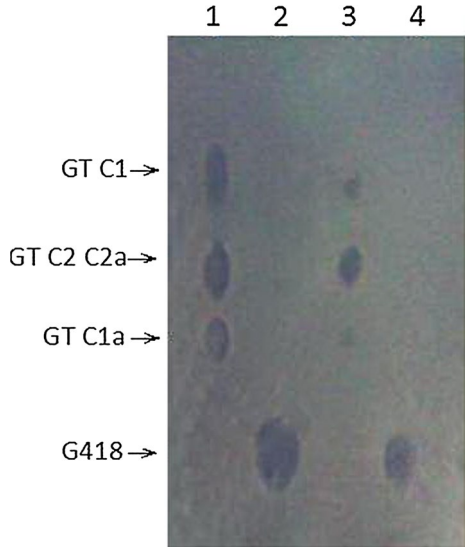

Fig. 4 Analysis of products by TLC combined with anti-microbiology assay. Authentic standard Gentamicin (*1*), G418 (*2*). The wild-type strain produced gentamicin (GT) C1a, C2, C2a and C1 (*3*). The *gac*J disruption strain SPU336 just produced an antibiotic has the same Rf value with G418 (*4*). Gentamicin C1a, C2, C2a, C1 and G418 were indicated by *arrows*

Analysis of the products of the *gac*J disruption strain

SPU336 was fermented to analyze its products with the wild-type strain as a control under identical conditions. As shown in Fig. 4, TLC coupled with an antimicrobial activity assay indicated that G418 became the main product of the mutant strain SPU336 and none of gentamicin C1a, C2, C2a or C1 could be detected in the mutant strains. The purified products of the wild-type strain and mutant were further analyzed by HPLC-ELSD. The main product of the *gac*J disruption strain SPU336 has the same retention time as the G418 standard (Fig. 5a). The structure of the main product was analyzed by Mass spectrometry; as shown in Fig. 5b, mass spectrometry results demonstrated that SPU336 accumulated G418, whose molecular weight was 496 (yield ions at m/z 497.282).

Complementation of *gac*J disruption strain SPU336

To eliminate the possibility of polar effects on the other genes, the *gac*J disruption strain was complemented by introducing an intact *gac*J gene under the strong promoter P*erm**, and the products of the complementary strain were analyzed with HPLC. As shown in Fig. 6, the complemented strain showed restored gentamicin C complex production, and G418 production levels were similar to the wild-type strain. This result demonstrated that the accumulation of G418 in the disruption strain was caused by the *gac*J disruption instead of a polar effect on other genes.

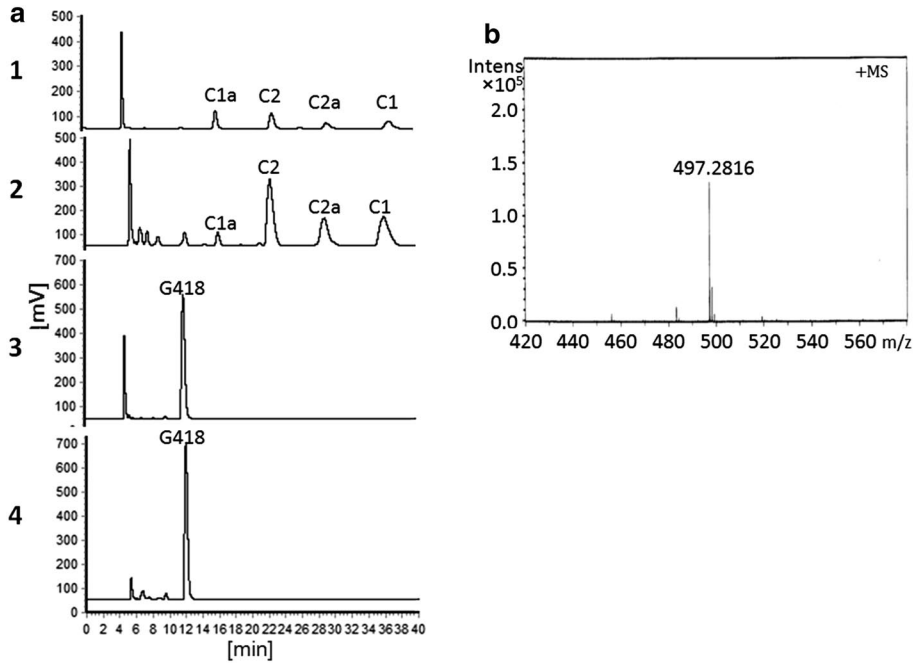

Fig. 5 HPLC and MS analyses of metabolites from *gac*J mutants. **a** HPLC analysis of authentic standard gentamicin (*1*), extract from wild-type strain (*2*), G418 (*3*) and extract from SPU336 (*4*), the antibiotics were isolated as described in the materials and methods. **b** Mass spectra of extract from SPU336

Improve production of G418 in *Micromonospora echinospora*

To determine G418 production, the fermentation was repeated three times for each strain. G418 was extracted from culture broth by adsorption onto a cation-exchange resin. By this means, recovery of G418 and gentamicin from broth was over 85 %, and the production of G418 was determined based on HPLC-ELSD. All *gac*J disruption strains have the same products and identical production profiles, but G418 production was highest in strain no. 9, which reached 680 ± 31 μg/mL, increasing by approximately 15-fold as compared to the wild-type strain.

To further improve G418 production, the *gac*J disruption strains were optimized by two rounds of random mutagenesis and selection. As shown in Fig. 7a, the production of G418 by strain no. 9 was improved by 15 % as a result of the first round of NTG-induced mutagenesis and a further 10 % using the second round of mutagenesis. Final production of G418 reached 861 ± 35 μg/mL, an improvement of 19-fold compared to the wild-type strain. Those results demonstrated that random mutagenesis was a feasible way to enhance production in a metabolically engineered strain.

Morphology and stability of high-yield mutants

The phenotype of the mutants was compared with the wild-type strain by culturing on solid medium. Mutants and wild-type strains all grow well between 28 and 37 °C, and the color of the colony surface is purple to black (Fig. 7b). Mutants have identical morphologies as compared to the wild-type strains.

The genetic stability of mutants was evaluated by serial subculture. HPLC analysis showed that the mutants still did not produce C1a, C2, C2a and C1 after five times subcultures, and the yield of G418 among the five generations ranged from 820 ± 29 to 881 ± 38 μg/mL. These results suggested that the mutant strain SPU338 was genetically stable and could apply to G418 production.

Discussion

In the gentamicin biosynthetic pathway, C-6′ amination is an essential step in the biosynthesis of the gentamicin C complex. The *gac*J disruption strains no longer accumulate products with C-6′ amination, suggesting that *gac*J encodes a dehydrogenase involved in C-6′ amination biosynthesis. Since C-6′ amination was blocked by disruption of *gac*J, G418 became the final product of the metabolic blocked gentamicin biosynthetic pathway. The production of G418 in the *gac*J disruption strain was improved by 15 fold compared with the wild-type strain.

The G418 production was further improved by NTG-induced random mutagenesis. Random mutagenesis is an effective approach to increase microbial productivity. The commonly used mutagens are ethyl methanesulfonate, NTG, UV and gamma irradiation. Among them, NTG is one of the most efficient mutagens for generating optimal yield from mutants [1]. Therefore, we choose NTG for random mutagenesis of the *gac*J disruption strain.

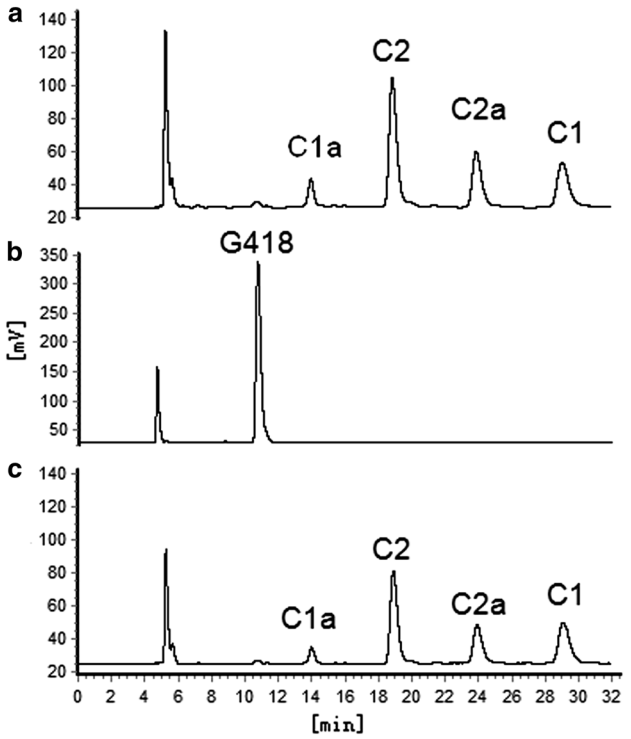

Fig. 6 HPLC analysis of *gac*J complementary strain. HPLC analysis of extract from wild-type strain (*1*), extract from SPU336 (*2*) and extract from *gac*J complementary strain (*3*)

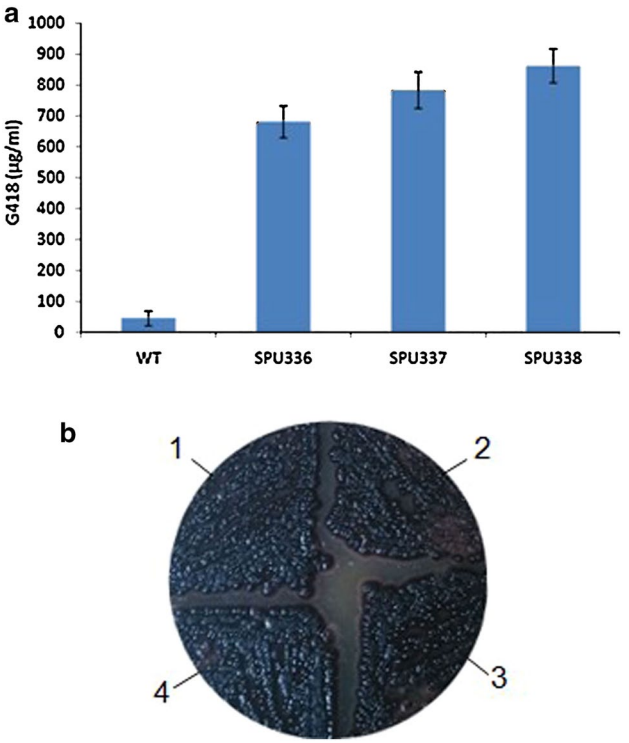

Fig. 7 Morphology and production analyses of *Micromonospora echinospora* wild-type and mutant strains. **a** Production analysis of mutants. The G418 was extracted from broth by cation-exchange resin; production was determined by HPLC; for each strain, the fermentation was repeated three times. **b** Morphology analysis of wild-type strain (*1*), *gac*J disruption strain SPU336 (*2*), mutant strain SPU337 (*3*) and G418 high-producing mutants SPU338 (*4*)

However, other mutagens might also workable, and a combination of different mutagens may increase production further. The two-step random mutagenesis improved G418 production by a further 26.6 %, demonstrating that strain improvement by random mutagenesis remains valuable for fine-tuning the strains produced by rational engineering, especially when the biosynthetic pathway and regulatory mechanisms are still obscure. Random mutagenesis and selection sometimes can achieve the same goal with rational engineering. For example, Medema MH and co-workers analyzed the transcriptome of a *Streptomyces clavuligerus* strain optimized for production of clavulanic acid by multiple rounds of mutagenesis and selection and discovered that the changes matched well with those that have been introduced separately by rational engineering [13].

The *gac*J disruption strains not only improved G418 production but also eliminated most of the impurities from the G418 product. Because antibiotic purification is a costly and time-consuming process, reduction or even elimination of impurities contained in fermentation broth will simplify the purification process. From an industrial perspective, simplification of the purification process can be as beneficial as enhancement of production.

Aminoglycosides are one of the most important types of antibiotics for human beings. For rational engineering of biosynthetic pathways to improve productivity and produce novel aminoglycosides, understanding of biosynthetic enzymes in these natural products biosynthesis is required. Many aminoglycoside biosynthesis enzymes have been studied so far, including aminotransferase, glycosyltransferase, methyltransferase, and 4-amino-2-hydroxy-butyrate biosynthesis enzymes [8–11, 19]. Those studies provided a great opportunity to improve antibiotic production or even construct strains producing new antibiotics. For example, by heterogeneously expressing the (2S)-4-amino-2-hydroxy-butyrate (AHBA) biosynthetic genes, Yoon et al. [16] generated strains that produced the semi-synthetic antibiotic amikacin and other AHBA modified antibiotic in vivo, which afforded a route to produce amikacin by fermentation instead of chemical synthesis. Furthermore, with the fast development of metabolic engineering and synthetic biology, more tools can be used by genetic engineers to create strains for producing valuable chemicals in an eco-friendly way [4, 17, 22].

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 8127341) and Scientific Research Foundation for Doctors of Liaoning Province (No. 20121103).

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