Microbiological Research 185 (2016) 36–44

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Biosynthesis of 3″-demethyl-gentamicin C components by *gen*N disruption strain of *Micromonospora echinospora* and test their antimicrobial activities *in vitro*

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ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form 19 January 2016
Accepted 24 January 2016
Available online 28 January 2016

Keywords:
3″-N-Demethyl gentamicin C components
Antimicrobial activity
Methyltransferase
Gene disruption

ABSTRACT

Gentamicin consists primarily of four components, which have different patterns of methylation at C-6′ position. The methyl groups have a significant impact on gentamicin antimicrobial activity. Sequence analysis predicted that GenN was a methyltransferase in the gentamicin biosynthetic pathway. To study the function of *gen*N, it was disrupted in *Micromonospora echinospora*. The *gen*N disruption strains produced 3″-N-demethyl-gentamicin C complex instead of the gentamicin C complex. In this study, 3″-N-demethyl gentamicin C1a was purified from the broth of disruption strain, and its structure was elucidated using MS and NMR. Besides 3″-N-demethyl products corresponding to gentamicin C1a, C2, and C2a, two 3″-N-demethyl products corresponding to gentamicin C1 were detected, which were concluded as C-6′ epimers originating from decreased substrate specificity of 6′-N methyltransferase. To explore the effects of 3″-N-methyl on gentamicin antimicrobial activity, antimicrobial activity of these demethyl gentamicin analogues were tested *in vitro*. 3″-N-Demethyl gentamicin components have identical activity with corresponding components of gentamicin. The results of bioassays showed that the 3″-N-methyl group has little impact on gentamicin activity. However, these highly bioactive compounds afforded a unique opportunity for creating new and high potent aminoglycoside antibiotics.

1. Introduction

Gentamicin is a 2-deoxystreptamine (2-DOS) containing aminoglycoside antibiotic used for treating many types of gram-negative bacterial infections. It also shows promise for treatment of inherited diseases associated with premature stop codons (Wilschanski et al., 2003). Commercial gentamicin is a mixture of congeners C1, C1a, C2, and C2. All the gentamicin C components contain 2-DOS and two sugars. Common methylated sugar of gentamicin complex was designated as garosamine. Another sugar was designated as purpurosamine (Copper et al., 1971). As shown in Fig. 1, gentamicin C components differ in the degree of methylation in purpurosamine.

Gentamicin C complex has four positions decorated by methylation, which are C-6′, 6′-N, 3″-N, and C-4″. The methyl groups have great impact on gentamicin antimicrobial activity and toxicity. The gentamicin C2 exhibited little cellular toxicity and no nephrotoxicity, but C1a, C2a, and C1 had 100% cellular toxicity at 24 h of exposure (Sandoval et al., 2006). Because the gentamicin C1a has similar activity with C2, but differ from C-6′ methylation, it is concluded that C-6′ methylation is not a determinant of gentamicin bioactivity. Addition of a methyl group at the 6′-amino group of gentamicin C2 reduces the 16S RNA affinity 20-fold. So gentamicin C1 is less potent than C2 and C1a (Yoshizawa et al., 1998). However, the influence of 3″-N methyl group on bioactivity of gentamicin is still obscure. Therefore, investigatation the biosynthesis of 3″-N methylation and its influence on antimicrobial activity of gentamicin will provide useful information for improving bioactivity of aminoglycoside antibiotics.

Many biosynthetic gene clusters of 2-DOS-containing aminoglycosides have been identified since the butirosin biosynthetic gene cluster was cloned and sequenced (Ota et al., 2000; Kharel et al., 2004a,b; Huang et al., 2005; Subba et al., 2005). Since then, the enzymatic steps that lead to 2-DOS, and thence to the pseudodisaccharide paromamine and neoamine were elucidated3 (Huang et al., 2007; Truman et al., 2007; Clausnitzer et al., 2011). Gentamicin is produced by *Micromonospora echinospora*. The gentamicin biosynthetic gene cluster has been cloned and sequenced by sev-

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http://dx.doi.org/10.1016/j.micres.2016.01.005
0944-5013/

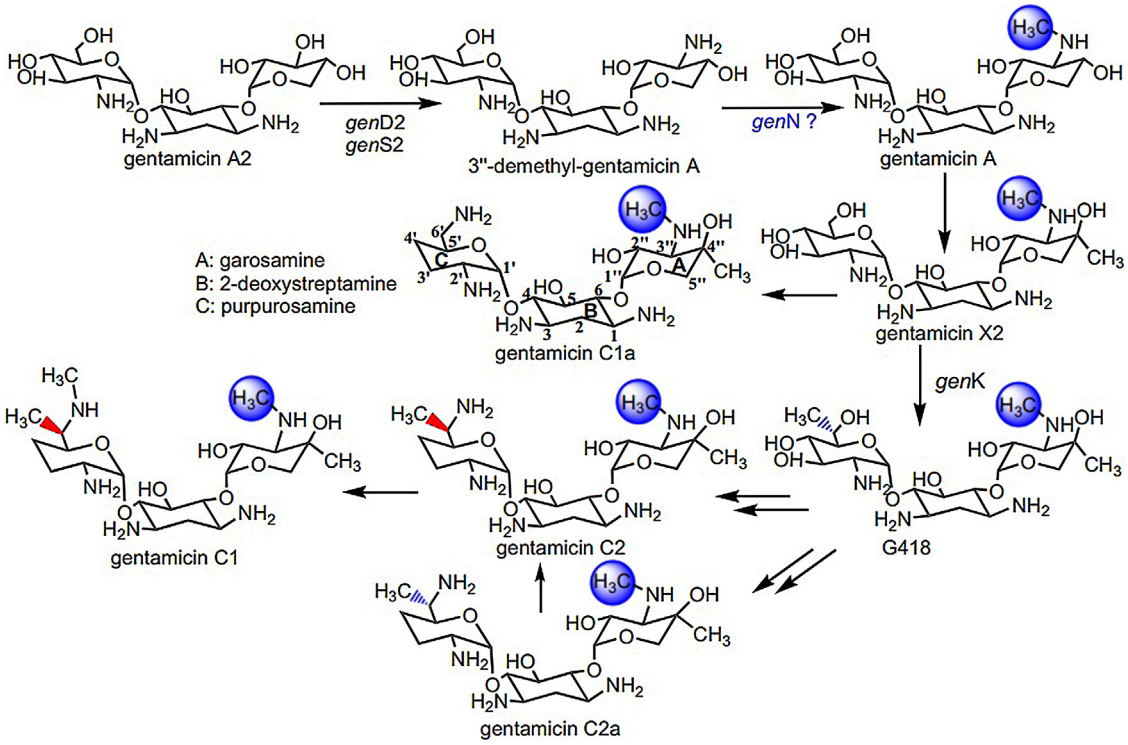

Fig. 1. Proposed biosynthetic pathway of gentamicin.

eral groups3 (Kharel et al., 2004c; Unwin et al., 2004; Aboshanab, 2007). Furthermore, biosynthesis of the first pseudo-trisaccharide intermediate gentamicin A2 has been studied by expressing a subset of the gentamicin biosynthetic genes in heterologous host strain *Streptomyces venezuelae* (Park et al., 2008). However, gentamicin A2 is just an intermediate of the gentamicin biosynthetic pathway. The biosynthesis of gentamicin also involved in several decorated steps such as methylation, amination, and deoxygenation (Fig. 1).

C-6′ methylation step in the gentamicin biosynthesis is catalyzed by GenK. This assignment was demonstrated by gene knockout and *in vitro* catalytic mechanism study (Hong and Yan, 2012; Karki et al., 2012; Kim et al., 2013; Li et al., 2013). The replacement of *gen*D1 by a thiostrepton resistance gene (*tsr*) results in accumulation of gentamicin A2, which abolish all the four methyl group, and authors interpreted that GenD1 catalyzes N-methylation at the 3″-N of gentamicin A2 (Kim et al., 2008). However, Huang et al. reported that GenN is the real 3″-N methyltransferase, while the GenD1 is methyltransferase act on C-4″ instead of 3″-N, this conclusion was consistent with this study (Huang et al., 2015). However, 3″-N-demethyl gentamicin C complex has not been prepared and studied previously because of their low production. In this work, five 3″-N-demethyl gentamicin C analogues were separated from broth of *gen*N disruption strains. To verify the exact chemical structure of the product from disruption strain, one purified product was analyzed using NMR. Furthermore, the five new gentamicin analogues afforded a unique opportunity for exploration of the effects of 3″-N-methyl on gentamicin antimicrobial activity.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and culture conditions

The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* Top10 was used as the cloning host, grown on Luria–Bertani (LB) liquid or solid medium. Liquid ATCC172 was employed for *M. echinospora* vegetative growth (Kim et al., 2008). The conjugal transfer was performed on MS agar (Kieser et al., 2000). Solid slanting medium was used for *M. echinospora* sporulation (Ni et al., 2011). The previously described media and culture conditions were used for gentamicin production (Ni et al., 2011).

2.2. Construction of genN disruption plasmid

DNA isolation and manipulation were performed as described by Sambrook (Sambrook and Russell, 2001). The *gen*N gene was analyzed with the BLAST program, and an in-frame deletion of a conserved 429-bp sequence was made. Primers were designed using the biosynthetic gene cluster sequence of gentamicin (GenBank accession number: AJ628149). Primers sequence are listed in Table 2. N1 and N2 were used to amplify a 1500-bp fragment containing the upstream sequence and the first 235-bp of *gen*N. PCR product was digested with *Hin*dIII and *Bam*HI, ligated to pKC1139 generating pMPN01. A second 1700-bp PCR product amplified with primers N3 and N4, containing the last 302-bp of the open reading frame and downstream sequence, was digested with *Bam*HI and *Eco*RI and then inseted into the same sites of pMPN01 to yield pMPN02.

2.3. Construction of genN-complemention plasmid

The gene complementation plasmid was prepared by cloning *gen*N in pEAP1 under the control of the *PermE** promoter. A 1000-bp PCR product amplified with primers N5 and N6, containing the intact *gen*N, was digested with *Bam*HI and *Hin*dIII; the resulting *Bam*HI-*Hin*dIII fragment was cloned into the same sites of pSPU241 to yield pAPN1. The 1.3-kb insert that contains the *PermE** and the intact *gen*N was recovered as a *Eco*RI fragment and inserted into the same site of pEAP1 to generate pEAPN.