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Isolation and Characterization of Kasugamycin Biosynthetic Genes from *Streptomyces kasugaensis* KACC 20262

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Abstract The biosynthetic gene cluster for the aminoglycoside antibiotic kasugamycin was isolated and characterized from the kasugamycin producing strain, *Streptomyces kasugaensis* KACC 20262. By screening a fosmid library using *kasA*, the gene encoding aminotransferase, we isolated a 22 kb DNA fragment. The fragment contained seventeen complete open reading frames (ORFs); one of these ORFs, *kasD*, was identified as the gene for dNDP-glucose 4,6-dehydratase, which catalyzes the conversion of dNDP-glucose to 4-keto-6-deoxy-dNDP-glucose. The enzyme showed a broad spectrum of substrate specificity. In addition, *ksR* was overexpressed in *E. coli* BL21 and proved to be a self-resistance gene against kasugamycin. These findings suggest that the isolated gene cluster is highly likely responsible for the biosynthesis of kasugamycin.

Key words: Kasugamycin, biosynthetic gene cluster, dNDP-glucose-4,6-dehydratase, resistance gene

Aminoglycosides are one of the earliest classes of antibiotics to be studied, and they have been among the most important clinical antibiotics for a long time. Even today, the importance of aminoglycosides has not diminished; for example, in the treatment of HIV infection [4, 16]. However, the biosynthetic pathways of only a few model compounds have extensively been studied by genetic and biochemical approaches [12, 17, 18, 28].

Kasugamycin is an aminoglycoside antibiotic produced by *Streptomyces kasugaensis* [26]. It is effective against *Piricularia oryzae* and widely used in agriculture to prevent the rice blast disease. Researchers have consequently studied the therapeutic effects of kasugamycin in treating respiratory and urinary tract infections in animals, as well as otological infections in humans caused by *Pseudomonas* [6, 22]. Kasugamycin inhibits protein biosynthesis, but does not cause codon misreading [23, 24]. Kasugamycin markedly inhibits the binding of fMet-tRNA to the 30S ribosomal subunit with mRNA. The antibiotic has a unique carboxyformidoyl group and D-*chiro*-inositol, which is devoid of the amino group and different from the inositol ring of other aminoglycosides. D-*chiro*-inositol also has potential in the treatment of Type 2 (adult-onset) diabetes [11].

The partial map of the kasugamycin biosynthetic gene cluster has previously been published [7, 8]. However, to facilitate the genetic analysis of kasugamycin biosynthesis and to elucidate the biosynthetic pathway of kasugamycin, it is necessary to isolate the complete biosynthetic gene cluster.

In a previous study, we isolated and characterized the biosynthetic genes of bluensomycin and spectinomycin [9, 10]. In the present study, we used a similar approach and isolated a 22 kb fragment from *S. kasugaensis* KACC 20262, which contained 17 putative open reading frames (ORFs). Functional analysis was performed on two of these ORFs in the gene cluster, which were identified to be the dNDP-glucose-4,6-dehydratase gene (*kasD*) and the antibiotic resistance gene (*ksR*). From these results, we postulate that the isolated kasugamycin gene cluster is very likely responsible for the biosynthesis of kasugamycin.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Streptomyces kasugaensis KACC 20262 strain was used to construct the genomic library. *E. coli* EPI100™-T1R was used as a host for the construction of pEpiFOS™-5 fosmid library. For DNA cloning and amplification, *E. coli* DH5αF' was used and, for overexpression of recombinant protein, *E. coli* BL21 (DE3) was used. pGEM-T easy (Promega Biotech.) was used for sequencing and subcloning. pET28a(+)

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and pET26b(+) (Novagen) were used for overexpression of *kasD* and *ksR*, respectively. *Escherichia coli* strains were cultured in LB medium supplemented with appropriate amounts of antibiotics whenever necessary. *S. kasugaensis* KACC 20262 was cultured in GYM broth (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$, pH 7.2 with KOH) at 28°C for 3 days and then processed to obtain genomic DNA.

General Methods for DNA Manipulation

General procedures for manipulating DNA were carried out according to the standard protocols [20]. DNA was isolated from agarose gels (BentechBio Co. Ltd., Korea) with the Qiagen kit (Chartworth, U.S.A.). Plasmid was purified using a AtManBio plasmid kit (AtManBio, Korea) in accordance with the manufacturer's instructions.

Construction of the *S. kasugaensis* Genomic Library for Isolation of the Kasugamycin Biosynthetic Gene Cluster

To construct the library, the chromosomal DNA isolated from *S. kasugaensis* KACC 20262 was sheared to approximately 40 kb fragments, and the ends were repaired to blunt 5'-phosphorylated ends. Then, the blunt-ended DNA was ligated to an pEpiFOS-5 vector (EpiFOS Fosmid Library Production Kit, EPICENTRE, U.S.A.) digested with *Eco*72 I. The ligated DNA was packaged and plated on EPI 100™ plating cells. To screen the cosmid library for kasugamycin biosynthetic genes, we used aminotransferase forward (AMTF) and reverse (AMTR) primers; AMTF 5'-TACC-ACGGCAACAGCGCGCTGA-3' and AMTR 5'-GAGC-ACCGCCGCCAGC GGAA-3'.

DNA Sequencing and Analysis

The nucleotide sequences of both strands were determined by an ABI PRISM 3700 DNA analyzer (Applied Biosystems, U.S.A.). The sequences were analyzed by codon preference [3]. Comparison of the nucleotide and amino acid sequences with its database was performed by the BLAST network service [1]. The protein sequences were aligned with the genetic computer group software package and CLUSTAL program [5].

Cloning and Expression of the *kasD* Gene

PCR was carried out to amplify *kasD* from the fosmid template that contained the kasugamycin biosynthetic gene cluster. The PCR product was sequenced to confirm the absence of mutation during the PCR reaction, and the amplified *kasD* gene was cloned into the *E. coli* expression vector pET28a(+) to give pETKASD. The plasmid was transformed into *E. coli* BL21 (DE3) for overexpression. The transformant was grown in LB medium supplemented with 50 μg ml^{-1} kanamycin at 37°C until OD_{600} reached to 0.6, then induced with 1 mM isopropyl β-D-thiogalactoside (IPTG), and allowed to grow at 28°C for 3 additional hours. Then, the cells were harvested and sonicated. The expressed protein was analyzed with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to determine the molecular weight and the existence of inclusion body [12]

Solubilization, Refolding, and Assay for the Activities of KasD

The inclusion body was isolated by centrifugation at 31,000 ×*g* for 10 min at 4°C and resuspended in 0.1 M Tris-HCl (pH 8.5) with a different concentration of urea (from 0.5 M to 6 M). To remove the insoluble cellular debris from the solubilized protein, the sample was centrifuged at 10,000 ×*g* for 20 min at 4°C [21]. Centriprep®YM-10 centrifugal filter devices (Millipore Co., U.S.A.) was used following the manufacturer's instructions for the refolding and concentrating of the protein.

To analyze the activities of the KasD protein, 100 μl reaction mixture was made by adding 100 mM Tris-HCl (pH 7.5), 5 mM NAD+, and 5 mM dTDP-glucose, along with varying amounts of the KasD protein (10 μg, 30 μg, 50 μg). After incubation at 37°C for 60 min, the reaction was terminated by the addition of 0.9 ml of 0.1 M NaOH. The amount of product was determined by measuring absorbance at 320 nm [27]. To determine the substrate specificity of KasD, dUDP-glucose, dADP-glucose, dTDP-glucose, and dGDP-glucose (Sigma-Aldrich Corp, U.S.A.) were tested as the substrates, and the products from the reaction were analyzed with the method described above.

Assay of the Resistance Gene

The *ksR* gene was amplified by PCR and cloned into the plasmid pET26b(+) to give pETKSR. The pETKSR was transformed into *E. coli* BL21. The strain that contained pETKSR and pET26b(+) was grown for 24 h at 37°C in LB broth, which was supplemented with up to 2,000 μg ml^{-1} of kasugamycin. The cell growth was scored by measuring absorbance at 600 nm.

Results and Discussion

Construction and Screening of a Fosmid Library for the Kasugamycin Biosynthetic Gene Cluster

Genomic library of *S. kasugaensis* was constructed in pEpiFOS-5 and approximately 2,000 clones were obtained. Fosmid isolated from each clone was screened by PCR amplification of *kasA*, by using AMTF and AMTR as primers. Three clones, namely pKAS168, pKAS341, and pKAS448, yielded an amplified DNA fragment with a proper size (about 910 bp). Enzyme digestion indicated that the size of the inserts were about 40 kb.

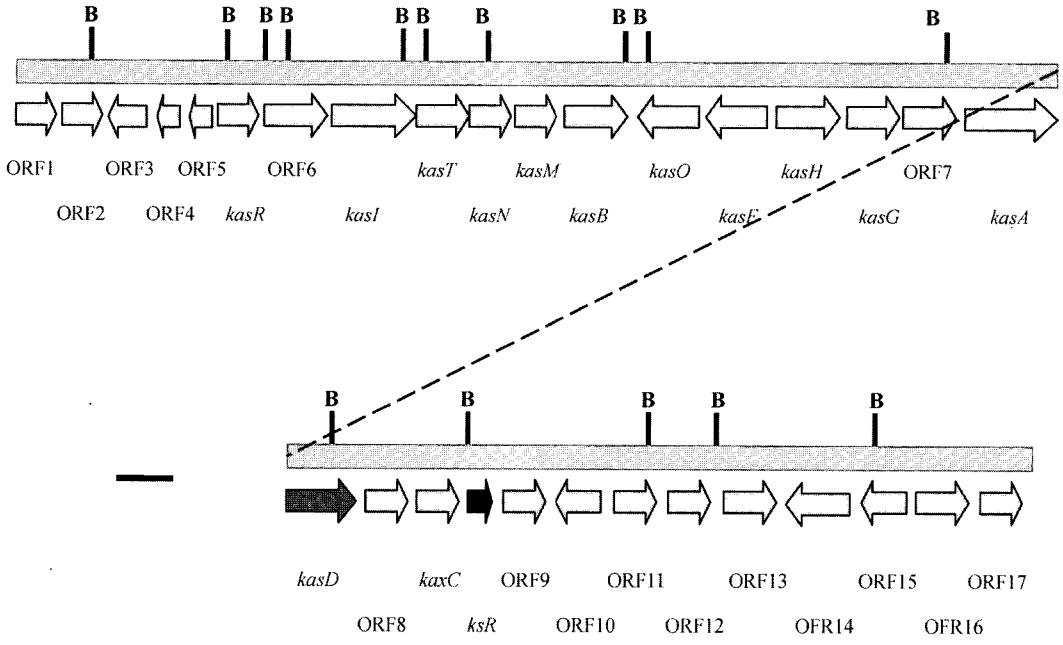

Fig. 1. Organization of the gene cluster for kasugamycin biosynthesis and speculated gene products.
ORF1: Putative protein -S- isoprenylcysteine methyltransferase; ORF2, 3, 4, 5, 6, 7, 8, 10: Unknown protein; *kasR*, Transcriptional regulator; *kasI*, Oxidase for inositol derivative; *kasT*, ATP-binding protein; *kasM*, *N*: Membrane protein; *kasB*, Glycin/D-amino acid oxidase; *kasO*, Monooxygenase; *kasE*, NDP-N-acetylglucosamine 2-epimerase; *kasH*, NDP-hexose-3-dehydratase; *kasG*, Glucosyl transferase; *kasA*, Amino transferase; *kasD*, dNDP-hexose-4,6-dehydratase; *kasC*, N-acetyl transferase; *ksR*, Kasugamycin acetyl transferase; ORF9, Putative acetyl transferase; ORF11, Unknown protein; ORF12, Putative sugar efflux transporter; ORF13, Chloramphenicol resistance protein; ORF14, Unknown protein; ORF15, Secreted protein; ORF16, Histidine kinase; ORF17, Response regulatory protein.

Sequence Analysis of the Kasugamycin Biosynthetic Gene Cluster

About 22 kb fragment of pKAS168 was sequenced and the results showed that it contained 17 ORFs that were likely to be involved in kasugamycin biosynthesis (Fig. 1). Six of the ORFs corresponded well to the gene order of the partial map of the kasugamycin biosynthetic genes reported by Ikeno *et al.* [8]; *kasI*, *kasB*, *kasO*, *kasE*, *kasH*, *kasG*, *kasA*, *kasD*, and *kasC* were proposed to be kasugamycin biosynthetic genes. The proteins encoded by these genes had the homologies of other antibiotic biosynthetic enzymes that act as oxidase, hydratase, aminotransferase, epimerase, glucosyl transferase, and N-acetyl transferase. NDP-hexose-3-dehydratase (KasH), amino transferase (KasA), glycine/D-amino acid oxidase (KasB), and epimerase (KasH) have been predicted to complete generation of kasugamine that is then connected to D-*chiro*-inositol by glycosyltransferase (KasG) to yield kasugamycin. The nucleotide sequence analysis of the isolated DNA region localized all the genes necessary for kasugamycin biosynthesis between *kasB* and *ksR*. In addition, the fragment contained two regulatory genes (*kasR* and *kasQ*), four resistance genes (*kasT*, *kasN*, *kasM*, and *ksR*), and two other ORFs whose functions are unknown. Moreover, in the downstream region of the *ksR* gene, 9 ORFs were identified. The related proteins such as the secretion proteins, ABC transporters, and membrane proteins are likely to participate in the secretion of kasugamycin outside of the cell.

dNDP-Glucose-4,6-Dehydratase Activity of KasD

KasD showed 37% similarity to the StrE coding for the dTDP-D-glucose-4,6-dehydratase of *Streptomyces griseus*. The dehydratase catalyzes a reaction to generate 4-keto-6-deoxy-dNDP-glucose from dNDP-glucose in the presence of NAD^+ as a cofactor. This reaction can be found in the biosynthetic pathway of many antibiotics that contain the 6-DOH (deoxyhexose) moiety [14, 19]. KasD was expressed and characterized *in vitro* by heterologous expression in *E. coli* BL21 (DE3). Overexpression of KasD resulted in the production of a 40 kDa insoluble protein, as determined by SDS-PAGE (Fig. 2). Solubilization of the insoluble protein was achieved under 5 M urea. Refolding and concentration of the solubilized protein were performed using Centriprep®YM-10 centrifugal filter devices (Millipore Co., U.S.A.).

As substrates, dTDP-glucose, dADP-glucose, dUDP-glucose, and dGDP-glucose were incubated with solubilized KasD in the presence of NAD^+ and the amounts of reaction products were then measured by spectrophotometry at 320 nm.

The results showed that the most preferred substrate of KasD was dTDP-glucose, followed by dUDP-glucose, dADP-glucose, and dGDP-glucose in decreasing order

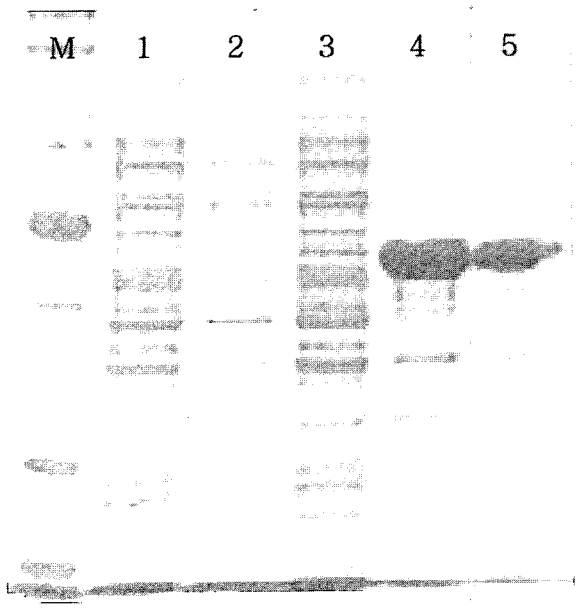

Fig. 2. SDS-PAGE of overexpressed KasD.
M: Marker. Molecular weight markers were myosin (200 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Lane 1, pET28a; lane 2, Supernatant of the cell lysate before induction; lane 3, Supernatant of cell lysate after 0.1 mM IPTG induction; lane 4, Debris of cell lysate after 0.1 mM IPTG induction; lane 5, KasD after solubilization and refolding.

(Fig. 3). We deduced that TDP-glucose is the main NDP-sugar in the biosynthesis of kasugamycin, and that the efficiency of the biosynthesis was guaranteed by its ability to utilize other NDP-glucoses.

On the basis of these findings, we postulated the pathway in order of dNDP-glucose→dNDP-4-keto-6-deoxy-glucose →dNDP-4-keto-6-deoxy-glucosamine→dNDP-4-keto-6-deoxy-N-acetyl-glucosamine. Because dTDP-glucosamine and dNDP-N-acetyl-glucosamine are not commercially available, it is difficult to prove whether the exact precursor is dNDP-glucose, dNDP-glucosamine, or dNDP-N-acetyl glucosamine. However, our proposed pathway differs from that of early research in which glucose was first converted to glucosamine [25]. Further studies are necessary to elucidate an exact pathway for kasugamycin biosynthesis.

Involvement of the *ksR* Gene in Self-resistance

The genes required for the biosynthesis of an antibiotic in streptomycetes are usually clustered and tightly linked to the genes that confer resistance to the antibiotic. Therefore, isolation of an antibiotic resistance gene is a straightforward step in studying the antibiotic biosynthesis.

Actinomycetes, which can produce aminoglycoside antibiotics (AGs), have been shown to possess AG-inactivating enzymes such as phosphotransferase (APHs), acetyltransferase (AACs), and aminotransferase (ANTs) [2]. The KsR protein contained the conserved domain (pfam00583: GNAT family) that is contained in the protein with N-acetyl transferase function [16]. In addition, the deduced protein showed significant homology to the acetyl transferase of *S. coelicolor* A3 (2) (79%) and that of *S. diastaticus* (63%). Therefore, we proposed that KsR protein could be involved in the self-resistance mechanism that modifies the structure of kasugamycin. To confirm this proposition, we conducted a resistance assay for this gene in *E. coli* BL21 (DE3). As shown in Fig. 4, *E. coli* BL21 transformant harboring pETKSR showed resistance to kasugamycin at a concentration of 1,200 μg ml^{-1}, while the strain that harbored pET26b showed resistance only to 200 μg ml^{-1}. No resistance was shown against other

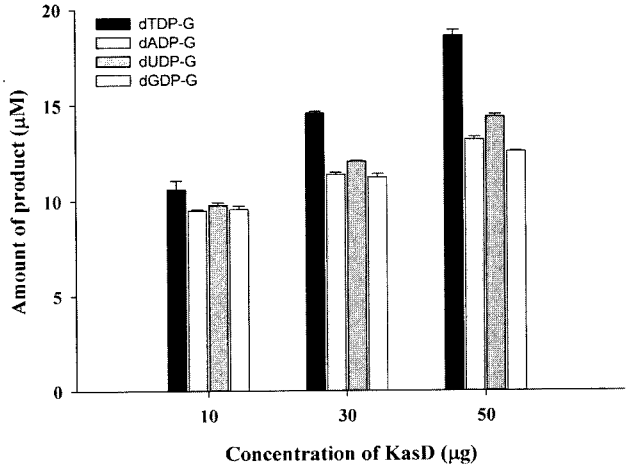

Fig. 3. Substrate specificity of KasD.
The amount of products compared to each substrate at various concentrations of purified KasD.

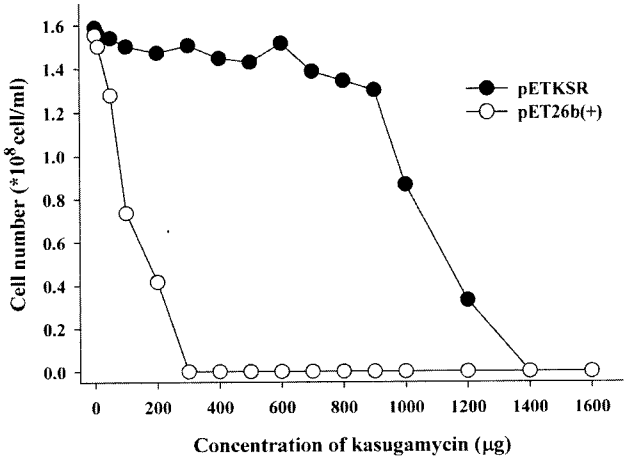

Fig. 4. Assay of self-resistance in *E. coli* BL21 (DE3).
The resistance was tested against kasugamycin in *E. coli* BL21 which harbors pETKSR and pET26b, respectively.

aminoglycoside antibiotics such as kanamycin, butirosin, spectinomycin, and streptomycin (data not shown).

The results indicate that the KsR protein is strongly involved in the self-resistance mechanism through the chemical modification of the antibiotic. Although the action mechanism is still unclear, we suggest that the proposed N-acetyl transferase might inactivate kasugamycin by modifying carbon 2 or 4 of the kasugamine group through acetylation.

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