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Genetic Organization of a 50-kb Gene Cluster Isolated from *Streptomyces kanamyceticus* for Kanamycin Biosynthesis and Characterization of Kanamycin Acetyltransferase

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Abstract A 50-kb chromosome DNA region was isolated from *Streptomyces kanamyceticus* by screening the fosmid genomic library, using the 16S rRNA methylase gene (*kmr*) as a probe. Sequence analysis of this region revealed 42 putative open reading frames (ORFs), which included biosynthetic genes such as genes responsible for 2-deoxystreptamine (2-DOS) biosynthesis as well as genes for resistance and regulatory function. Also, the kanamycin acetyltransferase gene (*kac*) was characterized by *in vitro* enzyme assay, which conferred *E. coli* BL21 (DE3) with 10, 50, and 80-times higher resistance to kanamycin A, tobramycin, and amikacin, respectively, than the control strain had, thus strongly indicating that the isolated gene cluster is very likely involved in kanamycin biosynthesis. This work provides a solid basis for further elucidation of the kanamycin biosynthesis pathway as well as the productivity improvement and construction of new hybrid antibiotics.

Key words: Aminoglycoside, 2-deoxystreptamine (DOS), kanamycin biosynthetic gene cluster, kanamycin acetyltransferase, self-resistance determinant, *Streptomyces kanamyceticus*

Studies on antibiotics biosynthesis and resistance have contributed greatly to human health care. Among diverse kinds of structure and activity of antibiotics, aminoglycoside antibiotics are potent, broad-spectrum antibiotics for the treatment of life-threatening infections [24]. Despite the fact that numerous bacterial species have developed resistance to these compounds, their strong bactericidal activity and the ability to interact synergistically with other antimicrobial agents, especially β-lactam, have retained their utility in clinical practice. Genetic and biochemical studies of streptomycin [5], bluensomycin [16], nebramycin [11], and spectinomycin [10, 14] have given comprehensive information about the biosynthesis, regulation, and resistance mechanisms [29]. Recently, sequence information of 2-deoxystreptamine (2-DOS)-containing aminoglycoside butirosin [28], gentamicin (available in GenBank database with the accession number AY524043) and tobramycin [18] has also been reported.

Kanamycin is a 2-DOS-containing aminoglycoside antibiotic produced by *Streptomyces kanamyceticus*, and has clinically been used as an effective agent for the treatment of streptomycin-resistant tuberculosis. Kanamycin A and B, two components in the kanamycin complex, also serve as the starting materials for semisynthetic amikacin, dibekacin, and arbekacin, which are potent chemotherapeutic agents against resistant bacteria, especially arbekacin, which is used as antimethicillin-resistant *Staphylococcus aureus* (MRSA) [20]. As shown in Fig. 1, kanamycin is built up from three subunits, including 2-deoxystreptamine, kanosamine, and the third an aminosugar, which distinguishes the three kanamycin components by different amino group substitutions (Fig. 1).

Although kanamycin is industrially and clinically important, its biosynthetic gene cluster and biosynthetic pathway have been very poorly studied. Based on feeding experiments and mutational biosynthesis with idiotrophs of *S. kanamyceticus*, biosynthesis of kanamycin has been proposed [27], however, the precise pathways related to both subunit biosynthesis and assembly still remain difficult to define. Furthermore, bacteria resistant to this antibiotic are continuously emerging, which has greatly impaired its clinical use. Genetic studies on kanamycin biosynthesis and regulation are of great importance, not only for improving the productivity of this important antibiotic, but also beneficial to the design of new antibiotics against resistant pathogens by combinational biosynthesis. The prerequisite for the elucidation of kanamycin biosynthesis

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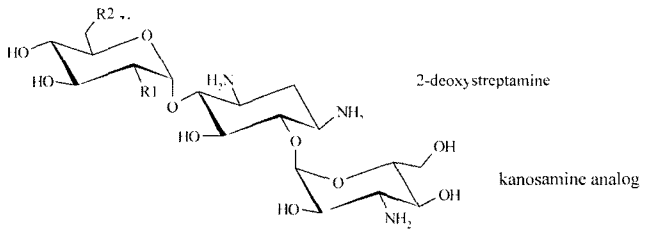

	R1	R2
Kanamycin A	OH	NH_2
Kanamycin B	NH_2	NH_2
Kanamycin C	NH_2	OH

Fig. 1. Chemical structures of kanamycin A, kanamycin B, and kanamycin C produced by *Streptomyces kanamyceticus*.
2-Deoxystreptamine (the middle ring) and kanosamine (the lower ring) moieties are indicated. The three components in the kanamycin complex differ from each other in the different substitution of R_1 and R_2 of 2-deoxystreptamine, which is 6-amino-6-deoxy-D-glucose in kanamycin A, 2,6-diamino-2,6-dideoxy-D-glucose in kanamycin B, and D-glucosamine in kanamycin C.

and genetic manipulations is the acquirement of the kanamycin gene cluster.

In this report, a 50-kb gene cluster was isolated from *Streptomyces kanamyceticus,* and the putative functions of the genes were deduced. The kanamycin self-resistance determinant, the kanamycin acetyltransferase gene (*kac*), which confers heterologous *E. coli* host with high kanamycin resistance, was characterized by *in vitro* enzyme assay. Because self-resistant determinant is an important component in a biosynthesis gene cluster, the cluster isolated is very likely involved in kanamycin biosynthesis.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

S. kanamyceticus ATCC 12853 was cultured in a YEME medium at 28°C for 48 h while shaking and was then used to isolate genomic DNA. pGEM T-easy and pET21a (+) vectors were used for gene cloning. *E. coli* DH5α F' and *E. coli* BL21 (DE3) were used as hosts for plasmid propagation and protein expression.

DNA Isolation, Manipulation, and Cloning

Experimental procedures were those described by Sambrook and Russell [31], Kwon *et al.* [21] and Kieser *et al.* [17]. Restriction enzymes were purchased from TaKaRa (Japan), and agarose gel was a product from BentechBio Co. Ltd. Korea. The genomic library was constructed, using an EPIFOS™ Fosmid Library Production Kit (Molecular Probes Inc., Oregon), and was operated according to the manufacturer's instructions. Screening of the library was performed by using the self-resistant gene *kmr* as a probe. PCR primers, *kmr*-F: 5'-CAGTTACGTTCCTCGTCAC-3' and *kmr*-R: 5'-GATCTGTACCTGCATGCCGC-3', were designed for amplification of the *kmr* gene within the genomic DNA. PCR conditions were 94°C for 5 min for the first cycle followed by 94°C for 1 min, 66°C for 45 s, 72°C for 45 s for the following 34 cycles, and 72°C for 10 min for the last cycle. The library clones were first grouped by mixing 200 μl of overnight cultures, 50 clones in each group, after which DNA was extracted and used as PCR templates. The positive groups were then subgrouped and PCR was performed until single clones with a positive *kmr* product could be isolated. Full sequencing of the fosmid with DNA inserts was achieved by utilizing a shotgun method.

Analysis of the DNA and Protein Sequence

ORFs were determined by Frame Plot 2.3.2 [13]. Amino acid sequences of potential gene products were compared with those in the GenBank by using BLAST (www.ncbi.nlm.nih.gov/BLAST). Multiple sequences alignments were carried out, using MultAlin [3].

Cloning and Purification of the Kanamycin Acetyltransferase (*kac*) Gene, Measurement of Kac Activity, and MIC Test of *E. coli* BL21 (DE3) Transformants

The primers, *kac*-F: 5'-ATA*gaattc*ATGCGGGCACATCGG-3' and *kac*-R: 5'-AGT*aagctt*GACCAGCTCTTCGGC-3', introducing *Eco*RI and *Hin*dIII sites (underlined) were used to amplify the *kac* gene, using the same PCR conditions mentioned above. The amplified product (530 bp) was digested with *Eco*RI/*Hin*dIII enzymes, and cloned into pET21a (+) vector, and then transformed into *E. coli* DH5α F'. After sequencing confirmation, the *kac* gene was transformed into *E. coli* BL21 (DE3), and protein expression was induced by adding IPTG at 1 mM final concentration. Overexpressed Kac was purified by Ni-NTA matrices (QIAGEN), and enzyme activity was measured by the discontinuous assay method [26]. MICs were examined for testing resistance to kanamycin, amikacin, gentamicin, tobramycin, and sisomicin according to the method of Magnet *et al.* [23]. All results were obtained by an average of three independent experiments.

RESULTS AND DISCUSSION

Construction of Genomic Library and Screening

The genomic library was established by using EPIFOS™ Fosmid as a construction vector. Physical shearing of DNA assured the construction of a complete and unbiased fosmid library, as compared with more biased libraries which result from the fragmentation of DNA by partial restriction endonuclease digestion. A total of 2,580 clones were obtained through *in vitro* packaging and plating and were subjected to PCR screening. Nine positive clones

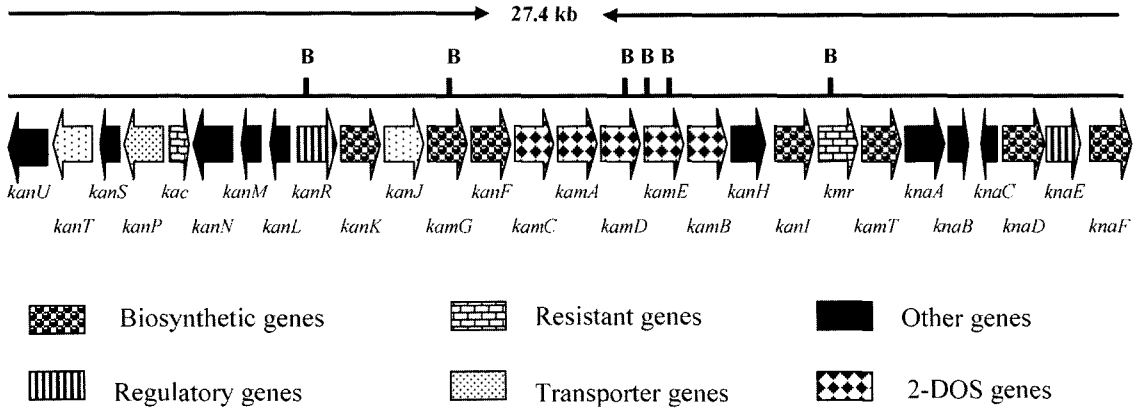

Fig. 2. Organization of the kanamycin gene cluster; B stands for restriction enzyme *Bam*HI.
Dotted line marks the additional region isolated in the two fosmid clones which was not described in this figure.

were found to contain the *kmr* gene by T-vector cloning and sequencing confirmation (Fig. 2), which were then sequenced at both ends to check the possible insert fragments by using the fosmid end-sequencing primers supplied with the kit. Physical mapping with *Bam*HI, *Apa*I, and *Sph*I showed that these 9 clones were all different from each other (data not shown). Among them, pFos1084 with the largest size was fully sequenced. Based on the results of

Table 1. Summary of putative kanamycin biosynthetic genes*.

ORF	Proposed function	Identity in amino acid sequence	Accession numbers of selected homologous proteins
kanU	Serine phosphatase	44%	ZP_00294301
kanT	Membrane efflux protein	76%	NP_630566
kanS	Unknown protein	85%	NP_630565
kanP	Membrane efflux protein	53%	NP_828653
kac	Kanamycin acetyltransferase	100%	BAD11815
kanN	Unknown protein	34%	CAD60534
kanM	Unknown protein	56%	CAD60534
kanL	Unknown protein	62%	CAD60534
kanR	Regulatory protein	43%	P08076
kanK	Dehydrogenase	69%	CAE22477
kanJ	Transport protein	44%	CAE22476
*kam*G	Glutamate-1-semialdehyde aminotransferase	34%	CAF34040
kanF	Dehydrogenase	62%	CAE22474
kamC	L-Glutamine: *scyllo*-inosose aminotransferase	75%	CAE22472
kamA	*scyllo*-Inosose synthase	68%	CAE22471
kamD	Dehydrogenase	54%	CAE22470
kamE	Glycosyltransferase	75%	CAE22469
kamB	L-Glutamine: *scyllo*-inosose aminotransferase	57%	AAR98552
kanH	Hypothetical protein	32%	EAA53554
kanI	NDP-sugar epimerase	29%	AAL14256
kmr	16s RNA methylase	100%	AB164229
kamT	Glycosyltransferase	51%	AAR98545
knaA	Hypothetical protein	70%	NP_962269
knaB	Translation initiation inhibitor	46%	NP_832452
knaC	Hypothetical protein	78%	NP_823084
knaD	Dehydrogenase	86%	NP_823085
knaE	TetR family transcription regulator	84%	NP_823086
knaF	Putative crotonyl CoA reductase	94%	NP_823087

*Functions of gene products were deduced by BlastP. Accession numbers of homologous proteins and identities of amino acid sequences were obtained from the GenBank database by BlastP (www.ncbi.nlm.nih.gov/BLAST/).

full sequencing, pFos850 that contained DNA fragment overlapping with pFos1084 in the opposite direction was also fully sequenced.

Sequence Analysis and Identification of the Kanamycin Gene Cluster

The contig delimited by pFos1084 and pFos850 spanned a 50-kb region of the *S. kanamyceticus* genome. High G/C content (ca. 69.8–73.7%) was found in all annotated ORFs, which is a characteristic of streptomycetes DNA. Sequencing results revealed 42 ORFs, and Table 1 summarizes the deduced function of 28 ORFs which were very likely to be involved in kanamycin biosynthesis and will be referred to as the kanamycin gene cluster in the following text. The organization of the gene cluster is illustrated in Fig. 2. Upstream of the gene cluster, there are 9 ORFs responsible for sugar transporter, sugar permease, sugar binding receptor, and alcohol dehydrogenase, as well as a SAM-dependent methyltransferase and one NAD^+ synthase. Downstream of the gene cluster, there are 5 ORFs encoding enzymes such as methyl malconyl-CoA mutase, and acyl dehydrates which are involved in fatty acid elongation. Therefore, these flanking 14 ORFs are apparently not involved in kanamycin biosynthesis, and were not studied further. The kanamycin gene cluster, which spanned a 27.4-kb region, includes genes that are very likely involved in 2-DOS biosynthesis and sugar modification; for example, aminotransferase, dehydrogenase, and glycosyltransferase, as well as genes for resistance, antibiotic transport, and regulatory function.

2-DOS-specific biosynthesis sequences were identified upstream of *kmr*. The sequences, made up of 5 ORFs (in the order of *kamC*, *kamA*, *kamD*, *kamE*, *kamB*), included genes for two L-glutamine *scyllo*-inosose (DOI) aminotransferases (*kamB* and *kamC*), one DOI synthase gene (*kamA*), one dehydrogenase (*kamD*), and one glycosyltransferase (*kamE*) (Fig. 2). These sequences matched perfectly with the 2-DOS gene cluster as reported in the tobramycin gene cluster in both the organization and similarity in amino acid sequence [18, 19], but was different from the gentamicin cluster which contains only one DOI aminotransferase. The two DOI aminotransferases are proposed to be responsible for the introduction of two amino groups into 2-DOS, and the conserved domain for the StrS family aminotransferase was found in these two aminotransferases. The most highly conserved peptide sequence motif, G-D-E-x_{77}-E-D-x10-G-x_3-G-x8-S-x_4-K-x_{5-6}-(E, Q)-G-G, was also identified in KamB and KamC by multiple alignment (Fig. 3), similar to the motif found in StsC [1], demonstrating the conserved active sites in this aminotransferase family. Because of the close similarity of DOI synthetase with DHQ synthetase [18], it can be concluded that the biosynthetic enzymes for both streptamine and the 2-DOS family of aminoglycosides were evolutionarily very conserved and might have been evolved from the same ancestor. The biosynthesis pathway of 2-DOS has not been fully demonstrated, but the putative pathway in *Bacillus circulans* and *Streptomyces tenebrarius* has been postulated [9, 18]. Based on the high homology with the known proteins, we propose the biosynthesis pathway of the 2-DOS moiety of kanamycin as outlined in Fig. 4.

In addition to genes involved in 2-DOS biosynthesis, other putative biosynthetic genes were also identified in the

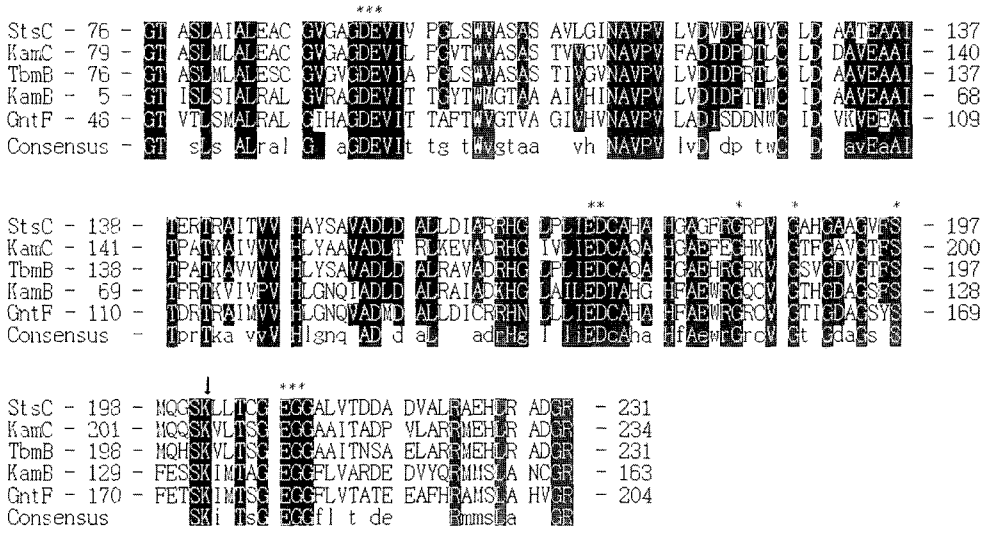

Fig. 3. Multi-alignment of two L-glutamine *scyllo*-inosose (DOI) aminotransferases, KamB and KamC, with three other Strs family aminotransferases, StsC (CAA70012), GntF (AAR98552), and TbmB (CAE22472).

Conserved motifs G-D-E-x_{77}-E-D-x10-G-x_3-G-x8-S-x_4-K-x_{5-6}-(E, Q)-G-G are indicated by asterisks, and the single conserved lysine residue is marked by an arrow.

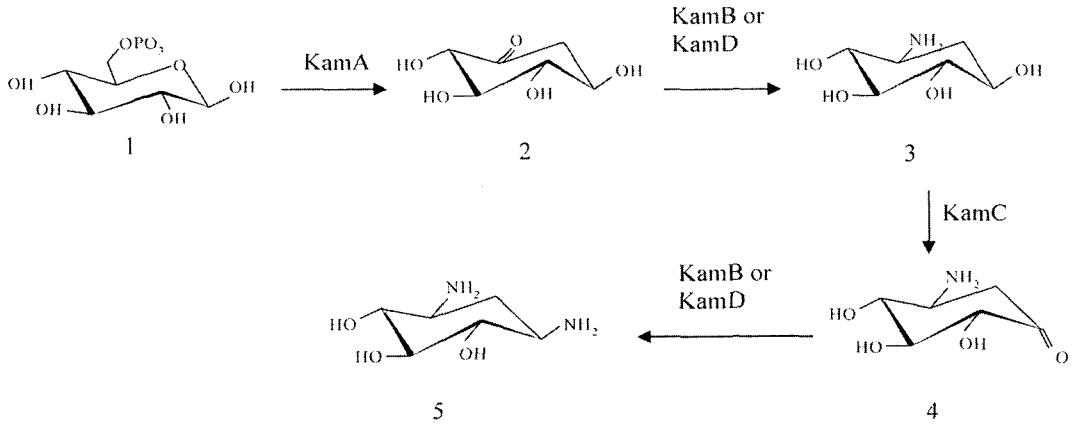

Fig. 4. Proposed biosynthetic pathway for kanamycin 2-deoxystreptamine biosynthesis.
1, D-glucose-6-phosphate; 2, 2-deoxy-*scyllo*-inosose; 3, 2-deoxy-*scyllo*-inosoamine; 4, 2-deoxy-3-amino-*scyllo*-inosose; 5, 2-deoxystreptamine.

kanamycin gene cluster. These genes encode many modification enzymes of aminocyclitol and deoxyaminosugars, such as dehydrogenases, aminotransferase, and glycosyltransferases. Conserved domains for dehydrogenase and oxidoreductase were identified in KamD, KanF, and KanK, but the products of these three genes belong to different protein families, which are the MviM family, Tdh family, and BetA family. Except for the putative function of KamD in 2-DOS biosynthesis, the function of KanF and KanK are still not clear, but the adjacent positions of *kanF* to the aminotransferase gene *kamG* (which belongs to the class III aminotransferase family) indicated that these two enzymes may catalyze a coupled transamination reaction for amino substitution in kanamycin biosynthesis [27]. A putative NDP-glucose 4-epimerase gene was found to be next to the *kmr* gene, and this gene may be involved in the biosynthesis of kanosamine moiety, as inferred by Arakawa *et al.* [2], implicating a similar pathway for 6-deoxysugar biosynthesis to those that were found in streptomycetes species [32]. Two glycosyltransferases, KamE and KamT, may be responsible for the glycosylation at C-6 and C-4 [27], respectively. Although the exact biosynthesis pathway for kanamycin biosynthesis still remains to be defined, disruption of the biosynthesis genes and analysis of the accumulated intermediates are expected to reveal a clear picture on the pathway for kanamycin biosynthesis.

The *kanR* gene, encoding a putative regulatory protein, was found to be located on the start side of the gene cluster; the gene product showed a high degree of homology with StrR (43% identity), the regulatory protein for streptomycin biosynthesis in *S. griseus* [6, 5]. KanR contains a predicted helix-turn-helix DNA binding motif (LRTVAKAAGISLSTAHDVRT), resembling that found in KasT, another member in the StrR family regulatory proteins [12]. Biosynthesis of streptomycin is regulated by the A-factor regulatory cascade [29], however, it is still unknown whether the transcription of *kan*R is also regulated by A-factor or related butyrolactones, although a recent study on the *S. kanamyceticus* strain, JCM4433, could not detect A-factor production [7]. By studying the protein binding with the promoter region of *kanR*, upstream regulators such as AdpA or ArpA are expected to be identified, and the regulation of kanamycin biosynthesis can finally be delineated.

A gene encoding the putative membrane efflux protein *kanP* was found adjacent to the *kac*, and another transporter gene *kanJ* was inside the 2-DOS gene cluster, and the products of both genes exhibited a 44% identity to TbmE, the transport protein in *S. tenebrarius*. These membrane transporters, together with the two resistant genes *kmr* and *kac*, which will be discussed in the following text, may be involved in the self-resistance of kanamycin producer.

Table 2. MIC test of various aminoglycosides for *E. coli* BL21 (DE3) containing either pET-21a (+) or the recombinant vector pET-21a (+):: *kac-his*$_6$*.

Plasmids contained in *E. coli* BL21 (DE3)	MIC (μg/ml)				
	Gentamicin	Amikacin	Kanamycin	Tobramycin	Sisomicin
pET-21a (+)	5	2.5	5	5	5
pET-21a (+) :: *kac-his*$_6$	5	200	250	50	5

*The MICs of selected aminoglycosides were performed by checking the dead curve of *E. coli* BL21 (DE3) strains containing either pET-21a (+) and pET-21a (+):: *kac-his*$_6$. MICs were determined after a 16 h incubation of two strains in 3 ml of LB broth containing various amounts of aminoglycosides at 37°C, 210 rpm shaking with an inoculum of 10^5 CFU per tube. The experiments were carried out 3 times independently in triplicates.

Overall, considerable similarities of the biosynthesis genes between the kanamycin gene cluster and those from the tobramycin (56–75%) and butirosin (31%–37%) producers were observed, and high homology was found particularly with the tobramycin producer, which might have resulted from the similar chemical structure and the close evolutionary relationship. By comparing the gene organization and the chemical structures, more information on the biosynthesis of kanamycin and other 2-DOS biosynthesis can be obtained.

Expression of *kac*-Conferred *E. coli* BL21 (DE3) with Hyper-Resistance to Various Aminoglycoside Antibiotics

S. kanamyceticus possesses two enzymes that are involved in self-resistance; 6'-*N*-acetyltransferase (Kac) and 16sRNA methylase [4, 25]. In the kanamycin gene cluster, they were found to be located on two opposite directions (Fig. 2). The *kac* gene was located upstream of the gene cluster, adjacent to the putative regulatory gene *kanR*. The DNA fragment containing the *kac* gene (deduced from the acquirement of aceltyltransferase activity in the *S. lividans* transformants) from *S. kanamyceticus* strain IFO13414 was earlier cloned, and an increased resistance to a number of aminoglycoside antibiotics was observed [15]. Nevertheless, the exact enzymatic reaction catalyzed by Kac has not yet been characterized because of no sequence information about *kac* at that time, although the biochemistry of acetyltransferases from bacterial pathogens has been robustly studied.

The *kac* gene was cloned into pET-21a (+) vector, and MICs of various aminoglycosides for *E. coli* BL21 (DE3), harboring either the pET-21a (+) vector without insert, or the recombinant vector pET-21a (+)::*kac-his*$_6$, were determined. The MICs obtained demonstrated that, under the experimental conditions where the *kac* gene was placed under the control of the strong T7 promoter, the Kac-His$_6$ protein was able to confer *E. coli* BL21 (DE3) with 10, 50, 80-times higher resistance to kanamycin A (up to 250 ug/ml) as well as tobramycin and amikacin than the control strain; however, no change to gentamicin and sisomicin was observed (Table 2). The Kac-His$_6$ protein was purified from a soluble fraction of crude cell of *E. coli* BL21 (DE3), harboring the recombinant vector pET-21a (+)::*kac-his*$_6$ (Fig. 5), and the purified Kac-His$_6$ protein was used in the *in vitro* enzyme assay. It showed strong acetylation activity to kanamycin, amikacin, and tobramycin, but not to gentamicin and sisomicin, being consistent with the resistance pattern obtained from the MIC test (Table 3).

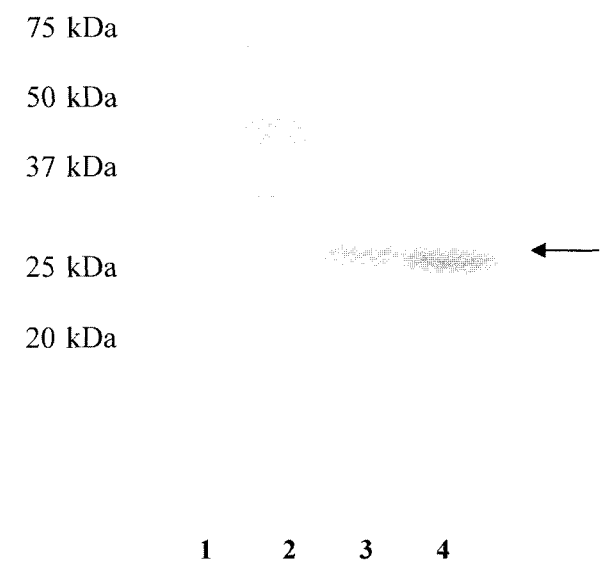

Fig. 5. SDS-PAGE of Kac.
Lane1, protein size marker; Lane 2, *E. coli* BL21 transformed with pET21 a (+) empty vector; lane 3, *E. coli* BL21 transformed with pET21 a (+) inserted with *kac*; lane 4, purified Kac, as indicated by the arrow.

The resistance of Kac to kanamycin, amikacin, and tobramycin, but not to gentamicin, represents typical characteristics of type I acetyltransferase AAC (6'), corresponding to the high homology of Kac with the AAC (6')-Ib subfamily of proteins [32] (Fig. 6). On the other hand, no resistance of Kac to sisomicin is different. The substrate binding feature of this special aminoglycoside acetyltransferase distinguishes Kac from both the AAC (6')-I and AAC (6')-II enzymes of bacterial pathogens studied so far [32]. The fact that kanamycin acetyltransferase confers to a heterologous host kanamycin resistance strongly indicates that the gene cluster isolated in this work is highly likely to be involved in kanamycin biosynthesis.

The specific action of aminoglycoside to discriminately bind to prokaryotic ribosomes to induce codon misreading and inhibit translocation has made it an important class of antibiotics in clinical applications [30]. Specifically, 2-DOS containing aminoglycoside antibiotics are being used as potent agents for hospital and opportunistic infection treatments [24, 20]. In the studies of this important antibiotic family, yield improvement and creation of new aminoglycoside antibiotics with lower toxicity and higher activity against

Table 3. Kanamycin acetyltransferase (Kac) activities using different aminoglycoside substrates*.

Kac activity	Gentamicin	Amikacin	Kanamycin	Tobramycin	Sisomicin
A (412)	0.124	0.325	0.598	0.368	0.095

*Kac activity was spectrophotometrically determined by assessing the increase of A_{412} due to the formation of 5-thio-2-nitrobenzoate. The assay mixtures contained 50 mM HEPES buffer (pH 7.5), 0.2 mM acetyl-CoA, 0.1 mM aminoglycoside, 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and 2 mg of the purified Kac-His$_6$. A_{412} was measured after incubation of 1-ml aliquot of a reaction mixture at 37°C for 30 min.

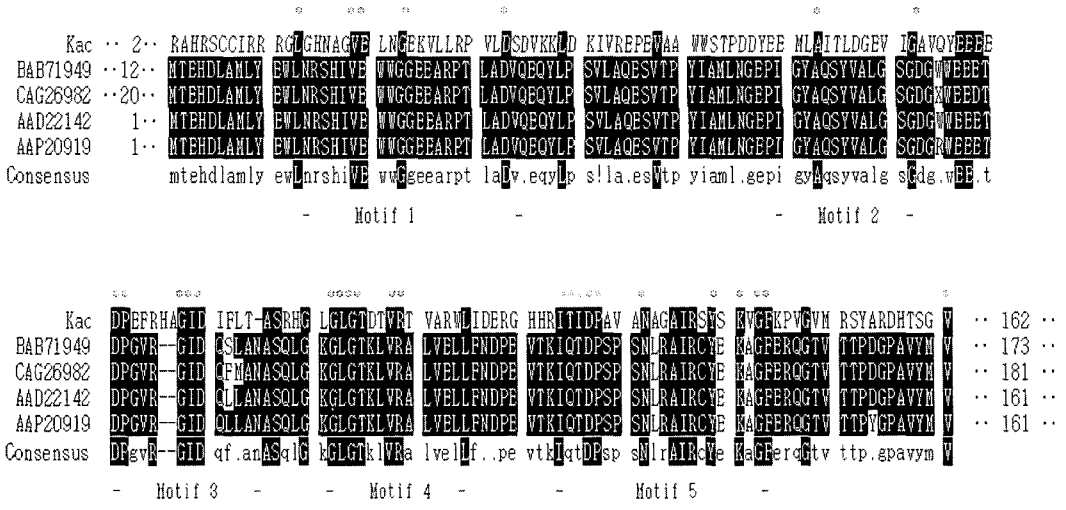

Fig. 6. Conserved motifs of Kac with various AAC (6')-Ib family proteins, revealed by multiple alignment of Kac with the acetyltransferases from *Serratia marcescens* (BAB71949), Gram-negative bacterium TR-59 (CAG26982), *Enterobacter aerogenes* (AAD22142), and *E. coli* (AAP2091).

Conserved motifs [see Ref. 12] are boxed and consensus amino acids in the motifs are denoted by asterisks. The numbers indicate amino acid locations in the proteins.

resistant pathogens are two main tasks. Studies on the putative pathway of the specific regulator gene *kanR* can be greatly helpful for production enhancement of kanamycin. Furthermore, genes encoding modification enzymes of aminocyclitol and deoxyaminosugars identified in the kanamycin gene cluster, such as dehydrogenase genes *kanF*, *kanK*, and *kamD*, aminotransferase gene *kanG*, and glycosyltransferase genes such as *kanE* and *kanT*, could be exploited to produce more effective therapeutic agents by combinatorial biosynthesis [26, 8]. The kanamycin gene cluster isolated in this work, therefore, would provide a basis for further studies not only on the elucidation of the kanamycin biosynthesis pathway and productivity improvement, but also on the construction of new hybrid antibiotics.

Acknowledgments

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