The Kanamycin Biosynthetic Gene Cluster from *Streptomyces kanamyceticus*

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Kanamycin is an aminoglycoside antibiotic produced by *Streptomyces kanamyceticus*[1]. Although it possesses broad range antibacterial activity, bacteria resistant to this antibiotic readily appear, and therefore its clinical use has been restricted to tuberculosis. Resistant bacteria inactivate kanamycin by modifying its specific amino- or hydroxy-group using acetyltransferase, phosphotransferase and nucleotidyltransferase[2]. Investigations concerning bacterial resistance mechanisms led to the development of semi-synthetic kanamycin derivatives such as dibekacin[3], amikacin[4] and arbekacin[5]. These derivatives show good activities against kanamycin-resistant bacteria and, except arbekacin, have been used as chemotherapeutic agents against resistant bacteria of clinical importance. Arbekacin has been used as an effective anti-MRSA (methicillin-resistant *Staphylococcus aureus*) orphan drug in Japan since 1990.

Combinatorial biosynthesis, which has mainly dealt with investigations concerning polyketide synthases and non-ribosomal peptide synthetases, is an attractive and potential technology for the production of modified or novel antibiotics[6]. The identification of biosynthetic gene clusters for other type secondary metabolites can offer additional possibilities for combinatorial biosynthesis. In the case of aminoglycosides, biosynthetic gene clusters have been isolated and characterized for streptomycin[7], fortimicin A[8], butirosin[9], tobramycin[10] and gentamicin (only sequence data available; AY524043). Concerning kanamycin, however, molecular biological studies have been restricted to its resistance genes[11~15] and little is known about kanamycin biosynthetic genes. In this report we describe the isolation of the kanamycin biosynthetic gene cluster from *S. kanamyceticus*.

Streptomyces kanamyceticus 21-18, a strain from our collection which is highly developed for kanamycin production, was cultivated in YEME liquid medium[16] at 28°C for 24 hours, after which time, genomic DNA was prepared using an ISOPLANT DNA extraction kit (Wako Pure Chemicals, Japan). Genomic DNA was then partially digested with *Mbo*I, dephosphorylated with calf intestinal alkaline phosphatase, and subsequently ligated to SuperCos 1 vector (Stratagene, USA) that had been digested with *Xba*I, dephosphorylated with bacterial alkaline phosphatase and then digested with *Bam*HI. Following packaging of the ligated DNA into the head of λ phage using a Gigapack III *in vitro* packaging kit (Stratagene, USA), a cosmid library was propagated using *Escherichia coli* XLI-Blue MRA. The *kmr* gene encoding 16S rRNA methyltransferase was amplified by PCR using two primers (forward; 5′-ATGTCGCAGTCCGCGTCC-3′, reverse; 5′-TCAGCCCTTCGTGACCACG-3′), and employed as a probe for the screening of the cosmid library using an ECL direct DNA/RNA detection system (Amersham Biosciences, USA) according to the manufacturer's instructions. From approximately 1,000 colonies that were screened, one positive clone was obtained. As determined from restriction endonuclease and partial nucleotide sequence analyses of a cosmid pKM9 prepared from the positive clone, this cosmid was found to contain only part of the target cluster genes. Then, we carried out chromosome walking in order to isolate an additional DNA fragment.

Southern blot analysis of chromosomal DNA revealed that a left-side DNA fragment of pKM9 (Fig. 1) hybridized to an approximate 10-kbp *Sph*I fragment. The genomic DNA was completely digested with *Sph*I, and the DNA fragments around 10 kbp were purified from a low melting-point agarose gel following electrophoresis. A genomic DNA library containing these DNA fragments was constructed in pUC119 and screened using the DNA fragment described above as a probe. Restriction enzyme analysis of 5 positive clones showed that they contained the same *Sph*I fragment. One of these was named pKM95 and used for further analysis.

The nucleotide sequence of the insert of pKM95, including the overlapping region to the *Nde*I site of pKM9, consisting of 25,467 bp, was determined and has been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB164642. Frame analysis of the nucleotide sequence performed using

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Fig. 1. Restriction enzyme map of the isolated DNA fragments and schematic representation of the ORFs identified on the kanamycin biosynthetic gene cluster.

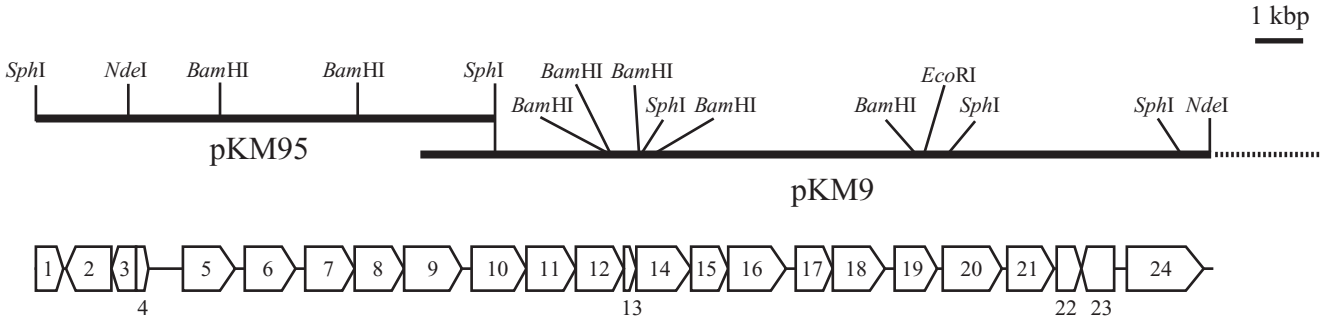
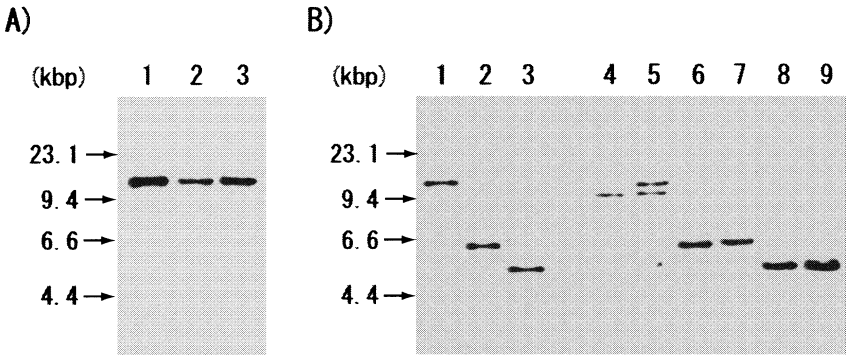


Table 1. Summary of genes identified on the kanamycin biosynthetic gene cluster.

Gene	Size (aa)	Proposed function	Closest homologue	ID/SM[a]	Accession number
orf1 (*kac*)	176	kanamycin resistance	aminoglycoside 6'-*N*-acetyltransferase	100/100	BAD11815
orf2	382	unknown	*S. cinnamoneus* Cinorf14	33/41	CAD60536
orf3	195	unknown	*S. coelicolor* conserved hypothetical protein	26/42	CAB86100
orf4	70	unknown	nothing		
orf5	369	transcriptional regulator	*S. griseus* StrR	43/53	CAA68515
orf6	343	dehydrogenase	*Micromonospora echinospora* GntP	42/59	AAR98562
orf7	413	transporter	*S. coelicolor* putative transport protein	32/47	CAB76879
orf8	392	aminotransferase	*Micromonospora echinospora* GntH	35/54	AAR98554
orf9	508	oxidoreductase	*Micromonospora echinospora* GntX	40/52	AAR98543
orf10	427	aminotransferase	*Micromonospora echinospora* GntA	59/70	AAR98547
orf11	390	2-deoxy-*scyllo*-inosose synthase	*Micromonospora echinospora* GntB	56/68	AAR98548
orf12	373	dehydrogenase	*Micromonospora echinospora* GntC	43/56	AAR98549
orf13	51	unknown	nothing		
orf14	418	glycosyltransferase	*Micromonospora echinospora* GntD	58/79	AAR98550
orf15	249	unknown	*Methanosarcina mazei* conserved protein	25/43	AAM31796
orf16	419	aminotransferase	*Micromonospora echinospora* GntF	58/73	AAR98552
orf17	285	unknown	*Magnaporthe grisea* hypothetical protein	32/48	EAA53554
orf18	352	unknown	nothing		
orf19 (*kmr*)	277	kanamycin resistance	16S rRNA methyltransferase	100/100	BAD11814, CAA75800
orf20	414	glycosyltransferase	*Micromonospora echinospora* GntZ	51/66	AAR98545
orf21	335	unknown	*Mycobacterium leprae* conserved hypothetical protein	61/75	CAC30300
orf22	132	unknown	*S. coelicolor* conserved hypothetical protein	78/86	CAA22726
orf23	223	unknown	*S. avermitilis* hypothetical protein	67/72	BAC69619
orf24	593	dehydrogenase	*S. avermitilis* putative 3-hydroxyacyl-CoA dehydrogenase	86/92	BAC69620

a: ID; % identity, SM; % similarity.

Fig. 2. Southern blot analyses of genomic DNAs of the wild type and high-producing strains using the insert DNA of pKM95 (A) and the 4.95-kbp *Sph*I fragment (B) as probes.



A: lane1; pKM95 digested with *Sph*I, lane2; genomic DNA of the wild type strain digested with *Sph*I, lane3; genomic DNA of the high-producing strain digested with *Sph*I. B: lane1; pKM9 digested with *Bam*HI, lane2; pKM9 digested with *Bam*HI and *Nde*I, lane3; pKM9 digested with *Sph*I, lane4; genomic DNA of the wild type strain digested with *Bam*HI, lane5; genomic DNA of the high-producing strain digested with *Bam*HI, lane6; genomic DNA of the wild type strain digested with *Bam*HI and *Nde*I, lane7; genomic DNA of the high-producing strain digested with *Bam*HI and *Nde*I, lane8; genomic DNA of the wild type strain digested with *Sph*I, lane9; genomic DNA of the high-producing strain digested with *Sph*I.

FramePlot[17] revealed that there are 24 open reading frames (ORFs) as shown in Fig. 1. The deduced gene products of the individual ORFs were analyzed using the BLAST program[18] (ver. 2.2.8) available through the National Center for Biotechnology Information, and the results are summarized in Table 1. *orf19* corresponded to the *kmr* gene used as a probe. *orf1* corresponded to the aminoglycoside 6′-*N*-acetyltransferase gene (*kac*)[11,12] which is one of the kanamycin resistance genes previously isolated from *S. kanamyceticus*. The product of *orf11* showed significant homology to 2-deoxy-*scyllo*-inosose synthase, a key enzyme involved in the biosynthesis of 2-deoxystreptamine[19]. In addition to these genes, several genes likely involved in kanamycin biosynthesis, such as aminotransferase and glycosyltransferase genes, were also found in the gene cluster. These facts indicated that the isolated gene cluster constitutes at least part of the kanamycin biosynthetic gene cluster, although further analyses, such as gene disruption and heterologous expression, would be required for the final confirmation.

The genomic DNAs of the wild type strain (NBRC 13414) and a high-producing strain 12-1, which is a parent of the high-producing strain 21-18 described above, were compared by Southern blot analysis using the DNA fragments contained in the isolated cluster as probes and an ECL direct DNA/RNA detection system. When the entire insert DNA of pKM95 was used as a probe (Fig. 2A), a single 10.3-kbp band corresponding to the probe was detected in both *Sph*I-digested genomic DNA samples. The intensity of the band is likely stronger with the high-producing strain DNA than with the wild type strain DNA. When the 4.95-kbp *Sph*I fragment containing *orf20-24* was used as a probe (Fig. 2B), two bands at positions 9.6- and 10.8-kbp were detected in the *Bam*HI digested genomic DNA sample of the high-producing strain, where the 10.8-kbp band corresponded to a *Bam*HI fragment derived from pKM9. On the other hand, only a single band at position 9.6-kbp was detected in the sample derived from wild type strain, while identical size bands were detected in both samples digested with *Sph*I or double-digested with *Bam*HI and *Nde*I. These results indicated that the high-producing strain had another copy of the biosynthetic gene cluster, the right-side end of which was different from the original cluster present in the wild type strain. Although cosmid clone pKM9 had the insert derived from the high-producing strain specific gene cluster given that a 10.8-kbp band corresponding to the high-producing strain specific gene cluster was detected in the *Bam*HI digested sample of pKM9, the nucleotide sequence determined in this study was based on the common region between the original and

high-producing strain specific gene clusters, from *Sph*I to *Nde*I. The increase in copy number of the biosynthetic gene cluster in the high-producing strain, although it remains unknown whether the entire cluster is present in an extra copy, might account for the improved production of kanamycin.

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References

1) UMEZAWA, H.; M. UEDA, K. MAEDA, K. YAGISHITA, S. KONDO, Y. OKAMI, R. UTAHARA, Y. OSATO, K. NITTA & T. TAKEUCHI: Production and isolation of a new antibiotic, kanamycin. J. Antibiotics A10: 181~188, 1957
2) KONDO, S. & K. HOTTA: Semisynthetic aminoglycoside antibiotics: Development and enzymatic modifications. J. Infect. Chemother. 5: 1～9, 1999
3) UMEZAWA, H.; S. UMEZAWA, T. TSUCHIYA & Y. OKAZAKI: 3′,4′-Dideoxy-kanamycin B active against kanamycin-resistant *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antibiotics 24: 485～487, 1971
4) KAWAGUCHI, H.; T. NAITO, S. NAKAGAWA & K. FUJISAWA: BB-K8, a new semisynthetic aminoglycoside antibiotic. J. Antibiotics 25: 695～708, 1972
5) KONDO, S.: K. IINUMA, H. YAMAMOTO, K. MAEDA & H. UMEZAWA: Syntheses of 1-*N*-{(S)-4-amino-2-hydroxybutyryl}-kanamycin B and -3′,4′-dideoxykanamycin B active against kanamycin-resistant bacteria. J. Antibiotics 26: 412～415, 1973
6) RODRIGUEZ, E. & R. MCDANIEL: Combinatorial biosynthesis of antimicrobials and other natural products. Curr. Opin. Microbiol. 4: 526～534, 2001
7) OHNUKI, T.; T. IMANAKA & S. AIBA: Self-cloning in *Streptomyces griseus* of an *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. J. Bacteriol. 164: 85～94, 1985
8) DAIRI, T.; T. OHTA, E. HASHIMOTO & M. HASEGAWA: Organization and nature of fortimicin A (astromicin) biosynthetic genes studied using a cosmid library of *Micromonospora olivasterospora* DNA. Mol. Gen. Genet. 236: 39～48, 1992
9) OTA, Y.; H. TAMEGAI, F. KUDO, H. KURIKI, A. KOIKE-TAKESHITA, T. EGUCHI & K. KAKINUMA: Butirosin-biosynthetic gene cluster from *Bacillus circulans*. J. Antibiotics 53: 1158～1167, 2000
10) KHAREL, M. K.; D. B. BASNET, H. C. LEE, K. LIOU, J. S.WOO, B. G. KIM & J. K. SOHNG: Isolation and characterization of the tobramycin biosynthetic gene cluster from *Streptomyces tenebrarius*. FEMS Microbiol. Lett. 230: 185～190, 2004
11) MURAKAMI, T.; C. NOJIRI, H. TOYAMA, E. HAYASHI, K. KATUMATA, H. ANZAI, Y. MATSUHASHI, Y. YAMADA & K. NAGAOKA: Cloning of antibiotic-resistance genes in *Streptomyces*. J. Antibiotics 36: 1305～1311, 1983
12) MATSUHASHI, Y.; T. MURAKAMI, C. NOJIRI, H. TOYAMA, H. ANZAI & K. NAGAOKA: Mechanisms of aminoglycoside-resistance of *Streptomyces* harboring resistant genes obtained from antibiotic-producers. J. Antibiotics 38: 279～282, 1985
13) NAKANO, M. M.; H. MASHIKO & H. OGAWARA: Cloning of the kanamycin resistance gene from a kanamycin-producing *Streptomyces* species. J. Bacteriol. 157: 79～83, 1984
14) DEMYDCHUK, J.; Z. OLIYNYK & V. FEDORENKO: Analysis of a kanamycin resistance gene (*kmr*) from *Streptomyces kanamyceticus* and a mutant with increased aminoglycoside resistance. J. Basic. Microbiol. 38: 231～239, 1998
15) JOE, Y. A. & Y. M. GOO: Kanamycin acetyltransferase gene from kanamycin-producing *Streptomyces kanamyceticus* IFO 13414. Arch. Pharm. Res. 21: 470～474, 1998
16) KIESER, T.; M. J. BIBB, M. J. BUTTNER, K. F. CHATER & D. A. HOPWOOD: Practical *Streptomyces* Genetics. p. 412, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, England, 2000
17) ISHIKAWA J. & K. HOTTA: FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. FEMS Microbiol. Lett. 174: 251～253, 1999
18) ALTSCHUL, S. F.; T. L. MADDEN, A. A. SCHAFFER, J. ZHANG, Z. ZHANG, W. MILLER & D. J. LIPMAN: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389～3402, 1997
19) KUDO, F.; H. TAMEGAI, T. FUJIWARA, U. TAGAMI, K. HIRAYAMA & K. KAKINUMA: Molecular cloning of the gene for the key carbocycle-forming enzyme in the biosynthesis of 2-deoxystreptamine-containing aminocyclitol antibiotics and its comparison with dehydroquinate synthase. J. Antibiotics 52: 559～571, 1999