Cryptic Kanamycin Resistance Gene in *Streptomyces griseus*

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Cryptic genes are phenotypically silent DNA sequences, which are not normally expressed during life cycles but capable of being activated as a rare event by mutation, recombination, insertion, deletion or other genetic mechanisms. These genes are different from nonfunctional gene defined as silent DNA sequence that cannot be reactivated. Cryptic genes are widespread among prokaryotic and eukaryotic microorganisms. Wild-type *Escherichia coli* K-12 has a cryptic *bgl* (β-glucosides resistance) operon[1-4] and an *argM* (acetylornithine-δ-transaminase) gene[5] which can be activated by transposition of an insertion sequence and mutation, respectively. Cryptic *trpE* (anthranilate synthase) gene of *Shigella dysenteriae*, which is a natural auxotroph requiring at least 12 amino acids, can be activated by mutation[7]. Strains of *Saccharomyces cerevisiae* have cryptic *SUC2* (invertase) gene which can be mutagenically activated[12]. Thus the activation of these cryptic genes does not involve the mediation of regulatory protein such as activator or repressor, but their own DNA structure changes which have a direct effect on their expression. From this point of view, the activation of cryptic genes will be the regulatory event which accompanies DNA structure changes such as site-specific inversion responsible for phase variation in *Salmonella*[13].

*Streptomyces* are noted for their genetic instability. Certain phenotypes such as the formation of aerial mycelium, antibiotics and pigments are lost at remarkably high frequencies. Such phenomena reported so far fall into negative phenotypic changes, but the activation of cryptic kanamycin resistance gene described below is phenotypically positive change. Therefore, considered that decrpytification of cryptic gene is another face of genetic instability in this group of microorganisms. Since cryptic genes do not normally express, in most cases their existence is not initially revealed by direct analysis of genomic DNA. For this reason, the existence of cryptic gene in actinomycetes has been missed so far.

Here I describe the first discovery of cryptic gene in actinomycetes, cloning and expression of a cryptic kanamycin resistance gene and its activated form of *Streptomyces griseus*, and sequencing of the resistance gene. Possible role of cryptic gene is also discussed.

Discovery of cryptic KM-resistance gene

*Streptomyces griseus* is generally sensitive to kanamycin (KM). However the generation of KM-resistant strains such as SS-1198PR and NP1-1PR has been found to occur through the protoplast regeneration of a streptomycin-producing (SM) soil-isolate *S. griseus* SS-1198 and its SM-nonproducing mutant NP1-1 at a frequency of 10^{-8}, respectively[11,12]. This phenomenon occurs reproducibly, and the frequency is too high to ignore. Biochemical analyses of these KM-resistant strains have demonstrated that the resistance was not due to the mutation of ribosome gene but to occurrence of aminoglycoside-inactivating enzyme activity, which was detected only little in the parental strain[10]. The structure of the inactivated KM was determined as 3-N-acetyl-KM indicating the acetylation of KM by an aminoglycoside-3-N-acetyltransferase, AAC(3)[13]. It was thus expected that a cryptic KM-resistance gene existed in *S. griseus*.

Cloning of *kan*

In order to analyze genetic mechanism(s) for the activation of cryptic KM-resistance gene, we cloned the resistance gene (*kan*) from SS-1198PR using *S. lividans* host-vector system[13]. It was revealed that *kan* lied within a 15 kb AcI fragment cloned as the plasmid pANT. Subcloning experiments localized the essential coding sequence of *kan* to the 1.5 kb MluI segment which was completely
contained within the 15 kb BglII fragment (unpublished data). When the 1.5 kb NsiI fragment was inserted into a vector in both directions, kan expressed equally, indicating the presence of kan own promoter in the fragment.

KM-resistant strains of S. griseus and S. lividans carrying the cloned kan were tested for their ability to grow in the presence of various aminoglycoside antibiotics (AGs). These strains were resistant not only to KM (1000 µg ml⁻¹) but also to gentamicin (200 µg ml⁻¹), dibekacin (400 µg ml⁻¹), and to a lesser extent (5-25 µg ml⁻¹), ribostamycin, paromomycin and neomycin. This result indicated that kan gave rise to multiple resistance to a certain range of AGs, resulting in the same additional resistance pattern in both S. griseus and S. lividans.

Detection of the cryptic KM-resistance gene in the wild-type strain

We carried out Southern hybridization experiment to detect the cryptic KM-resistance gene which should be present in the wild-type strain, SS-1198. A 1.3 kb BamH1 fragment, which contains kan, was labeled with [³²P]dCTP and used to probe SphI-digested total genomic DNAs of S. griseus strains. Hybridization yielded identical band patterns and identical 4.2 kb bands with comparable strength for all of the strains except NP1-1PR. This suggested that the activation of the cryptic KM-resistance gene was not due to insertion, deletion or other genomic rearrangements except NP1-1PR, because they would make structural changes of DNA sequences.

In contrast, the digest of NP1-1PR DNA was gave rise to slightly larger band (4.7 kb) with much stronger hybridization signal. Correspondingly, the same size band resulting from DNA amplification was seen in the agarose gel. These findings indicated that kan homologous sequence of NP1-1PR was amplified, and it was thought the possibility that genomic rearrangement participated in the process of the activation of the cryptic KM-resistance gene in NP1-1PR.

Cloning of kan’ and kan²

The amplified 4.7 kb SphI fragment containing kan homologous sequence of NP1-1PR was readily isolated from agarose gel because of a very high copy number. It was ligated to SphI-digested pIJ702 and introduced into S. lividans. Resistance levels to AGs conferred by the resultant plasmid (pANT6) were as low as (1/10-1/20) of those conferred by pANT3-1 consisting of pIJ702 and the 4.2 kb SphI fragment containing kan. Consequently the resistance determinant of NP1-1PR was designated kan’ to distinguish from kan.

We cloned kan homologous sequence (cryptic KM-resistance gene) of SS-1198 in Escherichia coli, because of its crypticity, by colony hybridization technique using a kan probe. Recombinant plasmid with the insert of a 4.2 kb SphI fragment (pANT4) appeared to contain the cryptic state gene (kan²) of SS-1198. The 1.8 kb BglII-BamH1 region containing kan² in pANT4 was compared with the corresponding fragments in pANT3 and pANT6 which contained kan and kan’, respectively. No difference in restriction sites and size was observed among these fragments. This confirmed the lack of DNA rearrangement in the process by which kan² was reactivated, and strongly suggested that kan² was converted from cryptic state to functional state by a mutation.

Introduction of kan² into S. lividans was not expected to confer any resistance to the host, and so we tried to transform S. lividans with kan². However repeated attempts did not succeed for unknown reason(s). It is possible that the DNA sequence containing kan² is extremely unstable in S. lividans, or that kan² is not silent in S. lividans and its product causes toxic effect.

It seems likely that the gene amplification of kan’ is an additional event after the activation of kan², because kan² and kan’ are clearly distinguishable from each other in their abilities to transform S. lividans. I would propose that in the strain NP1-1PR a mutation in kan² occurred first to give rise to kan’ which was then amplified.

Analysis of mRNA

Total RNA extracts prepared from S. griseus strains were analyzed to estimate concentration of specific mRNAs relating to kan (unpublished data). Slot hybridization with kan probe showed that little or no mRNA was detected from the KM-sensitive strain SS-1198, and that KM-resistant strains SS-1198PR and NP1-1PR clearly produced the specific mRNA. In particular, NP1-1PR yielded much stronger mRNA signal, which presumably reflected the higher level of the specific mRNA present due to the amplification of kan’ gene. These findings suggested that the activa-
tion of the cryptic KM-resistance gene was regulated at transcriptional level.

Resistance levels to AGs of *S. lividans* TK21 carrying *kan*<sup>20</sup> were much lower (1/20) than those of the strain carrying *kan*, whereas in *S. griseus*, the strain NPI-1PR carrying *kan*<sup>20</sup> showed high-level resistance to AGs as well as SS-1198PR carrying *kan*. This might suggest that high-level resistance of NPI-1PR was expressed by the overproduction of *kan*-directed mRNA as a result of gene amplification of *kan*.<sup>20</sup>

To determine 5' end of specific mRNA relating to *kan*, the total RNA extracts were subjected to S1 nuclease mapping (unpublished data). Two 5' end-labeled fragments as probes were prepared from the DNA segment containing *kan* and independently hybridized to the RNA extracts to determine the direction of transcription. Protected fragments were detected when one of the probes hybridized with RNAs that were isolated from KM-resistant strains of both *S. griseus* and *S. lividans*. Moreover, the detected fragments were identical in size with each other. This indicated that *kan* was transcribed into mRNA from the same position in both *S. griseus* and *S. lividans*. In contrast no protected fragment was observed when RNA prepared from SS-1198 carrying *kan*<sup>20</sup> was used.

These results suggested that transcription of *kan* was regulated in the same manner in both *S. griseus* and *S. lividans*, and *kan*-specific mRNA was not formed or accumulated in *S. griseus*. Consequently, it seems likely that a mutation which activated *kan*<sup>20</sup> is effective to transcription of the gene.

Nucleotide sequence of *kan*

Sequencing of the 1.5 kb MluI fragment containing *kan* revealed an open reading frame (ORF), which could generate an about 30,000 Dalton protein, with translational start codon ATG (unpublished data). This ORF is presumably translated to an AAC(3) which is responsible for KM resistance. Based on the results of S1 nuclease mapping, the transcriptional start point of *kan* was estimated to be approximately 90 bases upstream from the ATG (unpublished data). *E. coli* consensus promoter motif was found in -10 region, while no sequence homology occurred in -35 region. However it was interesting that the -35 region showed considerable similarity to that of *aph*<sup>E</sup>. Two inverted repeats, which would act as transcriptional terminator, were found at past the TGA termination codon.

It is expected that sequencing of *kan*<sup>20</sup> provides precise location of the mutation which is responsible for the activation of cryptic KM-resistance gene, but the work is not completed yet. The analysis of total RNA extract prepared from SS-1198 has shown that *kan*<sup>20</sup> was not transcribed into mRNA. Accordingly, we consider that *kan*<sup>20</sup> would be activated by the mutation at the 5' region of *kan*<sup>20</sup>, providing a promoter activity.

Possible role of cryptic gene

When I think about cryptic gene and its activation, I cannot help considering a problem. That is why cryptic gene has not been degenerated as unnecessary DNA sequences, in spite of its no contribution to host organism normally. A possible reason for this will be that cryptic gene might be necessary for adaptation to environmental changes. I believe that the presence of cryptic gene may be advantageous under a certain condition, which is probably unusual. Environmental changes may occur in a repetitive fashion. Regulation such as induction and/or repression of operon and allosteric control of enzyme activity must be regarded as a type of adaptation mechanisms to environmental changes which occur repeatedly during life cycles, that thought to be relatively short. Furthermore, cryptification and decryption of genes may be a regulation for adaptations to cyclic environmental changes repeated over long evolutionary span. Therefore I would consider that the activation of cryptic gene is a programmed event.

It has been reported that microorganisms acquired new metabolic capacity through genetic alterations which occur in response to selective pressure<sup>20</sup>. Whereas some of these cases are due to only reactivation of cryptic gene<sup>20</sup>. On the other hand, members of a specific group of bacteria differ from one another in terms of particular metabolic capacities. In some cases absence of a capacity results from absence of genetic information, in other cases lack of gene expression. Thus it is possible that we do not justice to the potential abilities of microorganisms. If we can recognize microorganisms have what capacity, improvement and/or development of useful microorganisms may be relatively easier than expected. That is because
activation of cryptic genes reported so far is directed by well known genetic mechanisms.

It will be feasible to activate cryptic gene artificially, for current gene technology is well developed. If so, it will lead us to discovery of a novel biologically active substance from actinomycetes. Additionally, expression of the activated gene can be enhanced by gene amplification, since this group of microorganisms seems to harbor additional DNA segments over several percent of the genome in length.

**SUMMARY**

Cryptic kanamycin resistance gene (kan*) and its functional forms (kan and kan') of *Streptomyces griseus* strains were genetically analyzed. Cloning of these genes cleared that resistance determinants (kan and kan) lied within 1.5 kb *MluI* fragments with no difference in restriction site and size compared to the corresponding fragment containing kan*. Gene amplification of kan' in a KM-resistant strain caused a highly elevated expression of kan' due to gene dosage effect. Sequencing of the 1.5 kb *MluI* fragment containing kan revealed an ORF which could generate an about 30,000 Dalton protein regarded presumably as an AAC(3). Analysis of mRNA revealed that activation of kan* was regulated at transcription level. Based on these results, we propose that the cryptic gene kan* was activated by a mutation at the 5' region, providing a promoter activity.

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**REFERENCES**


