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ABC Transporter Genes, *kasKLM*, Responsible for Self-resistance of a Kasugamycin Producer Strain

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We previously reported that a 7.6-kb DNA fragment from *Streptomyces kasugaensis* M338-M1, a kasugamycin (KSM) producer, included KSM acetyltransferase gene (*kac*^{338}) and some other genes possibly involved in KSM biosynthesis. As an extension of that study, a 10-kb *Sac*I-*Kpn*I DNA fragment, located 5~15-kb upstream of *kac*^{338}, was cloned and a 4.2-kb *Sac*I-*Eco*RI fragment therefrom was sequenced, revealing one incomplete (designated ORF J) and three complete open reading frames (designated *kasK*, *kasL* and *kasM*). The coding frames of *kasK*, *L* and *M* overlap one another with terminator/initiator ATGA sequence. RT-PCR analysis of a DNA region including *kasKLM* indicated the presence of one transcript that is long enough to span the three genes. The *kasK* gene potentially encodes an ATP-binding protein of the ATP-binding cassette (ABC) transporter superfamily. Homology search for the deduced KasK protein shows similarity to other ABC transporters involved in self-resistance of a mithramycin and possibly doxorubicin producer strain. The *kasL* and *kasM* genes encode different integral membrane proteins, both having six putative transmembrane helices. An expression plasmid for *kasKLM* (pTV-KLM) was constructed and these genes were expressed in *E. coli* JM109, which had been sensitive to KSM. The transformant acquired resistance to KSM, suggesting that KasK, L and M proteins as a set in *S. kasugaensis* M338-M1 pump out KSM to protect the producer from its toxic metabolite.

Organisms producing potentially autotoxic antibiotics possess basically three types of self-resistance mechanisms to avoid suicide: (1) modification of the target site that the antibiotic acts on, (2) intracellular inactivation of the antibiotic and (3) exclusion of the antibiotic from the cell[1]. The last resistance mechanism is designated "membrane-associated system" consisting of two classes. In one class, resistance is mediated by membrane proteins, which are believed to energize export of antibiotic molecules by proton-dependent transmembrane electrochemical gradients. The other class belongs to the ABC transporter superfamily[2] comprising many membrane-associated export and import systems, which are present both in prokaryotic and in eukaryotic cells. ABC transporters possess a highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily. They participate in the secretion of antibiotics from the producers, utilizing the energy from ATP hydrolysis to pump out the toxic metabolites across the membrane even against a concentration gradient. Some genes encoding ABC transporters have been cloned and characterized: *drrAB*[3] from *S. peucetius* (daunorubicin/doxorubicin resistance), *mtrAB*[4] from *S. argillaceus* (mithramycin resistance) and *OleC,C5*[5] from *S. antibioticus* (oleandomycin resistance). The mechanism of *drrAB* is similar to P-glycoprotein[6] for multidrug resistance of human cancers, suggesting that understanding the mechanisms of bacterial ABC transporters may shed light on how to solve the drug-resistance problems associated with some human diseases.

We previously cloned and characterized some KSM biosynthetic genes[7] from *S. kasugaensis* M338-M1[8]. In the present paper we report the cloning, characterization and functional analysis of ABC transporter type genes

(*kasKLM*) from *S. kasugaensis* M338-M1.

Materials and Methods

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this work are described in Table 1. *S. kasugaensis* M338-M1 has been maintained at the Institute of Microbial Chemistry. The following strains and plasmids were of commercial origins: *Escherichia coli* TH2[9] (TaKaRa, Code No. 9056), *E. coli* DH5α[10] (TOYOBO, Code No. DNA-903), *E. coli* JM109[10,11] (TaKaRa, Code No. 9052), cloning vector pKF 3[9,12] (TaKaRa, Code No. 3100), pUC118[13] (TaKaRa, Code No. 3318) and expression vector pTV118N[14] (TaKaRa, Code No. 3328). Other plasmids were produced in the present study.

Growth Conditions

S. kasugaensis M338-M1 was grown in TSB medium (DIFCO, 0370-17-3) and/or MR medium (KSM producing medium: 2% glycerol, 2% dextrin, 1% Bacto soyton, 0.3% yeast extract, 0.2% ammonium sulfate, 0.2% calcium carbonate pH 7.0) under shaking at 27°C for 24~96 hours. *E. coli* TH2 transformants were grown at 37°C in L-broth containing chloramphenicol (12 μg/ml) and streptomycin (50 μg/ml). *E. coli* DH5α and JM109 transformants were grown at 37°C in YT medium containing ampicillin (100 μg/ml).

Cloning and Sequencing

Isolation of genomic DNA from *S. kasugaensis* M338-M1 was conducted as described previously[15]. The *E. coli* TH2/pKF 3 cloning system was used to clone pSKE 4 and 5.

The cloned DNA region (4.2-kb *Sac*I-*Eco*RI fragment) was digested with appropriate restriction endonucleases, and the fragments were subcloned into pUC118 (pKS15, 22~25, Table 1) and sequenced with an automated laser fluorescence sequencer (ALF*red*™ DNA Sequencer, Amersham Pharmacia Biotech), using the ALFexpress™ AutoCycle™ Sequencing Kit (Amersham Pharmacia Biotech, Code No. 27-2693-02) according to the supplier's instructions. Sequencing primers used were M13 universal and reverse primers and some synthesized oligonucleotide primers (labeled with Cy5, purchased from Amersham Pharmacia Biotech).

Computer Analysis of DNA and Protein Sequences

DNA and protein sequences were analyzed with the DNASIS-Mac version 3.6 (Hitachi Software Engineering Co. Ltd.). Amino acid sequences of potential gene products were compared with those in the databases (SWISS-PROT and PIR) by means of FASTA[16] and BLAST[17].

Nucleotide Sequence Accession Number

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB033992.

Preparation of RNA

S. kasugaensis M338-M1 was cultured for 24 hours as described above. Approximately 1 g of wet mycelia was resuspended in 10 ml of the extraction buffer (see below) containing 4 M guanidinium thiocyanate. The mycelia were broken in a sonicator (Branson Sonifier 350) by 5 cycles of 30 seconds beating at 6 watts at 0°C and 30 seconds of chilling at 0°C. The cell lysate was treated as described in the instructions of RNA extraction kit (Amersham Pharmacia Biotech, 27-9270-01) to prepare total RNA.

RT-PCR

Reverse transcription (RT) and PCR amplification were performed with RNA LA PCR™ Kit (AMV) Ver. 1.1 (TaKaRa, RR012A) according to the instructions of the supplier. One μg of total RNA from *S. kasugaensis* M338-M1, which was used as template for cDNA synthesis, was treated with 7.5 units of RNase-free DNase I for 2 hours at 37°C to eliminate residual genomic DNA. 3′-pKS15 (5′-GCGACACAGACCTCCAGCCCCAGTT-3′, Fig. 4A) was used as a primer for first-strand cDNA synthesis. The RT reaction was performed with avian myeloblastosis virus reverse transcriprase for 30 minutes at 60°C. For the following PCR amplification, 5′-pKS23b (5′-TCCGGGATTTCCGAAGGAACGGCGT-3′) and the 3′-pKS15 were used as primers. A 100 μl PCR mixture contained 20 μl of the RT mix, 20 pmol each of the two primers, 2.5 units of LA *Taq* DNA polymerase (TaKaRa) and 1×LA PCR buffer. The PCR involved 30 cycles of denaturation (30 seconds at 97°C), annealing (1 minute at 65°C) and extension (1 minute at 72°C). Control amplification using equivalent amounts of RNA that had not been incubated with reverse transcriptase was conducted to confirm that genomic DNA was not contributing to the PCR amplification. The RT-PCR products were electrophoresed on a 0.6% agarose gel and visualized using ethidium bromide staining (Fig. 4B).

Table 1. Strains and plasmids.

Strains and plasmids	Genotype and genetic construct	Reference
Strains		
S. kasugaensis M338-M1	Kasugamycin producing strain	8
E. coli DH 5α	φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hidR17*(r_K^-, m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	10
TH 2	*supE44 hsdS20*(r_B^-, m_B^+) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 thi-1 trpR624*	9
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) / F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	10, 11
Plasmids		
pUC118	Cloning vector. Amp^r. 3.1-kb.	13
pTV118N	Expression vector derived from pUC118, containing a unique *Nco* I site. Amp^r. 3.1-kb.	14
pKF 3	Cloning vector. Sm^s, Cm^r. 2.2-kb.	9, 12
pSKE 4	pKF 3 derivative incorporating 8.4-kb *Kpn*I fragment from *S. kasugaensis* M338-M1. 10.6-kb.	This work
pSKE 5	pKF 3 derivative incorporating 5.5-kb *Sac*I fragment from *S. kasugaensis* M338-M1. 7.7-kb.	This work
pKS 15	pUC118 derivative containing 718 bp *Kpn*I-*Eco*RI fragment from pSKE 5. 3.9-kb.	This work
pKS 22	pUC118 derivative containing 577 bp *Sac*I-*Sma*I fragment from pSKE 5. 3.7-kb.	This work
pKS 23	pUC118 derivative containing 1135 bp *Sma*I-*Eco*RI fragment from pSKE 5. 4.3-kb.	This work
pKS 24	pUC118 derivative containing 846 bp *Eco*RI-*Sph*I fragment from pSKE 5. 4.0-kb.	This work
pKS 25	pUC118 derivative containing 965 bp *Sph*I-*Kpn*I fragment from pSKE 5. 4.1-kb.	This work
pTV-NE195	pTV118N derivative containing a PCR fragment (195 bp *Nco*I-*Eco*RI, a head region of *kasK*). Negative control for pTV-Kgal; *lacZ'* being fused out of frame.	This work
pTV-KLM	pTV118N derivative containing *kasKLM* (2.7-kb *Nco*I-*Eco*RI fragment) genes.	This work
pTV-Kgal	pTV118N derivative carrying a chimera gene consisting of a head region of *kasK* fused by *lacZ'* in frame.	This work
pTV-KLgal	pTV-KLM derivative in which *LM* region is replaced by a chimera gene consisting of a head region of *kasL* fused by *lacZ'* in frame.	This work
pTV-KLgal(-)	pTV-KLM derivative lacking 1.7-kb *Sph*I fragment. Negative control for pTV-KLgal; *lacZ'* being fused out of frame.	This work
pTV-KLMgal	pTV-KLM derivative carrying complete *kasKL* and a head region of *kasM* fused by *lacZ'* in frame.	This work
pTV-KLMgal(-)	pTV-KLM derivative lacking 0.7-kb *Kpn*I fragment. Negative control for pTV-KLMgal; *lacZ'* being fused out of frame.	This work

Subcloning of *kasK*, *L* and *M* into *E. coli* Expression Vector

To introduce a *Nco*I site including the start codon of the *kasK* gene, The 5′ region of the *kasK* gene (nt 1513~1708, Fig. 2) was amplified by PCR. 5′-ORF K (Sense: 5′-GGCCATGGTCGAGGTCAC-3′) and 3′-ORF K (Antisense: 5′-GGGAATTCTGACCAGGGT-3′) were used as PCR primers. PCR was performed using a MiniCycler™ (MJ Research). The reaction mixture contained 1 ng pKS23 (Table 1), 20 pmol of each primer, 50 μM each dNTP, 20 mM Tris-HCl pH 8.0, 25 mM KCl, 1.5 mM $MgCl_2$ and 0.05% Tween 20 in a final volume of 100 μl. Amplification involved 30 cycles of denaturation (30 seconds at 97°C), annealing (1 minutes at 65°C) and extension (1 minute at 72°C). The resulting PCR product was digested with *Nco*I/*Eco*RI and inserted into pTV118N, yielding pTV-NE195 (Table 1). The nucleotide sequences of the inserted DNA and its boundaries of pTV-NE195 were confirmed by the sequence analysis.

The 2.5-kb *Eco*RI fragment from pSKE 5 (Fig. 1) was subcloned into the *Eco*RI site of pTV-NE195, yielding pTV-KLM (Table 1, Fig. 5). The orientation of the inserted fragment was confirmed by sequence analysis.

Confirmation of Separate Translation of KasK, L and M Proteins

To confirm separate translational initiation of KasK, L and M proteins, pTV-KLM was modified to encode a fusion protein consisting of a short *N*-terminal peptide of either KasK, L or M and β-galactosidase α peptide (Fig. 5). To produce these derivatives, for example, a DNA region of nt 1513~1591 (Fig. 2), encoding the *N* terminal region of KasK, was PCR-amplified using as primers 5′-ORF K (Sense: 5′-GGCCATGGTCGAGGTCAC-3′) and 3′-ORF

K2 (Antisense: 5′-GGGAATTCGGTCCGCTCT-3′). The underlined sequences in the primers are *Nco*I site and *Eco*RI sites, respectively. The PCR product was digested with *Nco*I and *Eco*RI and inserted into pTV118N to obtain a recombinant plasmid (pTV-Kgal) that would encode a fusion protein consisting of Met^{1}~Pro^{26} of KasK and Asn^{4}~His^{106} of β-galactosidase α peptide. As a negative control, pTV-NE195 was used, which would encode a fusion protein consisting of Met^{1}~Ile^{65} of KasK and a frameshift (inactive) protein. Similarly, plasmids designed to encode a fusion protein consisting of Met^{1}~Ser^{17} of KasL and His^{19}~His^{106} of β-galactosidase α peptide (pTV-KLgal) and another fusion protein consisting of Met^{1}~Gly^{14} of KasM and Leu^{13}~His^{106} of β-galactosidase α peptide (pTV-KLMgal), and their negative controls (pTV-KLgal(−) and pTV-KLMgal(−), respectively), were also constructed (Table 1, Fig. 5).

β-Galactosidase Assay

E. coli JM109 transformants were cultured as described above. The expression of fused β-galactosidase genes was induced with 1 mM isopropylthiogalactopyranoside (IPTG) when the cell density reached 0.8~1.0 (OD_{660}). The cells were collected by centrifugation for 5 minutes at 5,000×*g*, resuspended in 5 ml of PBS and disrupted by sonication. The lysates were centrifuged for 5 minutes at 5,000×*g* to remove the cell debris. To measure the β-galactosidase activity of the fused proteins, 70 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside and 200 μl of PBS were added to the cell lysate (30 μl). The reaction was carried out for 30 minutes at 37°C and stopped by the addition of 0.5 ml of 1 M sodium carbonate. The absorbance at 420 nm by released *o*-nitrophenol was read.

Induction of KSM resistance in *E. coli* JM109

E. coli JM109 carrying pTV118N or pTV-KLM was grown at 37°C in YT medium containing ampicillin (100 μg/ml) under shaking. Growth was monitored by reading OD_{660} of the cultures. When the OD_{660} reached 0.5~0.7, 0.2 mM IPTG was added to the cultures and cultivation was continued at 37°C for 3 hours. Twenty μl portions of the cultures were inoculated into culture tubes containing 2 ml of YT medium with ampicillin (100 μg/ml) and KSM (200 μg/ml) and the growth was monitored by reading OD_{660} of the cultures at 37°C for 42 hours (Fig. 6).

Results

Cloning and Sequence Analysis

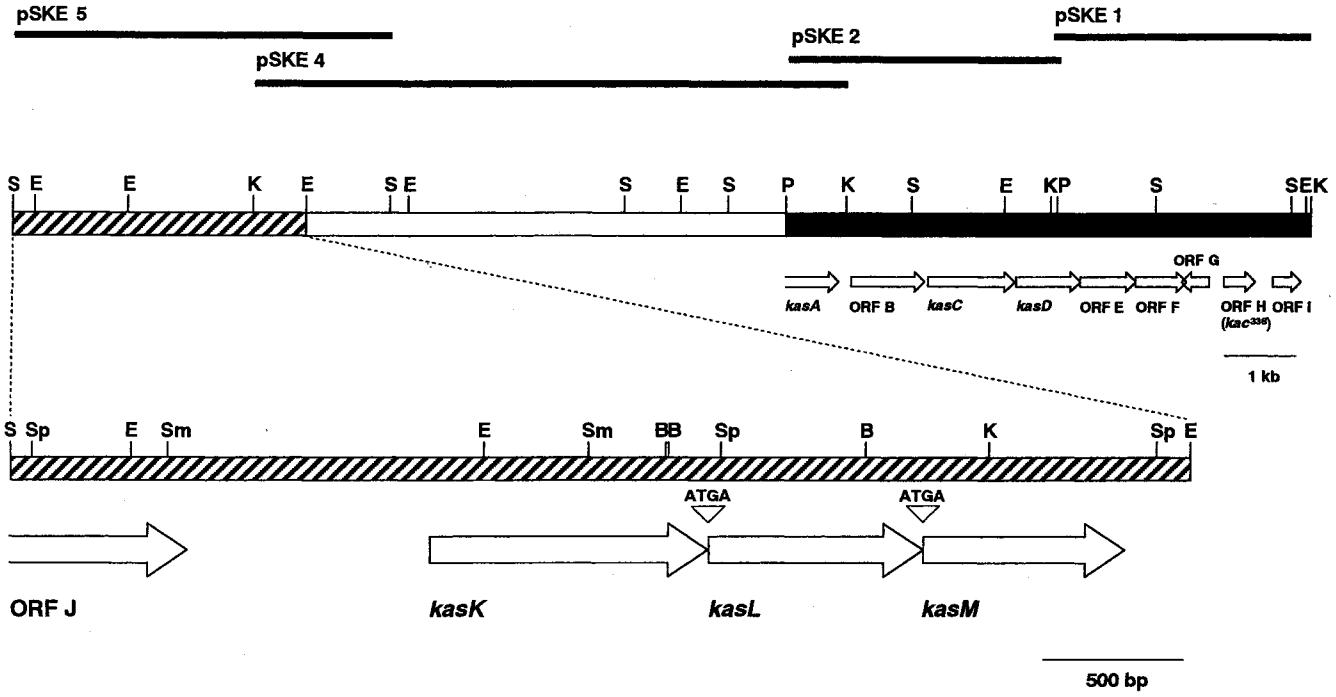
An 8.4-kb *Kpn*I fragment from *S. kasugaensis* M338-M1 chromosome was cloned using as a probe the 850 bp *Pst*I-*Kpn*I fragment from pSKE 2. The resultant recombinant plasmid was designated pSKE 4. A 5.5-kb *Sac*I fragment from *S. kasugaensis* M338-M1 chromosome was also cloned using as a probe the 594 bp *Kpn*I-*Sph*I fragment from pSKE 4. The plasmid was named pSKE 5 (Fig. 1). We sequenced the 4236bp *Sac*I-*Eco*RI DNA region from pSKE 5 (Fig. 1). The GC content of the region was calculated to be 67.7% from the sequence. Open reading frames (ORFs) were searched for based on the codon usage and third codon position bias which are characteristic of *Streptomyces* genes[18]. Within this region we recognized four ORFs, *i.e.* ORF J, *kasK*, *kasL* and *kasM*, though ORF J was incomplete. All these ORFs run in the same direction (Fig. 1). There were inverted repeat sequences downstream of both ORF J (nt 722~752, −32.30 kcal/mol) and *kasM* (nt 4062~4111, −63.80 kcal/mol), possibly functioning as transcriptional terminators (Fig. 2). In the intergenic space between ORF J and *kasK* (nt 646~1512), there is a relatively low GC content region for *Streptomyces* DNA (nt 1033~1303, GC%=57.2%), suggesting that DNA unwinds there to initiate transcription of the downstream genes.

There are three possible translational start points for *kasK i.e.*, nt 1297 ATG, nt 1468 ATG and nt 1513 ATG (Fig. 2). We favor the nt 1513 ATG as the translational start site for *kasK*, because the ATG is closely preceded by a potential ribosome binding site (RBS) (AAGGAG) and because the GC bias of third codon positions near that point is typical of *Streptomyces* codons. The coding frames of *kasK*, *L* and *M* overlap at their boundaries (ATGA), possibly suggesting translational coupling, as in many other bacterial genes[19,20]. The stop codon for the *kasK* is nt 2500 TGA, while the start for *kasL* is nt 2499 ATG, preceded by an RBS (GGGGG). The stop codon for *kasL* is nt 3270 TGA, while the start codon for *kasM* is nt 3269 ATG, preceded by an RBS (GGTGA). The stop codon for *kasM* is nt 3989 TGA.

Characterization of Putative KasK, L and M Proteins

The *kasK* gene is deduced to encode a protein (KasK) containing 329 amino acids with a molecular mass of 35,862 Da and a pI of 6.58. Kyte-Doolittle analysis[21] of KasK suggests it is hydrophilic (data not shown). The possible function(s) of KasK was deduced from the result

Fig. 1. Restriction map of the cloned DNA region from *S. kasugaensis* M338-M1, including the *kas* gene cluster.



On the restriction map, the striped region (left, also shown in an enlarged scale) is dealt with in the present paper. The open region (middle) remains to be studied. The solid region (right) has been reported[7]. The open arrows indicate the deduced ORFs and direction of transcription. There are two ATGA's at the boundaries between *kasK* and *kasL* and between *kasL* and *kasM*. Both ATGA are bifunctional; as the stop codon (TGA) for each preceding gene and as the initiation codon (ATG) for each following gene. The inserts of plasmids pSKE 1, pSKE 2, pSKE 4 and pSKE 5 are indicated above the map. Abbreviations: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; Sp, *Sph*I.

of a homology search using the FASTA and BLAST program. The deduced amino acid sequence of KasK showed 39% similarity with DrrA[3] protein which is responsible for self-resistance of *S. peucetius* to its own toxic metabolites, daunorubicin and doxorubicin. KasK was found to be similar to some other ATP-binding proteins; OleC[5] from *S. antibioticus* (39%) responsible for oleandomycin resistance, MtrA[4] from *S. argillaceus* (37%) for mithramycin resistance, NodI from *Rhizobium galegae* (37%) for oligosaccharide export (unpublished data, L. PAULIN *et al.*, Accession No. P50332), NatA[22] from *Bacillus subtilis* (36%) for extrusion of Na ion and TnrB2[23] from *S. longisporoflavus* (35%) for tetronasin resistance. The deduced amino acid sequence of KasK contains an ATP-binding motif[24] which is common to the large family of ABC transporters (Fig. 2).

The *kasL* and *kasM* encode proteins (KasL and KasM) containing 257 and 240 amino acids, with molecular masses of 27,497 and 26,431 Da and with pI's of 8.2 and 9.56, respectively. Kyte-Doolittle analysis of these putative proteins indicate that the two proteins would be extremely hydrophobic and both contain six transmembrane helices, therefore, would cross the cell membrane six times (Fig. 3). The deduced amino acid sequence of KasL showed 33% similarity with NodJ[25] from *Rhizobium leguminosarum* and 31% similarity with MtrB[4] from *S. argillaceus*. Comparison of KasM with other proteins also revealed 30% similarity with NodJ[25] from *Rhizobium leguminosarum*. These known proteins are membrane-spanning subunits in support of the above described ATP-binding subunits to form each transporter system.

Detection of mRNA of *kasK*, *L* and *M* by RT-PCR

We expect that *kasK*, *L* and *M* are transcribed into a polycistronic mRNA because these coding regions overlap

Fig. 2. Nucleotide sequence of a 4.2-kb *Sac*I-*Eco*RI region from pSKE 5 and deduced amino acid sequences of ORF J, *kasK*, *kasL*, and *kasM*.

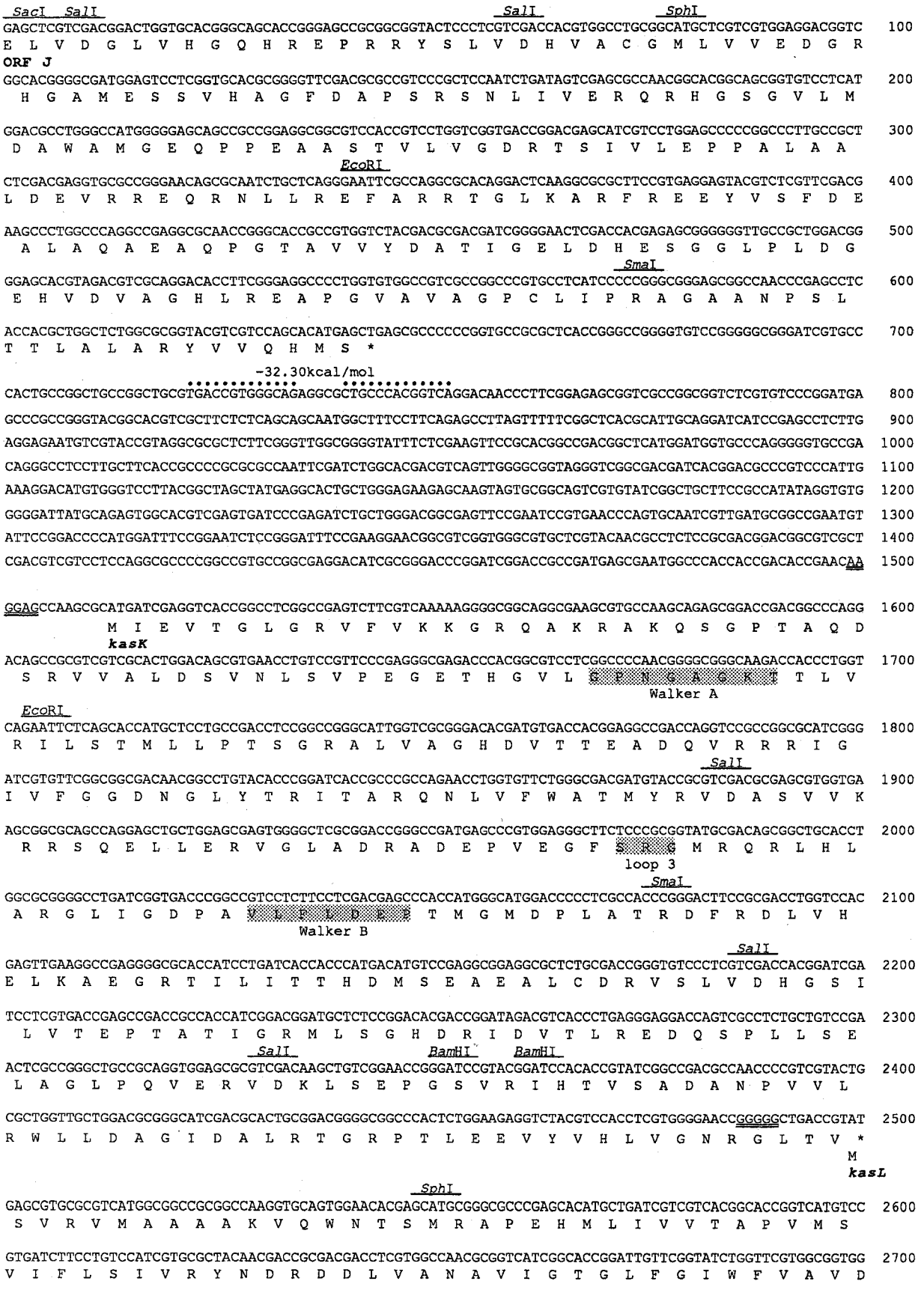
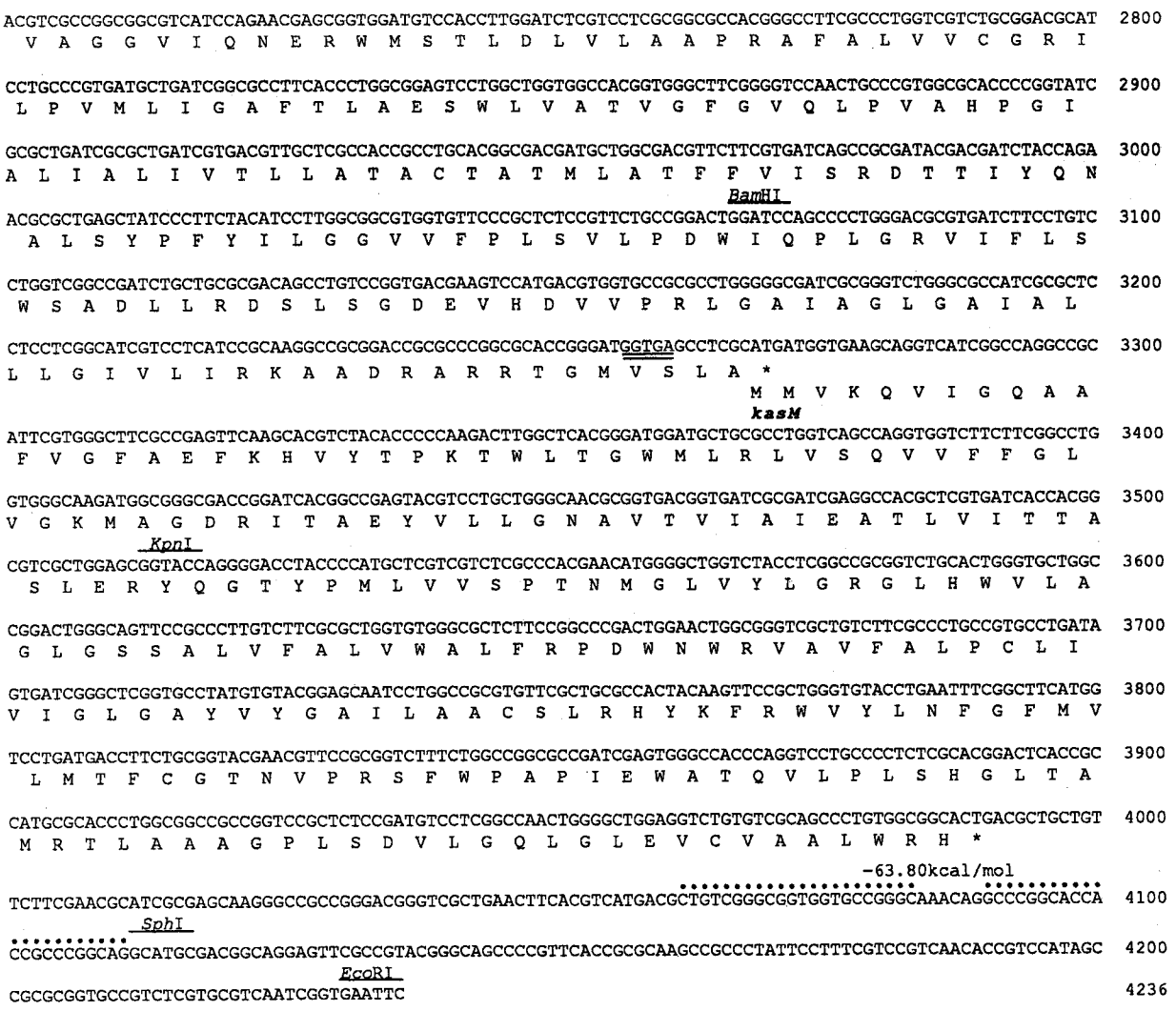


Fig. 2. (Continued)



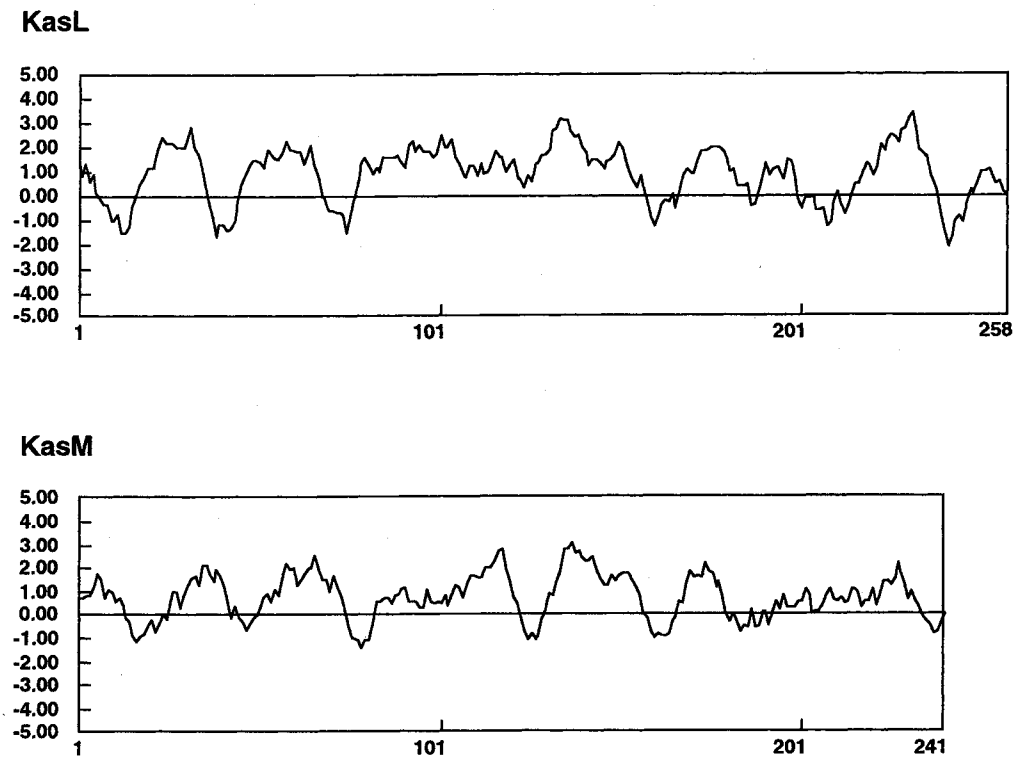
Shadowed amino acids represent the conserved motifs (Walker A, loop 3, and Walker B) for an ATP-binding cassette[21]. Double underlines and dots indicate putative ribosome binding site (RBS) and putative terminator of transcription, respectively.

one after another. To test the possibility, we analyzed the transcriptional product of *kasK*, *L* and *M* by RT-PCR. The hybridization positions and the orientations of the primers are shown in Fig. 4A, and the result of RT-PCR is shown in Fig. 4B. When the cDNA, synthesized with 3′-pKS15 primer, was used as the template for PCR, a product with the expected length was amplified (Fig. 4B, 2643bp fragment, Lane 2). This result suggests that *kasK*, *L* and *M* are transcribed as a polycistronic mRNA. The control run of PCR using equivalent amounts of RNA that had not been incubated with reverse transcriptase did not give any amplified product (Fig. 4B, Lane 3), indicating that the RNA template used for PCR amplification was not significantly contaminated with genomic DNA.

Confirmation of Codon-framing of *kasK*, *L* and *M*

We next tested if the codon-framings of *kasK*, *L* and *M* were correct. For this purpose, we constructed plasmids that included *N*-terminal 14~26 codons of either *kasK*, *L* or *M* fused in frame with β-galactosidase α peptide gene (Fig. 5). Translational initiation usually needs RBS that is located closely upstream of ATG. RBS from the expression vector, pTV118N, was expected to work for *kasK*, while nt

Fig. 3. Hydrophobicity analysis of the deduced KasL and KasM proteins.



Numerals on the absissas indicate the numbering of amino acids. Window size 10. Range of relative hydrophobicity 5 to -5.

2486 GGGGG and nt 3257 GGTGA (Fig. 2) might function as RBS for *kasL* and *M*, respectively. *E. coli* transformants harboring these plasmids showed β-galactosidase activity when induced with IPTG (Table 2). Introduction of a frameshift at the fusing point of any of the chimera genes resulted in loss of β-galactosidase activity of the transformants.

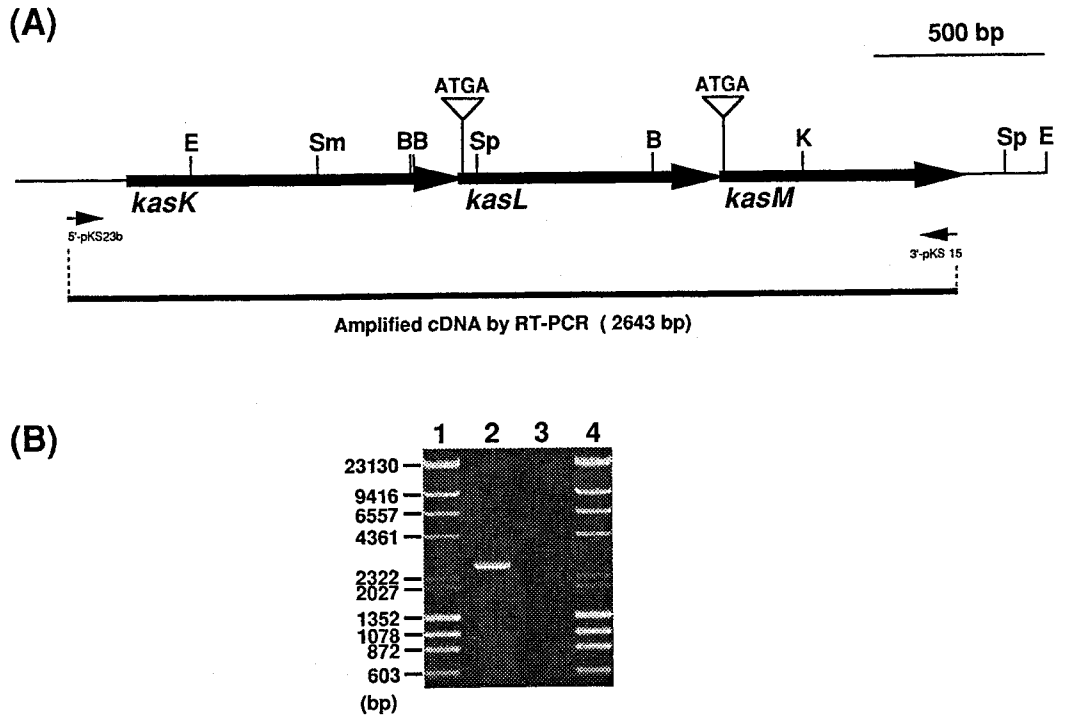
Resistance to KSM Induced by *kasK*, *L* and *M* in *E. coli* JM109

E. coli JM109 transformed with pTV-KLM acquired resistance to KSM (200 μg/ml) (Fig. 6) suggesting that the ABC transporter, consisting of the products of *kasK*, *L* and *M* genes, is functional in exporting KSM out of the cell.

Discussion

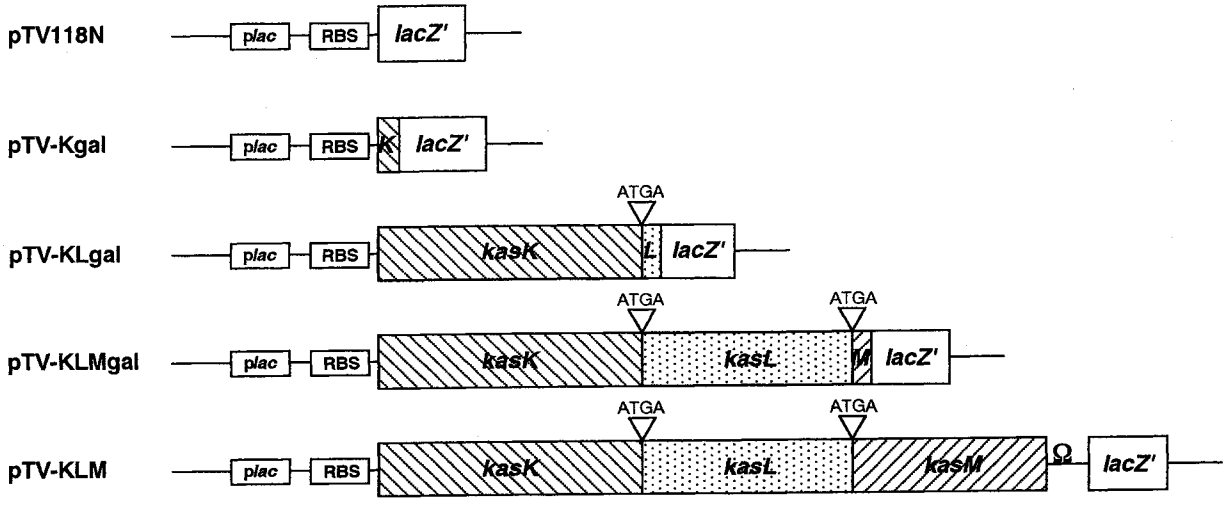
More than ten ABC transporter genes have been cloned from antibiotic-producing actinomycetes. SALAS *et al.* classified these genes into three groups, type I, II and III, on the basis of gene structures and arrangement[26]. A type I gene consists of two contiguous genes; the upstream one encodes a hydrophilic protein including an ATP binding site and the downstream one encodes a hydrophobic membrane-binding protein (*e.g.*, *drrAB*[3], *mtrAB*[4], *tmrB2B3*[23] *etc.*). A type II gene encodes a hydrophilic protein including two ATP binding sites (*e.g.*, *tlrC*, tylosin resistance[27]; *oleB*, oleandomycin resistance[28]; *lmrC*, lincomycin resistance[29] *etc.*). A type III gene encodes a membrane-crossing (6 times) protein including an ATP binding site on the carboxy terminal region (*e.g.*, *strVW*, streptomycin resistance[30]; *ble-orf7*, bleomycin resistance[31]). Our KSM transporter genes, *kasK*, *L* and *M*, should belong to type I because *kasL* and *M*, resembling each other and both encoding hydrophobic membrane-binding proteins, seem to have originated from a downstream gene of type I. Similar gene structures were reported for lantibiotic ABC transporters, such as those in

Fig. 4. Detection of the mRNA of *kasKLM* by RT-PCR.



(A) Diagram showing the positions and orientation of the oligonucleotide primers for RT-PCR. (B) Lane 1 and 4: Molecular size markers. Lane 2: The amplified 2643 bp fragment. Lane 3: Negative control (minus reverse transcriptase).

Fig. 5. Schematic representation of the fusion of *kasK*, *kasL* or *kasM* with *lacZ'*.

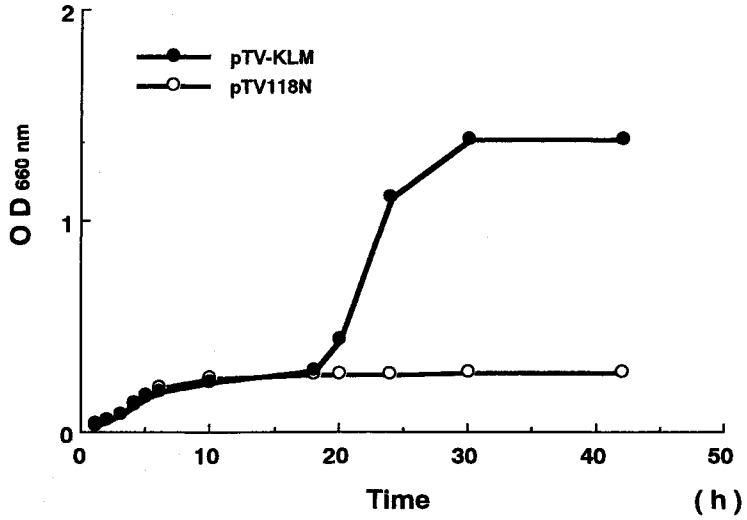


pTV118N is an expression vector. DNA segments including proposed translational start codons of *kasK*, *kasL* and *kasM* were fused in frame with *lacZ'*, to produce pTV-Kgal, pTV-KLgal and pTV-KLMgal, respectively. pTV-KLM is the parent clone. Ω indicates the putative terminator of transcription behind *kasM* (see the dots from 4062 to 4111 in Fig. 2).

Table 2. β-Galactosidase assay.

Plasmids	Specific activity (nmols/min/mg protein)	Colony color on X-gal plate
pTV118N	4363.5	Blue
pTV-Kgal	94.7	Blue
pTV-NE195	2.8	White
pTV-KLgal	25.1	Blue
pTV-KLgal (-)	2.0	White
pTV-KLMgal	65.6	Blue
pTV-KLMgal (-)	2.4	White
None	3.9	White

Fig. 6. KSM resistance of *E. coli* JM109 expressing *kasKLM* genes.



Proliferation profiles of *E. coli* JM109 harboring the pTV118N and pTV-KLM, grown in YT medium containing KSM (200 μg/ml).

Staphylococcus epidermidis (epidermin[32]), *Lactococcus lactis* (lacticin 481[33]), *etc.*

The *kasL* start codon overlaps with the *kasK* stop codon in nt 2499 ATGA, and *kasM* start codon overlaps the *kasL* stop codon in nt 3269 ATGA (Fig. 2). An overlapping ATGA was also found at the boundary of the *drrAB*[3] genes. Such a structure should induce a translational coupling[19,20] of *kasK*, *L* and *M*. Translational coupling refers to situations where translation of a gene in a polycistronic mRNA is more or less dependent on the translation of the upstream gene through the overlapping ATGA. As shown in Fig. 4B, we proved that the *kasK*, *L* and *M* genes were transcribed in a polycistronic mRNA. Studies are in progress to elucidate how the initiation of the transcription is controlled.

As shown in Fig. 6, KSM transporter conferred resistance to KSM on *E. coli* JM109 possibly by pumping out KSM that somehow permeated into the cells. We previously reported that *kac*[338], the gene encoding KSM acetyltransferase, is a gene responsible for self-resistance of

KSM producers[7]. All the KSM producer strains so far tested were positive with *kac*[338] in Southern analysis. The same was true with *kasK*, *L* and *M*. It is tempting to presume that KSM producers developed the double-safety system, one is inactivation and the other secretion, against their own toxic metabolite, KSM.

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