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 *SacI-KpnI* DNA fragment, located 5~15-kb upstream of *kac*338, was cloned and a 4.2-kb *SacI-EcoRI* 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 cell1. 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 superfamily2 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: *drrAB3* from *S. peucetius* (daunorubicin/doxorubicin resistance), *mtrAB4* from *S. argillaceus* (mithramycin resistance) and *OleC, C5* from *S. antibioticus* (oleandomycin resistance). The mechanism of *drrAB* is similar to P-glycoprotein6 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 genes7 from *S. kasugaensis* M338-M18. In the present paper we report the cloning, characterization and functional analysis of ABC transporter type genes.
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 (TaKaRa, Code No. 9056), *E. coli* DH5α (TOYOBO, Code No. DNA-903), *E. coli* JM109 (TaKaRa, Code No. 9052), cloning vector PKF39 (TaKaRa, Code No. 3100), pUC118 (TaKaRa, Code No. 3318) and expression vector pTV118N (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. The *E. coli* TH2/pKF 3 cloning system was used to clone pSKE 4 and 5.

The cloned DNA region (4.2-kb *Sacl*-EcoRI 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 (ALFred™ 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 and BLAST.

**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 mM 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 transcriptase for 30 minutes at 60°C. For the following PCR amplification, 5′-pKS23b (5′-TCCGGGTATTTCCGAAAGGAACGGCGGT-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* Tag 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.

<table>
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<tr>
<th>Strains and plasmids</th>
<th>Genotype and genetic construct</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong>&lt;br&gt;S. kasugaensis M338-M1</td>
<td>Kasugamycin producing strain</td>
<td>8</td>
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<tr>
<td><strong>E. coli</strong>&lt;br&gt;DH 5α</td>
<td>φ80lacZΔM15 Δ(lacZ/A-argF) U169 deoR recA1 endA1 hsdR17(rK&lt;sup&gt;−&lt;/sup&gt;, mK&lt;sup&gt;+&lt;/sup&gt;) phoA supE44 λ thi-1 gyrA96 relA1</td>
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<tr>
<td>TH 2</td>
<td>supE44 hsdR20=t&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;+&lt;/sup&gt;) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 met-1 thi-1 trpR624</td>
<td>9</td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) / F&lt;sup&gt;−&lt;/sup&gt;[traD36proAB&lt;sup&gt;+&lt;/sup&gt; lacF lacZAM15]</td>
<td>10, 11</td>
</tr>
</tbody>
</table>

| **Plasmids**<br>pUC118 | Cloning vector. Amp<sup>R</sup>. 3.1-kb. | 13        |
| pTV118N             | Expression vector derived from pUC118, containing a unique NcoI site. Amp<sup>R</sup>. 3.1-kb. | 14        |
| pKF3                | Cloning vector. Sm<sup>R</sup>, Cm<sup>R</sup>. 2.2-kb. | 9, 12     |
| pSKE 4              | pKF3 derivative incorporating 8.4-kb Kpnl fragment from S. kasugaensis M338-M1. 10.6-kb. | This work |
| pSKE 5              | pKF3 derivative incorporating 5.5-kb Sacl fragment from S. kasugaensis M338-M1. 7.7-kb. | This work |
| pKS 15              | pUC118 derivative containing 718 bp Kpnl-EcoRI fragment from pSKE 5. | 3.9-kb.   |
| pKS 22              | pUC118 derivative containing 577 bp Sacl-Smal fragment from pSKE 5. | 3.7-kb.   |
| pKS 23              | pUC118 derivative containing 1135 bp Smal-EcoRI fragment from pSKE 5. | 4.3-kb.   |
| pKS 24              | pUC118 derivative containing 840 bp EcoRI-Sphl fragment from pSKE 5. | 4.0-kb.   |
| pKS 25              | pUC118 derivative containing 965 bp Sphl-Kpnl fragment from pSKE 5. | 4.1-kb.   |
| pTV-NE195           | pTV118N derivative containing a PCR fragment (195 bp NcoI-EcoRI, 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 NcoI-EcoRI 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 Sphl 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 Kpnl 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 NcoI 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'-GGCCATGTTGGTTGTTAACTCAGAC-3') and 3'-ORF K (Antisense: 5'-GGCCATGTTGGTCAGGTTAC-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<sub>2</sub>, 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 minute at 65°C) and extension (1 minute at 72°C). The resulting PCR product was digested with NcoI/EcoRI 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 EcoRI fragment from pSKE 5 (Fig. 1) was subcloned into the EcoRI 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'-GGCCATGTTGGTTGTTAACTCAGAC-3') and 3'-ORF
K2 (Antisense: 5'-GGGAATTCGGTCCGCTCT-3'). The underlined sequences in the primers are NcoI site and EcoRI sites, respectively. The PCR product was digested with NcoI and EcoRI and inserted into pTV118N to obtain a recombinant plasmid (pTV-Kgal) that would encode a fusion protein consisting of Met1-Pro26 of KasK and Asn4~His106 of β-galactosidase α peptide. As a negative control, pTV-NE195 was used, which would encode a fusion protein consisting of Met1~Ile65 of KasK and a frameshift (inactive) protein. Similarly, plasmids designed to encode a fusion protein consisting of Met1~Ser17 of KasL and His19~His106 of β-galactosidase α peptide (pTV-KLgal) and another fusion protein consisting of Met1~Gly14 of KasM and Leu13~His106 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 (OD660). The cells were collected by centrifugation for 5 minutes at 5,000Xg, resuspended in 5ml of PBS and disrupted by sonication. The lysates were centrifuged for 5 minutes at 5,000Xg to remove the cell debris. To measure the β-galactosidase activity of the fused proteins, 70μl of 4mg/ml o-nitrophenyl-β-D-galactopyranoside and 200 μl of PBS were added to the cell lysate (30μl). 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 420nm 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 OD660 of the cultures. When the OD660 reached 0.5~0.7, 0.2mm 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 2ml of YT medium with ampicillin (100 μg/ml) and KSM (200 μg/ml) and the growth was monitored by reading OD660 of the cultures at 37°C for 42 hours (Fig. 6).

Results

Cloning and Sequence Analysis

An 8.4-kb KpnI fragment from S. kasugaensis M338-M1 chromosome was cloned using as a probe the 850bp PstI-KpnI fragment from pSKE 2. The resultant recombinant plasmid was designated pSKE 4. A 5.5-kb SacI fragment from S. kasugaensis M338-M1 chromosome was also cloned using as a probe the 594bp KpnI-SphI fragment from pSKE 4. The plasmid was named pSKE 5 (Fig. 1). We sequenced the 4236bp SacI-EcoRI 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. 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, ~32.30kcal/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. 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 (GTTGA). 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 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 reported7). 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, BamHI; E, EcoRI; K, KpnI; P, PstI; S, Sall; Sm, Smal; Sp, SphI.

of a homology search using the FASTA and BLAST program. The deduced amino acid sequence of KasK showed 39% similarity with DrrA3) 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; OleC5) from *S. antibioticus* (39%) responsible for oleandomycin resistance, MtrA4) from *S. argillaceus* (37%) for mithramycin resistance, NodI from *Rhizobium galegae* (37%) for oligosaccharide export (unpublished data, L. Paulin et al., Accession No. P50332), NatA22) from *Bacillus subtilis* (36%) for extrusion of Na ion and TnrB23) from *S. longisporoflavus* (35%) for tetronasin resistance. The deduced amino acid sequence of KasK contains an ATP-binding motif24) 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 NodJ25) from *Rhizobium leguminosarum* and 31% similarity with MtrB4) from *S. argillaceus*. Comparison of KasM with other proteins also revealed 30% similarity with NodJ25) 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 SacI-EcoRI region from PSKE 5 and deduced amino acid sequences of ORF J, kasK, kasL, and kasM.
Fig. 2. (Continued)

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Shadowed amino acids represent the conserved motifs (Walker A, loop 3, and Walker B) for an ATP-binding cassette21). 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.

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. 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., drrAB3, mtrAB4, tnrB2B323 etc.). A type II gene encodes a hydrophilic protein including two ATP binding sites (e.g., tlrC, tylosin resistance27; oleB, oleandomycin resistance28; imrC, lincomycin resistance29 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 resistance30; ble-orf7, bleomycin resistance31 etc.). Our KSM transporter genes, kasK, L and M, should belong to type I because kasL and M, resembling each other and both encoding hydrophilic 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

pTV-Kgal

pTV-KLgal

pTV-KLMgal

pTV-KLM

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).
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 genes. Such a structure should induce a translational coupling 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 kacm, the gene encoding KSM acetyltransferase, is a gene responsible for self-resistance of

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<th>Plasmids</th>
<th>Specific activity (mmol/mg protein)</th>
<th>Colony color on X-gal plate</th>
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<tr>
<td>pTV118N</td>
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<td>pTV-Kgal</td>
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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\textsuperscript{29}), Lactococcus lactis (lacticin 481\textsuperscript{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\textsuperscript{19} genes. Such a structure should induce a translational coupling 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
KSM producers\(^7\). All the KSM producer strains so far tested were positive with \(kac\)\(^38\) 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.

Acknowledgments

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