A 7.6 kb DNA Region from *Streptomyces kasugaensis* M338-M1 Includes Some Genes Responsible for Kasugamycin Biosynthesis

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A 7.6 kb *Pst*I-*Kpn*I DNA fragment including a sequence highly similar to kasugamycin acetyltransferase gene (*kac*) was isolated from *Streptomyces kasugaensis* M338-M1 and sequenced. Nine open reading frames (ORFs), designated as ORF A, B, C, D, E, F, G, H and I, were recognized in this region, although ORF A was incomplete. ORF G runs in the opposite direction to the others. The amino acid sequence deduced from ORF H showed 98% similarity to that of the kasugamycin acetyltransferase from *S. kasugaensis* MB273-C4, another kasugamycin (KSM) producer. Transformation of *E. coli* JM109 with ORF H made the strain highly resistant to KSM. The deduced amino acid sequences of the ORF A, C and D products were similar, respectively, to glucosyltransferase I from *E. coli* (26%), β-alanine : pyruvate transaminase from *Pseudomonas putida* (32%) and dTDP-D-glucose 4,6-dehydratase (StrE) from *Streptomyces griseus* (37%). The *strE*-like ORF (ORF D) seems to be the gene responsible for formation of the 6-deoxy structure of the kasugamine moiety. ORF A and ORF C are also likely to have roles in KSM biosynthesis. Taken together, our analyses strongly suggest that this DNA region includes at least a part of the gene cluster of KSM biosynthesis.

Kasugamycin (KSM) is an aminoglycoside antibiotic produced by *Streptomyces kasugaensis* M338-M1[1], effective against *Piricularia oryzae* and widely used in agriculture in Japan to prevent the rice blast. From *S. kasugaensis* MB273-C4, another KSM producing strain, a gene coding for the enzyme that acetylates the 2′-NH_2 of KSM and thereby inactivates the antibiotic was cloned and named *kac* by HIRASAWA *et al.* (JP. A-05-23187, 1993). In our previous paper[2] we described that a *kac*-like sequence was found in the genome of every KSM producer tested and that spontaneous deletion of the sequence paralleled the loss of KSM productivity of *S. albulus* MF861-C4[3], a third KSM producer whose genome was somehow unstable. The results suggested that *kac* is a self-resistance gene for KSM producers and located close to other KSM biosynthetic genes.

In the present paper we report the cloning and sequence analysis of a 7.6kb *Pst*I-*Kpn*I DNA fragment including a *kac* congener from *S. kasugaensis* M338-M1. We propose that at least three ORFs in this DNA region would be assigned to KSM biosynthesis.

Materials and Methods

Bacterial Strains, Plasmids, Fermentation Media and Genetic Manipulations

Streptomyces kasugaensis M338-M1[1], isolated as a KSM producer at the Institute of Microbial Chemistry in 1963, was grown in TSB medium at 27°C for 2 days and processed to obtain genomic DNA as described previously[2].

Escherichia coli DH5α (TOYOBO, Code No. DNA-903) was grown at 37°C in YT and/or 2×YT medium containing ampicillin (100 μg/ml). *E. coli* TH2 (TaKaRa, Code No. 9056) and *E. coli* JM109 (TaKaRa, Code No. 9052) were grown at 37°C in L-broth (Bacto tryptone 10 g, Bacto yeast extract 5 g, NaCl 5 g in 1 liter water, pH 7.5) containing chloramphenicol (12 μg/ml) and streptomycin (50 μg/ml for *E. coli* TH2) or ampicillin

Table 1. Plasmids prepared in this study.

Plasmid	Relevant properties	reference
pUC 118	3162 bp; Amp^r, *E. coli* plasmid	26
pSKE 1	6.9 kb; pUC118 containing 3.7 kb *Kpn*I DNA fragment from *S. kasugaensis* M338-M1	This work
pKF 3	2247 bp; Sm^s, Cm^r, *E. coli* plasmid	27, 28
pSKE 2	6.3 kb; pKF 3 containing 4.1 kb *Pst*I DNA fragment from *S. kasugaensis* M338-M1	This work
pTV 118N	3163 bp; Amp^r, derived from pUC118	29
pTV 273kac	3.6 kb; pTV 118N containing an amplified kac^{273} structural gene, 440 bp *Nco*I-*Bam*HI DNA fragment from *S. kasugaensis* MB273-C4	This work
pTV 338kac	3.6 kb; pTV 118N containing an amplified kac^{338} structural gene, 437 bp *Nco*I-*Bam*HI DNA fragment from *S. kasugaensis* M338-M1	This work

(100 μg/ml for *E. coli* JM109). Plasmids used and constructed in this work are described in Table 1. For cloning of pSKE 1 and pSKE 2, *E. coli* DH5α/pUC118 (TaKaRa, Code No. 3318) and *E. coli* TH2/pKF3 (TaKaRa, Code No. 3100) were used as host strain and cloning vector, respectively.

DNA Sequencing

The DNA fragments from *S. kasugaensis* M338-M1 cloned in pSKE 1 and pSKE 2 were digested with appropriate restriction endonucleases, and were subcloned in the pUC118. The resulting subclones were sequenced with an automated laser fluorescence sequencer (ALF*red*™ DNA Sequencer, Pharmacia). Sequencing reactions were done using the Cy5™ AutoCycle™ Sequencing Kit (Pharmacia, Code No. 27-2693-02) according to the supplier's instructions. The sequencing reactions were analyzed with an ALF*red*™ DNA Sequencer on a 6 M urea- 6% polyacrylamide gel in 1.2 × Tris-borate-EDTA (120 mM Tris, 99.6 mM Borate, 1.2 mM EDTA), and the running buffer was 0.6 × Tris-borate-EDTA (spacer 0.35 mm, 47°C, 10 hours). Sequence primers used were M13 universal and reversal primers from the Cy5™ AutoCycle™ Sequencing Kit and synthesized oligonucleotide primers (labeled with Cy5).

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 BLAST[4].

Expression of ORF H (kac^{338}) in *E. coli* JM109

5'-KAC primer (Sense: 5'-GGCCATGGCGCGCTGGGCCGGGACA-3') and 3'-KAC primer (Antisense: 5'-GGGGATCCTCGTTACAGGGCGATCA-3') were used for amplification of both *kac* structural genes. PCR amplification was performed using a MiniCycler™ (MJ Research). Each reaction mixture contained 50 ng genomic DNA, 20 pmols each primer, 50 mM 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. After addition of 2 U $Vent_R$ (exo^-) DNA polymerase (New England BioLabs, Code No. 257S), the DNA template was denatured 98°C for 2 minutes. Amplification was carried out by 30 cycles of annealing, extension (2 minutes at 72°C) and denaturation (30 seconds at 98°C). The resulting PCR products were digested with *Nco*I/*Bam*HI (double digestion) and cloned into pTV118N (TaKaRa, Code No. 3328) to create pTV273kac and pTV338kac. With these plasmids (pTV118N, pTV273kac and pTV338kac), *E. coli* JM109 was transformed.

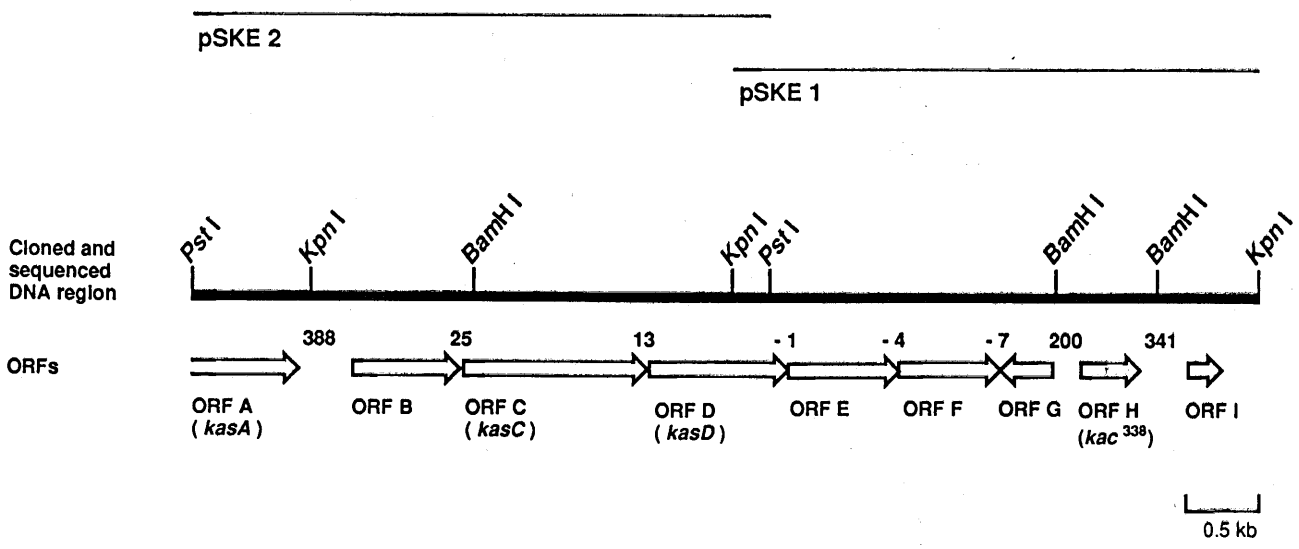
Single colonies were used to inoculate 2 ml L-broth containing ampicillin (100 μg/ml). After 16 hours of growth at 37°C, 1 ml of each culture was used to inoculate 9 ml L-broth containing ampicillin (100 μg/ml) and growth was continued at 37°C for 10 hours. At an OD_{620} of 0.5 isopropylthiol-β-galactoside (IPTG) was added to give a final concentration of 1 mM, and growth was continued for an additional 3 hours. These cultures (each 5 ml) were plated on a YT-1.5% agar plate and incubated at 37°C for 16 hours.

Nucleotide Sequence Accession Number

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nu-

Fig. 1. Restriction map of a 7.6 kb *Pst*I-*Kpn*I DNA region, including the kasugamycin acetyltransferase gene (*kac*^{338}) from *S. kasugaensis* M338-M1.

The ORFs within this region are indicated by open arrows. Numerals on the arrow heads indicate untranslated spaces. Minus indicates overlapping.



cleotide sequence databases with the accession number AB005901.

Results

Cloning and Sequencing Analysis of the 7.6 kb *Pst*I-*Kpn*I DNA Region

A 3.7 kb *Kpn*I fragment from *S. kasugaensis* M338-M1 was cloned using as a probe a 734 bp *Bam*HI fragment including *kac* gene (*kac*^{273}) from *S. kasugaensis* MB273-C4 (JP. A-05-23187). The recombinant plasmid clone was designated pSKE 1 (Fig. 1). A 4.1 kb *Pst*I fragment from *S. kasugaensis* M338-M1 was also cloned using as a probe a 271 bp *Kpn*I-*Pst*I fragment from pSKE 1. The plasmid clone was named pSKE 2 (Fig. 1).

We sequenced the entire 7581 bp *Pst*I-*Kpn*I DNA region from pSKE 1 and pSKE 2 (Fig. 2). The GC content of the entire region was 71.6%. Within this region we recognized nine open reading frames (ORFs), *i.e.* ORF A, B, C, D, E, F, G, H and I, all with high GC contents (80.3~90.9%) in the deduced 3rd codon positions[5], though ORF A was incomplete (Fig. 1, 2). These ORFs all run in the same direction except for ORF G (Fig. 1). Translational start codons were predicted from end-to-end similarity to other authentic proteins.

As potential ribosome binding site (RBS) candidates, there were GAAAGG preceding ORF C (1918~1923) and ORF D (3241~3246) and GAAA preceding ORF H (6306~6309). These sequences are complementary to the 3′ end of streptomyces 16S rRNA[6~8]. No other ORFs had RBS candidates (Fig. 2).

There were inverted repeat sequences downstream of ORF A (822~880, −36.18 kcal/mol), ORF G (5549~5608, −32.34 kcal/mol) and ORF I (7359~7406, −36.70 kcal/mol), possibly functioning as transcriptional terminators (Fig. 2). Noncoding regions were 388 bp between ORF A and B, 25 bp between B and C, 13 bp between C and D, 200 bp between G and H, and 341 bp between H and I. Some genes showed overlapping sequences; ORF D and E by 1 bp, E and F by 4 bp, and F and G by 7 bp (Fig. 1, 2).

Possible functions of the nine ORF products were deduced from the results of a homology search using the BLAST program[4]. The ORF B, E, F, G, and I showed no significant similarities with any proteins in the databases.

Possible Roles of Some ORFs in KSM Biosynthesis

ORF H

ORF H showed 98% base-sequence homology with *kac*^{273}, hence ORF H was denoted *kac*^{338} hereafter. The base sequences of *kac*^{273} and *kac*^{338}, with the amino acid sequences of their deduced proteins (Kac^{273} and Kac^{338}), are shown in Fig. 3.

Compared with *kac*^{273}, *kac*^{338} showed four base substitutions at G114A, G115A, C340T and C378T

Fig. 2-1. Nucleotide sequence of the 7.6 kb *Pst*I-*Kpn*I DNA region from *S. kasugaensis* M338-M1 and deduced amino acid sequence corresponding to ORFs.

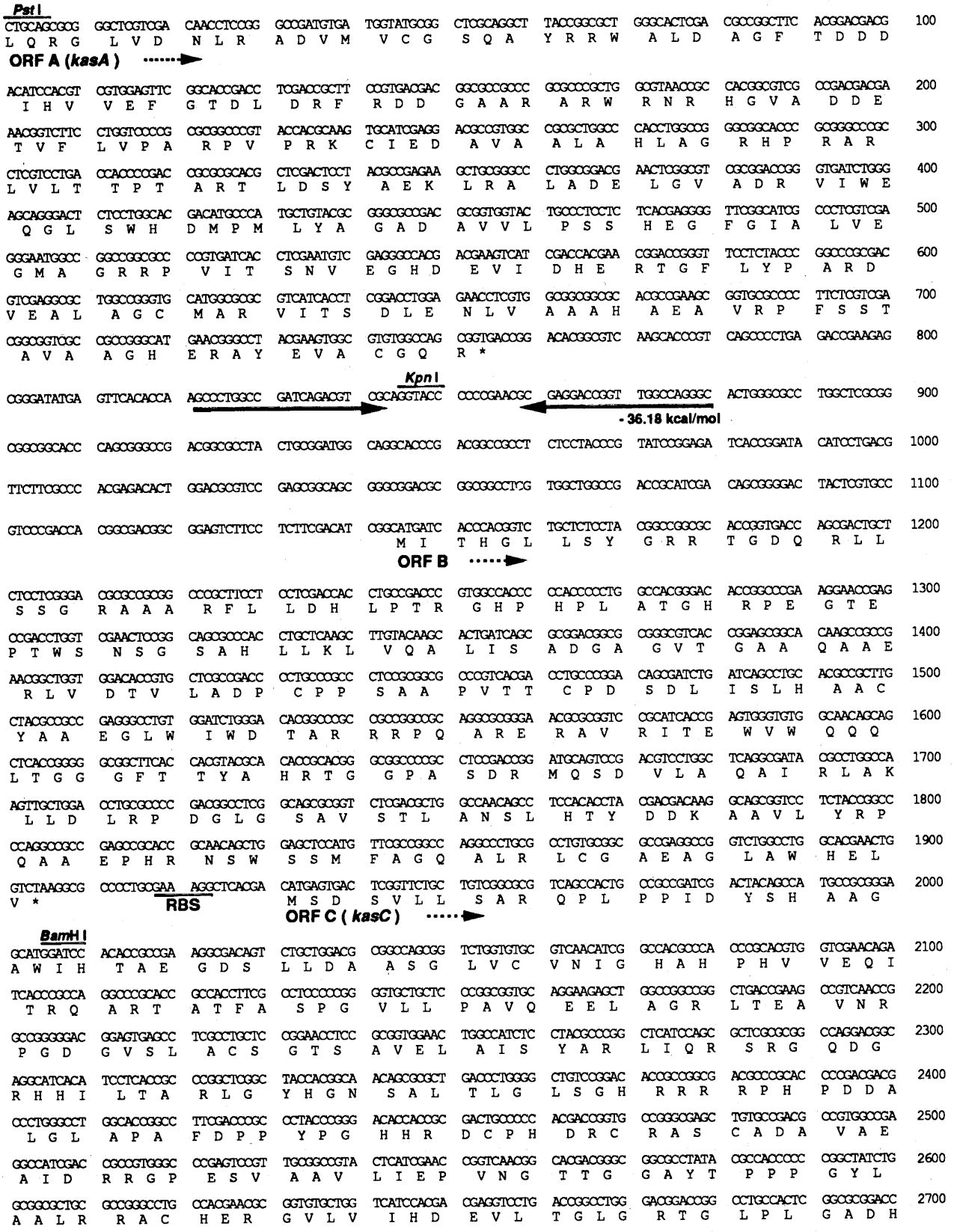


Fig. 2-2. Nucleotide sequence of the 7.6 kb *Pst*I-*Kpn*I DNA region from *S. kasugaensis* M338-M1 and deduced amino acid sequence corresponding to ORFs.

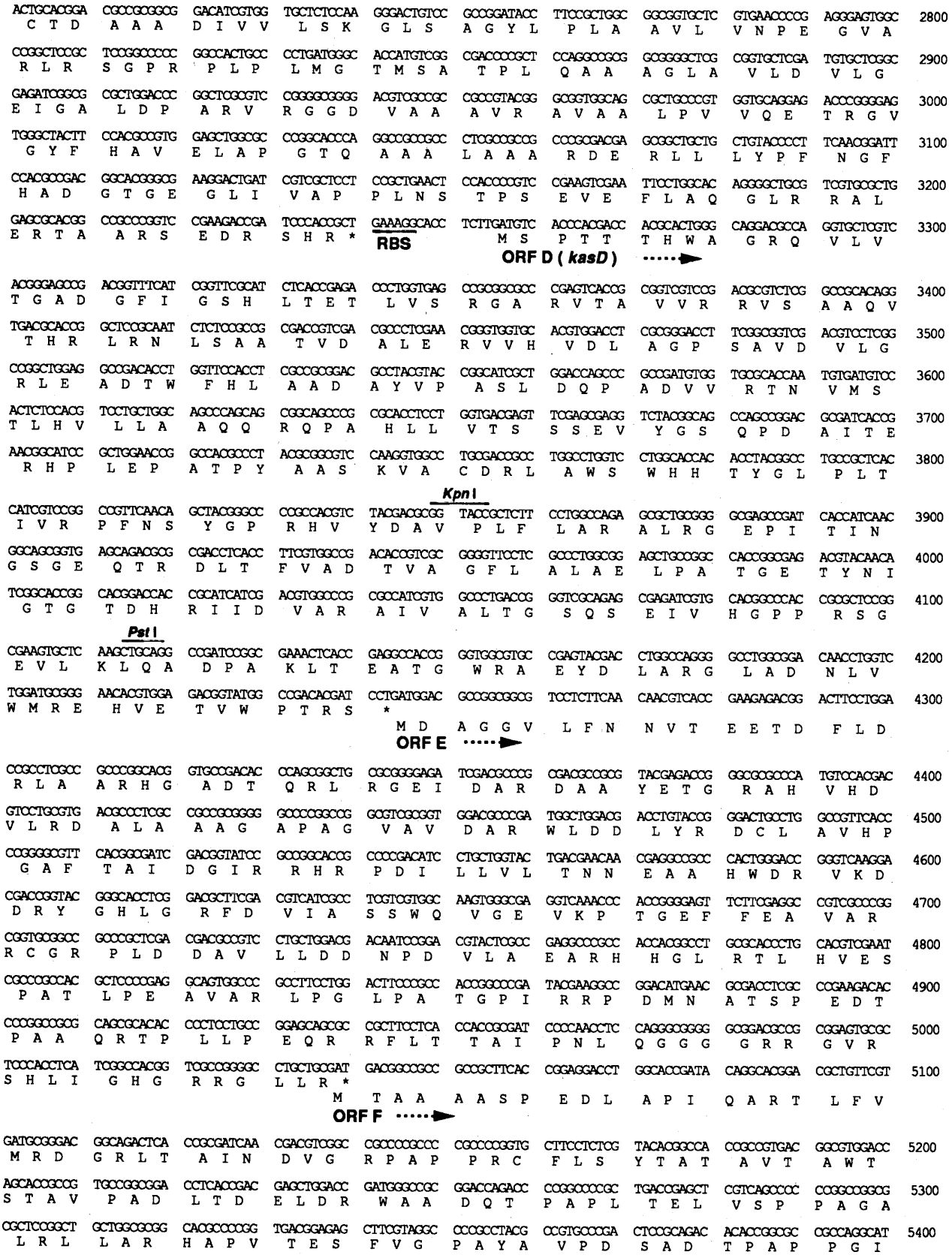
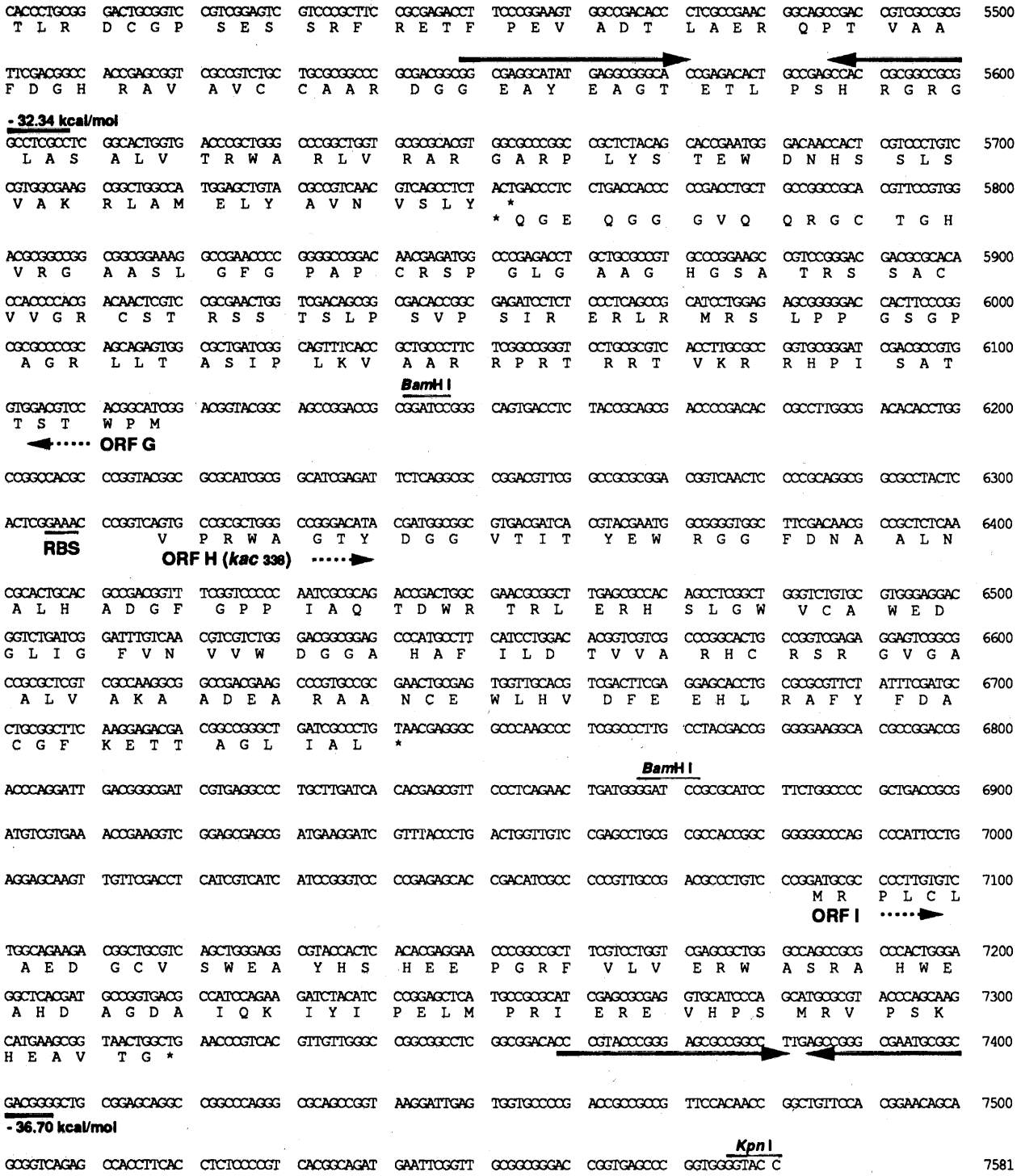
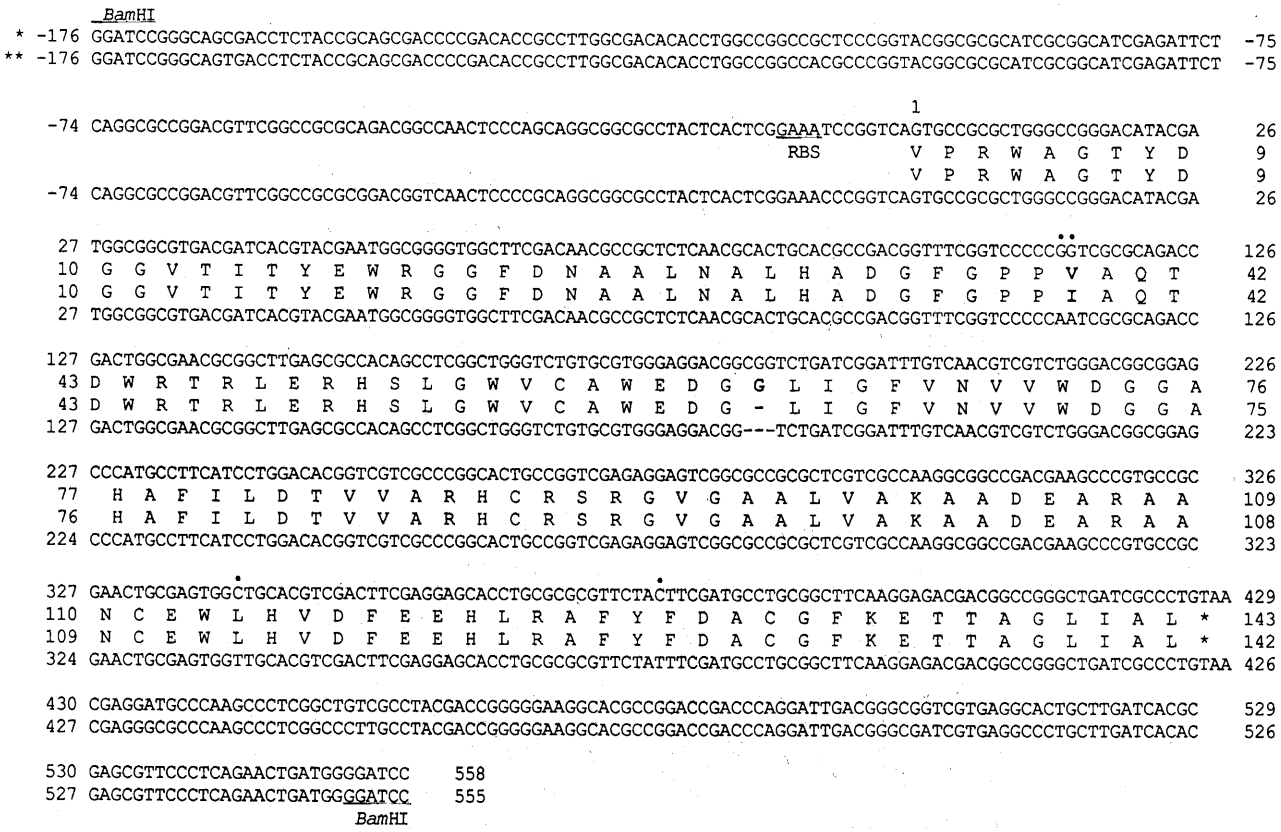


Fig. 2-3. Nucleotide sequence of the 7.6 kb *Pst*I-*Kpn*I DNA region from *S. kasugaensis* M338-M1 and deduced amino acid sequence corresponding to ORFs.



(G115A alone caused a missense mutation, while the others silent), and deletion of three sequential bases, CGG (186~188). In the deduced protein (Kac^{338}), consequently, there were an amino acid substitution of Val39Ile and a deletion of Gly^{63} (Fig. 3). The amino acid sequence homology between Kac^{273} and Kac^{338} was 98%. Then we tested if *kac*^{338}, as well as *kac*^{273}, could transform an *E. coli* strain to become resistant to KSM. The transformants with *kac*^{338} or *kac*^{273}, but not with the control plasmid, showed resistance to KSM at concentrations above 800 μg/ml (Fig. 4). The result strongly suggested that *kac*^{338} endowed the KSM producer

Fig. 3. Comparisons of nucleotide and deduced amino acid sequences of *kac* genes from *S. kasugaensis* MB273-C4 and M338-M1.



*: *S. kasugaensis* MB273-C4. **: *S. kasugaensis* M338-M1. · denotes a base change in the structure genes. —denotes deletion. Amino acid change is shown in bold face.

(*S. kasugaensis* M338-M1) with the self resistance to its own antibiotic, as proposed by HIRASAWA *et al.* with *kac*^{273} (JP. A-05-23187).

ORF D

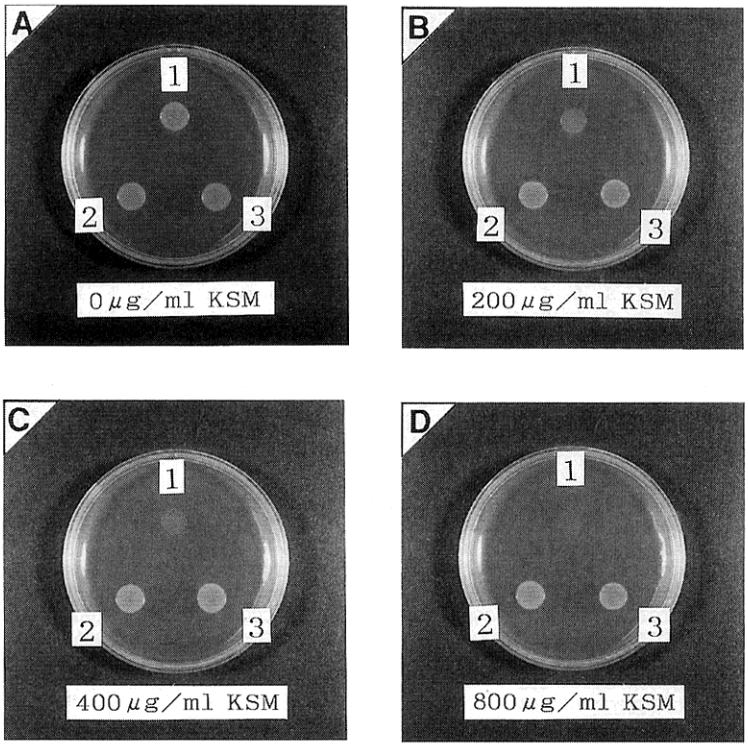
A deduced protein encoding ORF D (OrfD) spanned 329 codons whose sequence showed 37% and 36% similarities with StrE[9] from *Streptomyces griseus* and with RfbB[10] from *Xanthomonas campestris*, respectively (Fig. 5). There were additional similarities of OrfD to StrE: (1) the two were close in length, *i.e.* 329 codons (OrfD) *vs.* 328 codons (StrE), and (2) OrfD and StrE shared a highly preserved NAD(P) binding motif[11] near their *N*-terminals (Fig. 6). *strE*, as well as *rfbB*, codes for dTDP-D-glucose 4,6-dehydratase, that is the gene responsible for formation of the 6-deoxy structure of these antibiotics, *i.e.* streptomycin for StrE[9], granaticin for GraE[12], and mithramycin for MtmE[13]. W. PIEPERSBERG *et al.*[14] have proposed that *strE*-like genes are typically present member in gene clusters for the biosynthesis an antibiotics that contain a 6-deoxy-hexose (6-DOH) moiety and that cloning the gene will provide a probe to find out the gene cluster. The presence of the 6-DOH moiety in KSM, as well as the close location of ORF D to *kac*^{338}, strongly suggests that the DNA region we analyzed should coincide at least in part with the gene cluster for KSM biosynthesis. We therefore designated ORF D as *kasD*.

ORF C

A deduced product of ORF C (OrfC), as long as 436 codons, preceded *kasD* by a narrow margin of 13 bp. OrfC showed 32% similarity with β-alanine : pyruvate transaminase[15] of *Pseudomonas putida* (Fig. 5) and included a sequence, Val^{237} through Leu^{277}, that was a pyridoxalphosphate attachment site motif (Fig. 7) in ornithine aminotransferase[16]. The role of Orf C for KSM biosynthesis should be to convert carbonyl group (C-4′ position of kasugamine moiety) to amino group, and therefore we designated ORF C as *kasC*.

Fig. 4. Comparison of KSM resistance.

1: Transformed with empty vector, pTV118N.
2: Transformed with pTV273 kac.
3: Transformed with pTV338 kac.



ORF A

ORF A, possibly corresponding to a part of a gene, was located to one end of the DNA region we analyzed. The 3rd codon positions showed high GC content throughout the ORF (251 codons, determined so far), as did other streptomyces genes. Neither an initiation codon nor an RBS was recognized yet, however. The ORF A product showed 26% similarity with glucosyltransferase I[17] from *E. coli* (Fig. 5), suggesting a possible role in kasugamine addition during KSM biosynthesis. We therefore designated ORF A as *kasA*.

Discussion

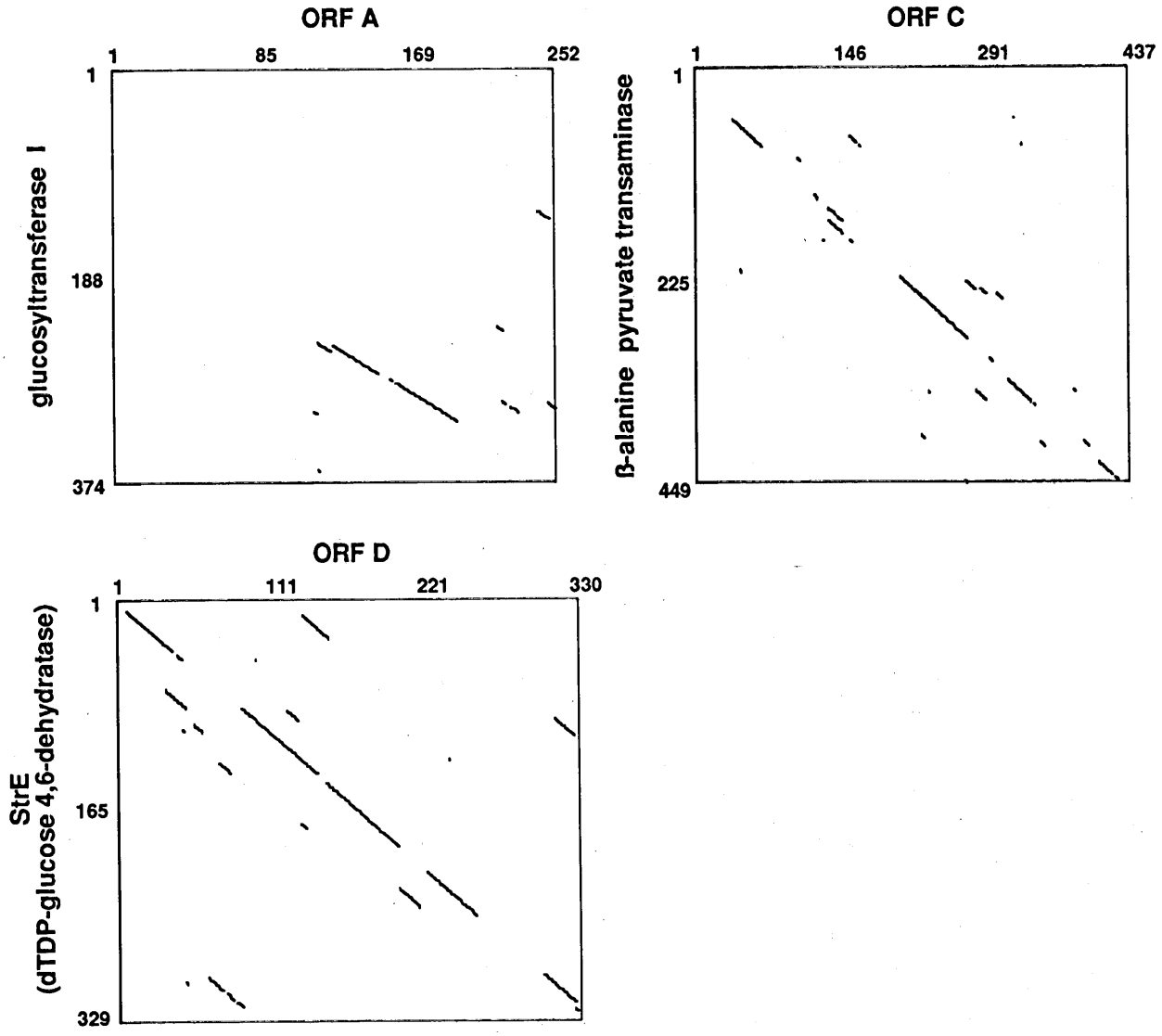
Biosynthesis of KSM was studied by FUKAGAWA *et al.* in 1968 by mainly determining the incorporation of radioactive precursors into KSM or its moieties[18~23]. The results showed that (1) D-glucosamine was efficiently incorporated into the kasugamine moiety[21], (2) D-glucosamine was readily converted to UDP-*N*-acetylglucosamine (UDP-GlcNAc), which accumulated in mycelia, and (3) a carboxyformidoyl group was selectively added to the 4′-amino of the kasugamine moiety. These results suggested that UDP-GlcNAc was an early precursor of KSM biosynthesis and, together with other results, led FUKAGAWA *et al.* to propose a tentative biosynthetic route of KSM (Fig. 8, from the thesis of Y. FUKAGAWA, partly modified by S. IKENO).

The deduced product of *kasD* (KasD) possibly catalyzes Step 1 in view of its similarity with dTDP-D-glucose 4,6-dehydratase (*strE* gene product, StrE) including an NAD(P) binding motif (Fig. 5, 6). *strE*-like genes cloned so far share about 60% homology. The low similarity (37%) between KasD and the StrE may reflect the structural difference between the substrates for the two enzymes. It is likely, as FUKAGAWA *et al.* proposed also, that the biosynthetic route of KSM, a secondary metabolite, diverges from the primary metabolism of sugars at UDP-GlcNAc.

kasC, the upstream neighbor of *kasD*, should code for a pyridoxalphosphate binding protein and transaminase that catalyzes Step 2 (Fig. 8).

ORF B, *kasC*, *kasD*, ORF E and ORF F are located very closely one after another and some are even

Fig. 5. Dotplot.



Deduced amino acid sequences of the ORF A, ORF C and ORF D products were compared with glucosyltransferase I from *E. coli*, β-alanine : pyruvate transaminase from *Pseudomonas putida* and the StrE (dTDP-glucose 4,6-dehydratase) from *S. griseus*, respectively. These data were compared using a window of 30 and a stringency of 8.

Fig. 6. Comparison of the *N*-terminal sequences.

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KasD     1  MSPTTTHWAG RQVLVT-GADG FIGSHLIETL VSRGARVIAV VRRVSAAQVT   50
RfbB     1  M---AT-W------LVIRGA-G FIGANFVLEA VSRGIRVVN- ---LDALTYA   37
StrE     1  M---TTH------LLVT-GAAG FIGSQYVRTL LGPGGPPDVV VTALDALTYA   42
            *   .*..      *** ** * ***.    .. .... ..  . .    *

KasD    51  HRLRNLSAAT   60
RfbB    38  GNLNTLASLE   47
StrE    43  GNPDNLAAVR   52
             . .* .
```

Highly conserved amino acid residues among NAD(P) binding proteins were indicated asterisks (*).

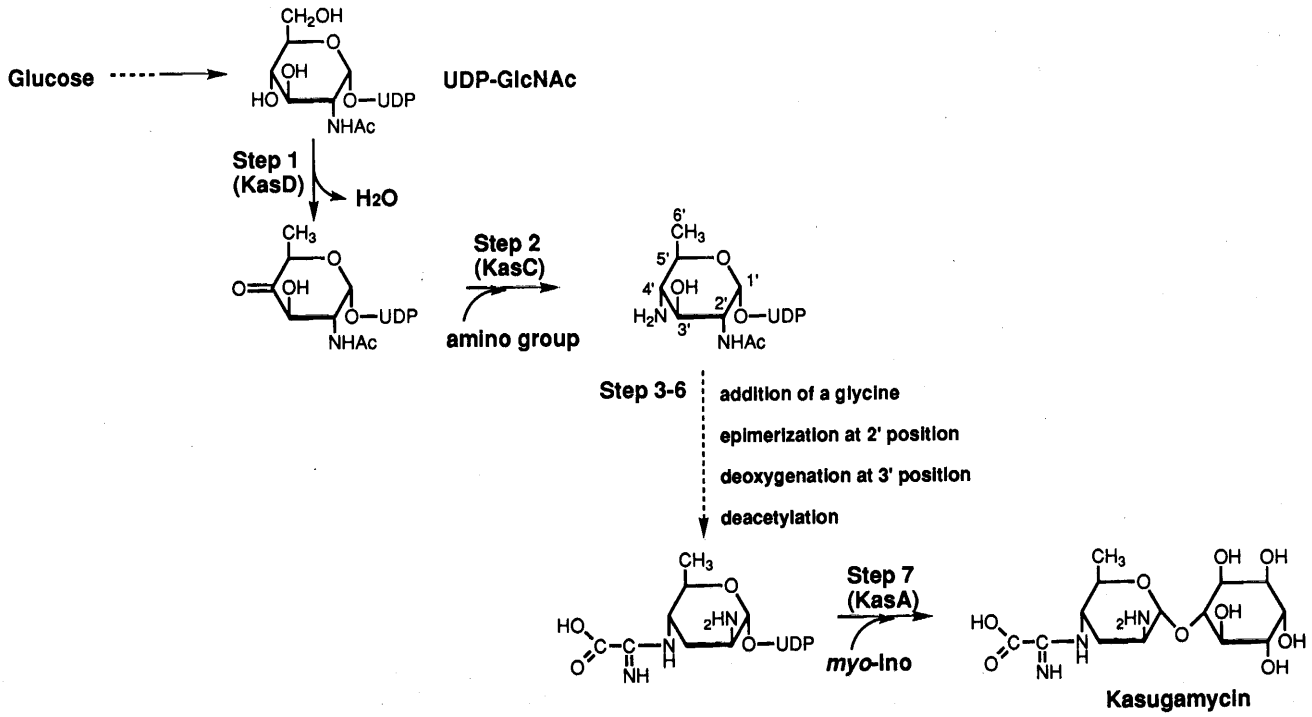
Fig. 7. Comparison of the pyridoxal phosphate attachment site of the ORF C protein with those of Class-III aminotransferases.

```
                    **   *   ** *                *       *       *
1)      237  VIHDEVLTGL  GRTGLPLGAD  HCTDAAA--D  IVVLSKGLSA  G  275
2)      256  LVFDEVITGF  GRTGSMFGAD  SFGVT---PD  LMCIAKQVTN  G  293
3)      245  LMFDEVQVGM  GRSGKLWGYE  YLGV-E--PD  IFTSAKGLGG  G  282
4)      239  LILDEAQTGV  GRTGTMFACQ  RDGVT---PD  ILTLSKTLGA  G  276
5)      237  LIVDEIQTGI  GRTGELLCYD  HYK-AFAKPD  IVLLGKALSG  G  276

Consensus    LLXDEVXXGL  GRXG-----X(12-16)----D  IXXXSKXXXA  G
             VV   I  PI   S A                   L   A    T  A
             II   L  V    A S                   V   G    S
             MM   M  M      D                   M        G
             FF   A  F                          F        N
             YY      Y                          Y
             WW      W                          W
                     A
                     G
```

1) ORF C protein from *Streptomyces kasugaensis* M338-M1. 2) β-Alanine pyruvate aminotransferase from *Pseudomonas putida*. 3) *N*-Acetylornithine aminotransferase from *Anabaena* sp. 4): 2,2-Dialkylglycine decarboxylase from *Pseudomonas cepacia*. 5) Ornithine aminotransferase from *Saccharomyces cerevisiae*. The highly conserved amino acid residues are indicated with asterisks.

Fig. 8. A tentative pathway of KSM biosynthesis.



overlapping (Fig. 2). In a gene cluster for biosynthesis of an antibiotic, most genes are separated with close margins or even overlapping and some are transcribed into a polycistronic mRNA. One bp overlapping like —TGATG—and four bp overlapping like—ATGA— are common in streptomyces and other bacteria and the overlapping genes are often found in operons[24]. The close location of many ORFs we observed seems not unusual, therefore.

Preliminary experiments using RT-PCR suggested that

ORF B, *kasC*, *kasD*, ORF E and ORF F were transcribed concurrently, possibly into a polycistronic mRNA, while ORF G appeared to be expressed in a reverse direction (data not shown).

None of our ORFs included TTA[25], ruling out the possible direct control by *bldA*. Construction of blocked mutants for the ORFs and determination of biological activities of gene products are in progress.

Acknowledgments

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