

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-M1¹⁾, 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'-NH₂ of KSM and thereby inactivates the antibiotic was cloned and named *kac* by HIRASAWA *et al.* (JP. A-05-23187, 1993). In our previous paper²⁾ 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-C4³⁾, 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 7.6 kb *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-M1¹⁾, 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 previously²⁾.

Escherichia coli DH5 α (TOYOBO, Code No. DNA-903) was grown at 37°C in YT and/or 2 \times YT medium containing ampicillin (100 μ g/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 μ g/ml) and streptomycin (50 μ g/ml for *E. coli* TH2) or ampicillin

Table 1. Plasmids prepared in this study.

Plasmid	Relevant properties	reference
pUC 118	3162 bp; Amp ^r , <i>E. coli</i> plasmid	26
pSKE 1	6.9 kb; pUC118 containing 3.7 kb <i>KpnI</i> DNA fragment from <i>S. kasugaensis</i> M338-M1	This work
pKF 3	2247 bp; Sm ^s , Cm ^r , <i>E. coli</i> plasmid	27, 28
pSKE 2	6.3 kb; pKF 3 containing 4.1 kb <i>PstI</i> DNA fragment from <i>S. kasugaensis</i> M338-M1	This work
pTV 118N	3163 bp; Amp ^r , derived from pUC118	29
pTV 273kac	3.6 kb; pTV 118N containing an amplified <i>kac</i> ²⁷³ structural gene, 440 bp <i>NcoI-BamHI</i> DNA fragment from <i>S. kasugaensis</i> MB273-C4	This work
pTV 338kac	3.6 kb; pTV 118N containing an amplified <i>kac</i> ³³⁸ structural gene, 437 bp <i>NcoI-BamHI</i> DNA fragment from <i>S. kasugaensis</i> 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 (ALFredTM DNA Sequencer, Pharmacia). Sequencing reactions were done using the Cy5TM AutoCycleTM Sequencing Kit (Pharmacia, Code No. 27-2693-02) according to the supplier's instructions. The sequencing reactions were analyzed with an ALFredTM 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 Cy5TM AutoCycleTM 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 BLAST⁴⁾.

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

5'-KAC primer (Sense: 5'-GGCCATGGCGCGCTGGGCCGGGACA-3') and 3'-KAC primer (Antisense: 5'-GGGGATCCTCGTTACAGGGGCGATCA-3') were used for amplification of both *kac* structural genes. PCR amplification was performed using a MiniCyclerTM (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 MgCl₂ 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 *NcoI/BamHI* (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.

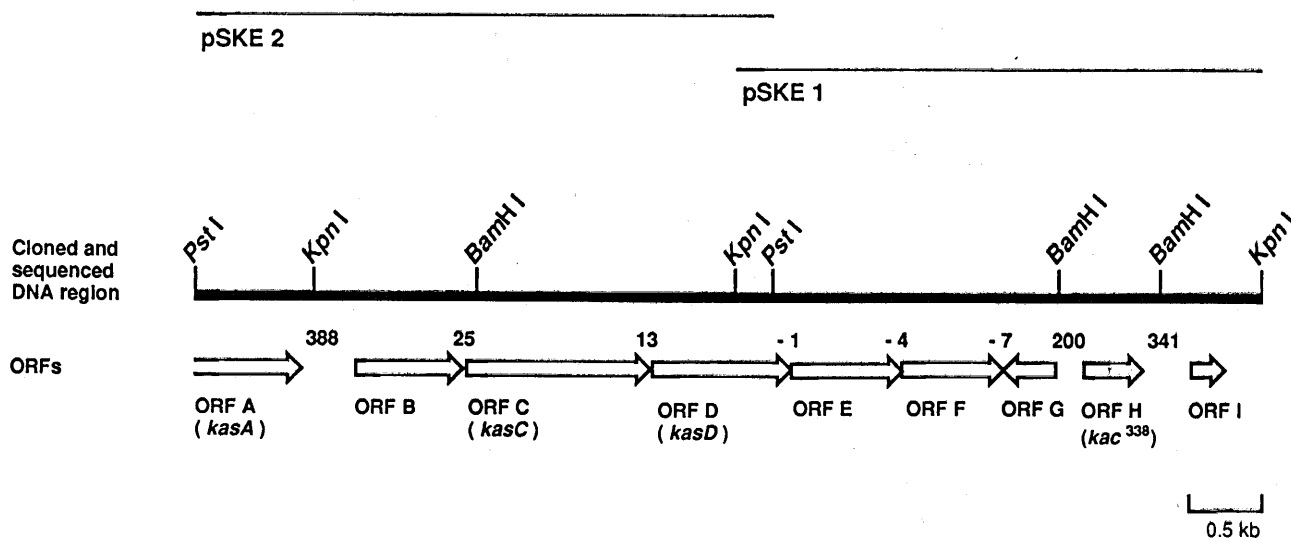
Single colonies were used to inoculate 2 ml L-broth containing ampicillin (100 µg/ml). After 16 hours of growth at 37°C, 1 ml of each culture was used to inoculate 9 ml L-broth containing ampicillin (100 µg/ml) and growth was continued at 37°C for 10 hours. At an OD₆₂₀ of 0.5 isopropylthiol-β-galactoside (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 positions⁵⁾, 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 program⁴⁾. 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 (Kac²⁷³ and Kac³³⁸), 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.

<i>Pst</i>I										
CTGCAGCGG	GGCTCGTGA	CAACCTCCGG	GOOGATGTA	TGGTATGGG	CTCGCAGGT	TACCGCGCT	GGCACTCGA	CGCGGCTTC	ACGGACGAG	100
L Q R G	L V D	N L R	A D V M	V C G	S Q A	Y R R W	A L D	A G F	T D D D	
ORF A (<i>ksa</i>A) -----▶										
ACATCCAGT	CGTGGAGTC	GCCACCGACC	TGACCCGCT	CGTGAAGAC	GGCGCCGCC	GCGCCGCTG	GGTAAOCCG	CACCGCTCG	CCGACGAGA	200
I H V	V E F	G T D L	D R F	R D D	G A A R	A R W	R N R	H G V A	D D E	
AACGGTCTC	CTGGTCCCG	CGCGGCCGT	ACCACGCAAG	TGCATCGAG	ACGCGTGGC	CGCGCTGGC	CACCTGGCG	GGCGCACCC	GGCGCCCGC	300
T V F	L V P A	R P V	P R K	C I E D	A V A	A L A	H L A G	R H P	R A R	
CTCGTCTGA	CCAACCCGAC	CGCGGCGAG	CTGACTCTT	ACGCGAGAA	GCTGGGGCC	CTGGGGGAG	AACTGGGGT	CGCGACCGG	GTGATCTGG	400
L V L T	T P T	A R T	L D S Y	A E K	L R A	L A D E	L G V	A D R	V I W E	
AGCAGGACT	CTCCTGGAC	GACATGCCA	TGCTGTACG	GGGCGGAC	CGGTGGTAC	TGCCCTCTC	TCAAGAGGG	TTCGGCATG	CCCTGCTGA	500
Q G L	S W H	D M P M	L Y A	G A D	A V V L	P S S	H E G	F G I A	L V E	
GGAAATGGC	GGCGGGGCG	CGTGTATC	CTGAAATGC	GAGGGCAG	ACGAAGTAT	CGACCAGAA	CGAACGGGT	TCCTTACCC	GGCCCGGAC	600
G M A	G R R P	V I T	S N V	E G H D	E V I	D H E	R T G F	L Y P	A R D	
GTGAGGGCG	TGGCCGGTG	CATGGCGCC	GTCATCACT	CGAACCTGA	GAACCTCGT	GGCGGGGCG	ACCGGAAGC	GGTGGCCCG	TTCCTGCTG	700
V E A L	A G C	M A R	V I T S	D L E	N L V	A A A H	A E A	V R P	F S S T	
CGCGGTGGC	CGCGGGCAT	GACCGGCT	ACGAAGTGG	GTGTGGCCG	CGGTGACCG	ACAAGGGTC	AAGCACCGT	CAGCCCTGA	GACCGAAGG	800
A V A	A G H	E R A Y	E V A	C G Q	R *					
<i>Kpn</i>I										
CGGATATGA	GTCACACCA	AGCCCTGGC	GATCAGAGT	CGCAGGTAC	CCCCGAAGC	GAGGACCGT	TGCCAGGGC	ACTGGGGCC	TGGCTCGGG	900
CGCGGCACC	CAGCGGGCG	ACGGCCCTA	CTGGGATGG	CAGCACCCG	ACGGCCGCT	CTCCTAACC	TATCCGAGA	TCACCGATA	CATCCTGAG	1000
TTCCTGGCC	ACGAGACACT	GAAGCGTCC	GAGCGGACG	GGCGGACGC	GGCGCGCTG	TGGCTGGCG	ACCGCATGA	CAGCGGGAC	TACTGTGCC	1100
GTCCGACCA	CGCGAAGGC	GGAGTCTCC	TCTTCGACAT	CGGCATGAT	ACCCAGGTC	TGCTCTCTA	CGCGGGGCG	ACCGGTGAC	AGGACTGCT	1200
				M I	T H G L	L S Y	G R R	T G D Q	R L L	
ORF B -----▶										
CTCCTCGGA	CGCGCCCGG	CCCGCTTCT	CTCGAACAC	CTGGGACCC	GTGGCCACC	CCAACCCCTG	GCCACGGGAC	ACCGCCCGA	AGGAAACGAG	1300
S S G	R A A A	R F L	L D H	L P T R	G H P	H P L	A T G H	R P E	G T E	
CCGACCTGG	CGAACTCCG	CAGCGCCAC	CTGCTCAAG	TGTGACAAG	ACTGATCAG	CGCGACGGG	CGGGCTCAC	CGGAGGGCA	CAAGCCCGG	1400
P T W S	N S G	S A H	L L K L	V Q A	L I S	A D G A	G V T	G A A	Q A A E	
AACGGCTGT	GGACACCGT	CTCGCGACC	CTGCCCCGC	CTGGCGGGG	CCGTCACGA	OCTGCCCGA	CAGGATCTG	ATCAGCCTC	ACCGCCCTG	1500
R L V	D T V	L A D P	C P P	S A A	P V T T	C P D	S D L	I S L H	A A C	
CTACCGCC	GAGGGCCGT	GGATCTGGD	CACGCCCGC	CGCGCGGG	AGGGCGGGG	ACGGCGGTC	CGCATCACG	AGTGGGTGT	GCAACAGCAG	1600
Y A A	E G L W	I W D	T A R	R R P Q	A R E	R A V	R I T E	W V W	Q Q Q	
CTCACCGGG	GGCGCTTAC	CACGTAAGC	CACCGCAGG	GGCGCCCGC	CTCGAACGG	ATGCGTCCG	ACGTCTGGC	TCAGCGATA	CGCTGGCCA	1700
L T G G	G F T	T Y A	H R T G	G P A	S D R	M Q S D	V L A	Q A I	R L A K	
AGTGTCTGA	CCTGGCCCG	GACGGCTCG	GCAGCGGGT	CTGAACTGT	GCCAACAGC	TCCACACCTA	CGAAGACAAG	GCAGCGTCC	TCTACCGCC	1800
L L D	L R P	D G L G	S A V	S T L	A N S L	H T Y	D D K	A A V L	Y R P	
CCAGCGCC	GAGCGCACC	GCAACAGCT	GAGCTCATG	TTCGCGGGC	AGCCCTCGG	OCTGTGGGC	GCAGGGCGG	GTCTGGCTG	GCAAGACTG	1900
Q A A	E P H R	N S W	S S M	F A G Q	A L R	L C G	A E A G	L A W	H E L	
GTCTAAGCG	CCCTGGGAA	AGCCTCAAGA	CATGATGAC	TGGTCTTGC	TGTGGGGCG	TCAGCCACTG	CGCGGATCG	ACTACAGCA	TGCGCGGGA	2000
V *		RBS	M S D	S V L L	S A R	Q P L	P P I D	Y S H	A A G	
ORF C (<i>kas</i>C) -----▶										
<i>Bam</i>H I										
GCATGGATC	ACACCGCGA	AGGGACAGT	CTGCTGGAG	CGGCAGCGG	TCTGGTGTG	GTCACATCG	GCCACGCCA	CCCGCACGT	GTGCAACAG	2100
A W I H	T A E	G D S	L L D A	A S G	L V C	V N I G	H A H	P H V	V E Q I	
TCACCGCCA	GGCCGCACC	GCCACCTTC	CTCCCGCGG	GGTGTGCTC	CGCGGGTGC	AGGAAGAGCT	GGCGCGCGG	CTGACCGAG	CCGTCAACG	2200
T R Q	A R T	A T F A	S P G	V L L	P A V Q	E E L	A G R	L T E A	V N R	
CGCGGGGAC	GGAGTGAGC	TGGCTCTCT	CGGAACTCC	GGGTGGAAC	TGGCCATCT	CTACCGCGG	CTCATCCAG	GCTCGCGGG	CCAGGAGGC	2300
P G D	G V S L	A C S	G T S	A V E L	A I S	Y A R	L I Q R	S R G	Q D G	
AGGCATACA	TCCTCACCG	CGGCTCGGC	TAACACGCA	ACAGCGGCT	GACCTGGGG	CTGTCCGGC	ACCGCGCGG	ACGCGCGAC	CCGACGAGC	2400
R H H I	L T A	R L G	Y H G N	S A L	T L G	L S G H	R R R	R P H	P D D A	
CCCTGGGCT	GGCACCGCC	TTCGACCGC	OCTACCGGG	ACACCGCGC	GACTGCCCC	ACGACCGGT	CCGGGCGAG	TGTGGCGAG	CCGTGGCGA	2500
L G L	A P A	F D P P	Y P G	H H R	D C P H	D R C	R A S	C A D A	V A E	
GGCATGAC	CGCGTGGGC	CGAGTCCGT	TGGCGCGTA	CTCATGAA	CGTCAACGG	CAOGAAGGG	GGCGCTATA	CGCCACCCG	CGCTATCTG	2600
A I D	R R G P	E S V	A A V	L I E P	V N G	T T G	G A Y T	P P P	G Y L	
CGCGGCTGC	GGCGGCGTG	CCAAGAACG	GGTGTCTGG	TCATCCAGA	CGAGTCTCT	ACCGGCTGG	GACGACCGG	OCTGGCCTC	GGCGGGGAC	2700
A A L R	R A C	H E R	G V L V	I H D	E V L	T G L G	R T G	L P L	G A D H	

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.

ACTGCAAGGA C T D	CCCGCGGGG A A A	GACATCGTG D I V V	TGCTCTCAA L S K	GGACTGTCC G L S	GCCGATAAC A G Y L	TTCCGCTGGC P L A	GGCGTGCTC A V L	GTGAADCCG V N P E	AGGGAGTGGC G V A	2800
COGGCTCCG R L R	TCGGGCCCC S G P R	GGCCACTGCC P L P	CCTGATGGC L M G	ACCATGTCCG T M S A	CGACCCCGCT T P L	CCAGGCGCG Q A A	GCGGGCTCG A G L A	CGGTGCTCGA V L D	TGTGCTCGC V L G	2900
GAGATGGCG E I G A	CCCTGGACCC L D P	GGCTCGGTC A R V	CGGGCGGGG R G G D	AGTTCGGCC V A A	CGCGTACGG A V R	GCGTGGCAG A V A A	CGCTGCCCCT L P V	GGTGCAGGAG V Q E	ACCCGGGAG T R G V	3000
TGGGCTACTT G Y F	CCACGGGTG H A V	GAGCTGGCC E L A P	CGGCAOCCA G T Q	GCCCGGGCC A A A	CTGGCGCGC L A A A	CCCGGACGA R D E	GCGGCTGCTG R L L	CTGTACCCCT L Y P F	TCAACGGATT N G F	3100
CCACGGGAC H A D	GGCAOGGGG G T G E	AAGGACTGAT G L I	CGTCCCTCT V A P	CCGCTGAACT P L N S	CCACCCCGTC T P S	CGAAGTCGA E V E	TTCTGGCAC F L A Q	AGGGCTGGC G L R	TCGTGGCTG R A L	3200
GAGCGCACG E R T A	CCGCCCCGTC A R S	CGAAGACCGA E D R	TCOACCCGCT S H R *	<u>GAAAGGCAAC</u> RBS	TCCTGATGTC M S	ACCCACGACC P T T	ACGCACTGG T H W A	CAGGACGCCA G R Q	GGTCTGGTC V L V	3300
ORF D (kasD) -----▶										
ACGGGAGCG T G A D	ACGGTTTCAT G F I	CGGTTCGCAT G S H	CTCAOCCGAG L T E T	CCCTGGTGG L V S	CCGCGCGCC R G A	CGAGTCACG R V T A	CGGTGCTCG V V R	ACGGTCTCG R V S	GCCGACAGG A A Q V	3400
TGACGCAOCC T H R	GCTTCGCAAT L R N	CTCTTCGGCG L S A A	CGACCGTCCA T V D	CGCCCTGGAA A L E	CGGTTGGTGC R V V H	ACGTGGACCT V D L	CGGGGACCT A G P	TGGCGCTCG S A L R	ACGTCTCGG T L G	3500
COGGCTGGAG R L E	GOOGACACCT A D T W	GGTTCACCT F H L	CGCGGGGAC A A D	GCCTAAGTAC A Y V P	CGGCATCGCT A S L	GGACAGCCG D Q P	GCGATGTTG A D V V	TGGCAOCAA R T N	TGTGATGTC V M S	3600
ACTTCCACG T L H V	TCCTGCTGGC L L A	AGCCAGCAG A Q Q	CGGCAGCCG R Q P A	CGCAOCTCT H L L	GGTGAOAGT V T S	TCGAGCGAG S S E V	TCTACGGCAG Y G S	CCAGCCGGAC Q P D	GCGATCACG A I T E	3700
AACGGCATCC R H P	GCTGGAACCG L E P	GCCAGCCCT A T P Y	ACCGGGGTC A A S	CAAGTGGCC K V A	TCGACCGCC C D R L	TGGCTGGTC A W S	CTGGCAOCC W H H	ACCTACGGC T Y G L	TGCGCTCAC P L T	3800
KpnI										
CATGCTCCG I V R	CCGTTCAACA P F N S	GCTACGGCC Y G P	CGCCACGTC R H V	TACGACGGG Y D A V	TACCCCTCTT P L F	CCTGGCCAGA L A R	GCGTGGGGG A L R G	GCGAGCCGAT E P I	CAOATCAAC T I N	3900
GGCAGCGGTG G S G E	AGCAGACCGG Q T R	CGACCTCAC D L T	TTGTTGGCG F V A D	ACACGTTCC T V A	GGGTTCTCT G F L	GCCCTGGCG A L A E	AGCTGGCCG L P A	CACCGCCGAG T G E	ACGTACAACA T Y N I	4000
TGGCAOCCG G T G	CAOCCACAC T D H	CGCATCATG R I I D	ACGTGGCCG V A R	CGCATGTG A I V	GCCTGACCG A L T G	GGTCCAGAG S Q S	CGAGATGCTG E I V	CAOCCOCCAC H G P P	CGCGCTCCG R S G	4100
PstI										
CGAAGTCTC E V L	AAGCTGACG K L Q A	CGATCCGGC D P A	GAAACTCAC K L T	GAGGCACCG E A T G	GGTGGGTCG W R A	CGAGTAOGAC E Y D	CTGGCCAGG L A R G	GCCTGGCGGA L A D	CAACCTGGTC N L V	4200
TGGATGGGG W M R E	AACACGTGGA H V E	GAOGTATGG T V W	COGACAOGAT P T R S	CCTGATGGAC *	GOOCCGGCG M D	TCCTCTCAA A G G V	CAACGTACC L F N	GAAGAGACG N V T	ACTTCTGGA E E T D	4300
ORF E -----▶										
COGCTCGCC R L A	GCCCGCACG A R H G	GTCCGACAC A D T	CCAGCGCTG Q R L	CGCGGGGAG R G E I	TOGAGCCCG D A R	CGACCCCGG D A A	TACGAGACG Y E T G	GGCGGGCCA R A H	TGTCACGAC V H D	4400
GTCCTGGTG V L R D	AOCCTCGC A L A	CGCCCGGGG A A G	GCCCCGGCG A P A G	GGTTCGGGT V A V	GGACCCCGCA D A R	TGGCTGGAG W L D D	ACCTGTACG L Y R	GGACTGCCTG D C L	CGGTTACCC A V H P	4500
COGGGGGTT G A F	CAOCCGATC T A I	GACGGTATCC D G I R	GOOCCACCG R H R	CCOCCACATC P D I	CTGCTGTAC L L V L	TGACGAACAA T N N	CGAGCCCGC E A A	CACTGGGAC H W D R	GGTCAAGGA V K D	4600
CGACCGGTAC D R Y	GGCCACCTCG G H L G	GACGCTTCGA R F D	CGTCATCGC V I A	TOGTTGGCC S S W Q	AAGTGGCCGA V G E	GGTCAAACCC V K P	ACCGGGGAGT T G E F	TCTTCGAGGC F E A	CGTCCCGCG V A R	4700
CGGTCCGGC R C G R	GCCCGCTCGA P L D	CGACCGGTC D A V	CTGCTGGAG L L D D	ACAATCCGGA N P D	CGTACTCGCC V L A	GAGCCCGCC E A R H	ACCACCCCT H G L	GCCACCCCTG R T L	CAOCTGAAT H V E S	4800
CGCCCGCAC P A T	GCTCCCGAG L P E	GCAGTGGCC A V A R	GCCCTCTGG L P G	ACTTCCCGC L P A	ACCGCCCGCA T G P I	TACGAAGGC R R P	GGACATGAAC D M N	GCGACCTGC A T S P	COGAAGCAC E D T	4900
CCCGGGCGG P A A	CAGCGCACAC Q R T P	CCCTCTGCC L L P	GGAGCAGCC E Q R	CGCTTCTCA R F L T	CCACCCGAT T A I	CCCAACCTC P N L	CAGGGCGGG Q G G G	GCGGACCCG G R R	CGGAGTCCG G V R	5000
TCOACCTCA S H L I	TOGGCCACG G H G	TOGCGGGCC R R G	CTGCTGGAT L L R *	GAGCCCGCC M T A A	GOOCCITCAC A A S P	CGGAGCACT E D L	GGCACOGATA A P I	CAGGCACGGA Q A R T	CCCTGTCTG L F V	5100
ORF F -----▶										
GATGCGGGAC M R D	GCCAGACTCA G R L T	COGCGATCAA A I N	CGACGTGGC D V G	CGCCCGGCC R P A P	CGCCCGGGT P R C	CTTCTCTCG F L S	TACAGGCCA Y T A T	CGCCGTGAC A V T	GGGTGGAAC A W T	5200
AGCACCGCG S T A V	TGCGCGGGA P A D	CCTCAOCCG L T D	GAGCTGGAC E L D R	GATGGGGCC W A A	GGACCAGAC D Q T	CGCCCGCCG P A P L	TGACCGAGCT T E L	CGTCAGCCCC V S P	CGCGCGGGG P A G A	5300
CGCTCCGGCT L R L	GCTGGCGGG L A R	CACGCCCGG H A P V	TGACGGAGAG T E S	CTTGTAGGC F V G	CCCGCTACG P A Y A	CCGTGCCGA V P D	CTCCCGAGAC S A D	ACACCGCCG T P A P	CGCCAGGCAT P G I	5400

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.

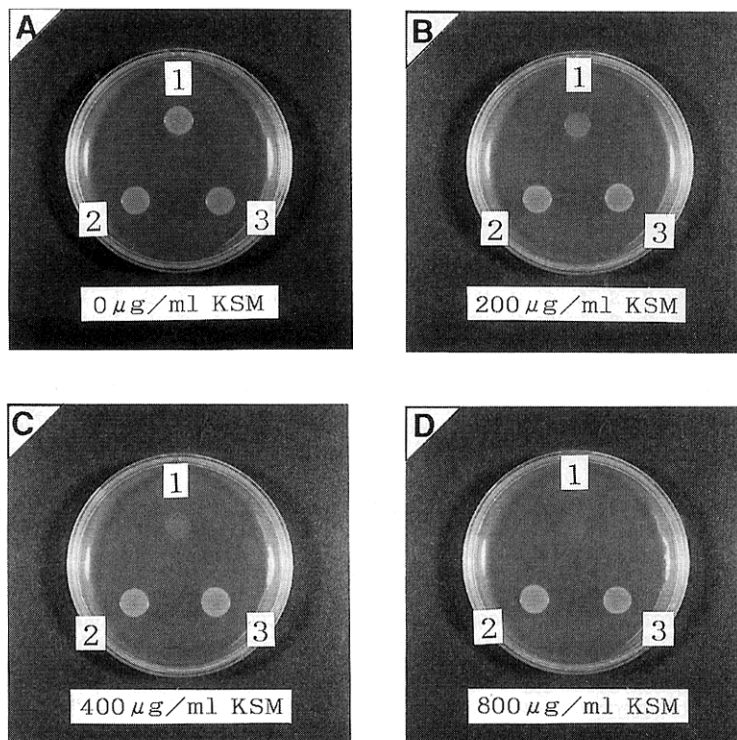
CACCOCTGOGG T L R	GACTGOGGTC D C G P	CGTGGAGTIC S E S	GTCOCGCTIC S R F	CGCGAGAAGT R E T F	TCCCGAAGT P E V	GGCGGACACC A D T	CTCGCGAACC L A E R	GGCAGCGGAC Q P T	CGTGGCGGCG V A A	5500
TTTCGAGGOC F D G H	AOCGAGCGGT R A V	CGCGTCTGTC A V C	TGCGGGGGCC C A A R	GCGAGGGGG D G G	CGAGGCATAT E A Y	GAGGGGGCA E A G T	CGGAGACT E T L	GCCGAGCCAC P S H	CGCGGGGGG R G R G	5600
-32.34 kcal/mol										
GCCTCGOCTC L A S	GGCACTGGTG A L V	AOCGCTGGG T R W A	CCCGGCTGGT R L V	GCGGCGAGT R A R	GGCGCCCGC G A R P	CGCTCTACAG L Y S	CACCGAATGG T E W	GACAACCCT D N H S	CGTCCGCTC S L S	5700
CGTGGGAGG V A K	CGGCTGGCCA R L A M	TGGAGCTGTA E L Y	CGCGCTCAAC A V N	GTCAGCTCT V S L Y	ACTGACCTC * Q G E	CTGACCCAC Q G G	CGGACTGCT G V Q	GCCGCGCCA Q R G C	CGTTCGCTG T G H	5800
ACGGGGGGG V R G	CGGGGAAAG A A S L	GCGGAACCC G F G	GGGGCGGAC P A P	AACGAGATGG C R S P	CCCGAGACT G L G	GCTGGCGGT A A G	GCCGGGAGC H G S A	CGTCCGGGAC T R S	GACGGGACA S A C	5900
CCACCCCAAG V V G R	ACAACCTGTC C S T	CGCGAATGG R S S	TGCGAGCGG T S L P	CGACCCCGC S V P	GAGATCTCT S I R	COCTCAGCG E R L R	CATCTGGAG M R S	AGCGGGGAC L P P	CACCTCCCG G S G P	6000
CGCGCCCGC A G R	AGCAGAGTGG L L T	CGCTGATCG A S I P	CAGTTTCAAC L K V	GCTGCOCTC A A R	TGGCGGGGT R P R T	OCTGGCGTC R R T	ACCTTGGCC V K R	GGTGGGGAT R H P I	CGACCCGCT S A T	6100
BamHI										
GTTGAGTTC T S T	AOGCATGGG W P M	AOGGTACGG G F G	AGCCGACCG G F G	CGGATCGGG G F G	CAGTACCTC G F G	TACCGCAGG G F G	AOCGAGAC G F G	CGCTTGGCG G F G	ACACACTGG G F G	6200
ORF G										
CGGOCACGC C G G	CGGTACGGC C G G	GCGCATCGG C G G	GCATCGAGAT C G G	TCTCAGGGC C G G	CGGAGTTGG C G G	GCCGGCGGA C G G	CGGTCAACTC C G G	CCCGCAGGG C G G	GCGCTACTC C G G	6300
ACTCGGAAC RBS	COGTCAGTG V P W A	COGCTGGG P R W A	CCGGACATA G T Y	CGATGGGGC D G G	GTGACGATCA V T I T	CGTACGAATG Y E W	GCGGGTGGC R G G	TTGACAACG F D N A	COGCTCAA A L N	6400
ORF H (kac 338)										
CGCACTGCAC A L H	GCGAGCGTT A D G F	TGGTCCCGC G P P	AATCGGCGAG I A Q	ACGACTGGC T D W R	GAACGGGGT T R L	TGAGCGCAC E R H	AGCCTGGCT S L G W	GGTCTGTGC V C A	GTGGGAGGAC W E D	6500
GGTCTGATG G L I G	GAITTTGCAA F V N	CGTCTCTGG V V W	GAAGGGGAG D G G A	CCCATGCTT H A F	CACTCTGGAC I L D	AOGTGTGGC T V V A	CCCGGACTG R H C	COGCTGAGA R S R	GGAGTGGGG G V G A	6600
COGCTCTGT A L V	CGCAAGGGG A K A	GCGGAGGAG A D E A	CCCGTGGGC R A A	GAAGTGGAG N C E	TGGTTGCAAG W L H V	TGACTTCGA D F E	GGAGCACTG E H L	CGCGGTTCT R A F Y	ATTTGATGC F D A	6700
CTGGGCTTC C G F	AAGGAGCGA K E T T	CGCGGGGCT A G L	GATCGOCTG I A L	TAACGAGGC *	GCCCAAGCC *	TGGGCTGTG *	CCTACGACG *	GGGGAGGCA *	CGCGGACCG *	6800
BamHI										
ACCCAGGATT A C C	GAGGGCGAT G A G	CGTGGGCCC G A G	TGCTTGATCA G A G	CAAGAGGTT G A G	COCTCAGAAC G A G	TGATGGGGT G A G	CCGCGCATCC G A G	TTCTGGCCCC G A G	GCTGACGGG G A G	6900
ATGTGTGAA A T G	ACCGAAGTIC G A G	GGAGCGAGG G A G	ATGAAGATC G A G	GTTTACCTG G A G	ACTGGTGTIC G A G	CGAGCTGGC G A G	GCCACCGGC G A G	GGGGGCCAG G A G	COCATTCTG G A G	7000
AGGAGCAAGT A G G	TGTTGACCT G A G	CATGTCATC G A G	ATCCGGTCC G A G	CGGAGGAC G A G	CGACATGGC G A G	CCGTTGGCG G A G	ACGCGCTGC G A G	COGATGGCC M R	COCTTGTGC P L C L	7100
ORF I										
TGGCAGAAGA A E D	CGGCTGGTC G C V	AGCTGGGAG S W E A	CGTAACACTC Y H S	ACAAGGAA H E E	CCCGCGGCT P G R F	TGTOCTGGT V L V	CGAGGCTGG E R W	GCCAGCGCG A S R A	COCACTGGGA H W E	7200
GGCTCAGAT A H D	GOGGTGAAG A G D A	CCATCCAGAA I Q K	GATCTACATC I Y I	COGGAGCTCA P E L M	TGCGCGCAT P R I	CGAGGCGAG E R E	GTGCATCCA V H P S	GCATGCGGT M R V	ACCCAGCAAG P S K	7300
CATGAAGCG H E A V	TAAGTGGCTG T G *	AACCGTTCAC T G *	GTTGTGGGC T G *	CGGGGCTC T G *	GGCGGACAC T G *	CGTACCGGG T G *	AGCGCGGCT T G *	TTGAGCGGG T G *	CGAATGGGC T G *	7400
GACGGGCTG -36.70 kcal/mol	CGGAGCAGG C G G	CGGCGAGGG C G G	CGCAGCGGT C G G	AAGGATTGAG C G G	TGGTGGCGG C G G	AOCGCGCGG C G G	TTCACAACC C G G	GGCTGTGCA C G G	CGGACAGCA C G G	7500
KpnI										
GCGTCCAGAG G C G	CCACTTCAC C C A	CTCTCCCGT C C C	CACGGCAGAT C A G	GAATTGGTT G A A	GCGGGGGAC G C G	CGGTGAGCC G C G	GGTGGGTAC C			7581

(G115A alone caused a missense mutation, while the others silent), and deletion of three sequential bases, CGG (186~188). In the deduced protein (Kac³³⁸), consequently, there were an amino acid substitution of Val39Ile and a deletion of Gly⁶³ (Fig. 3). The amino acid sequence homology between Kac²⁷³ and Kac³³⁸ 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. 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 I¹⁷⁾ 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 moiety²¹⁾, (2) D-glucosamine was readily converted to UDP-N-acetylglucosamine (UDP-GlcNAc), which accumulated in mycelia, and (3) a carboxyformidoyl group was selectively

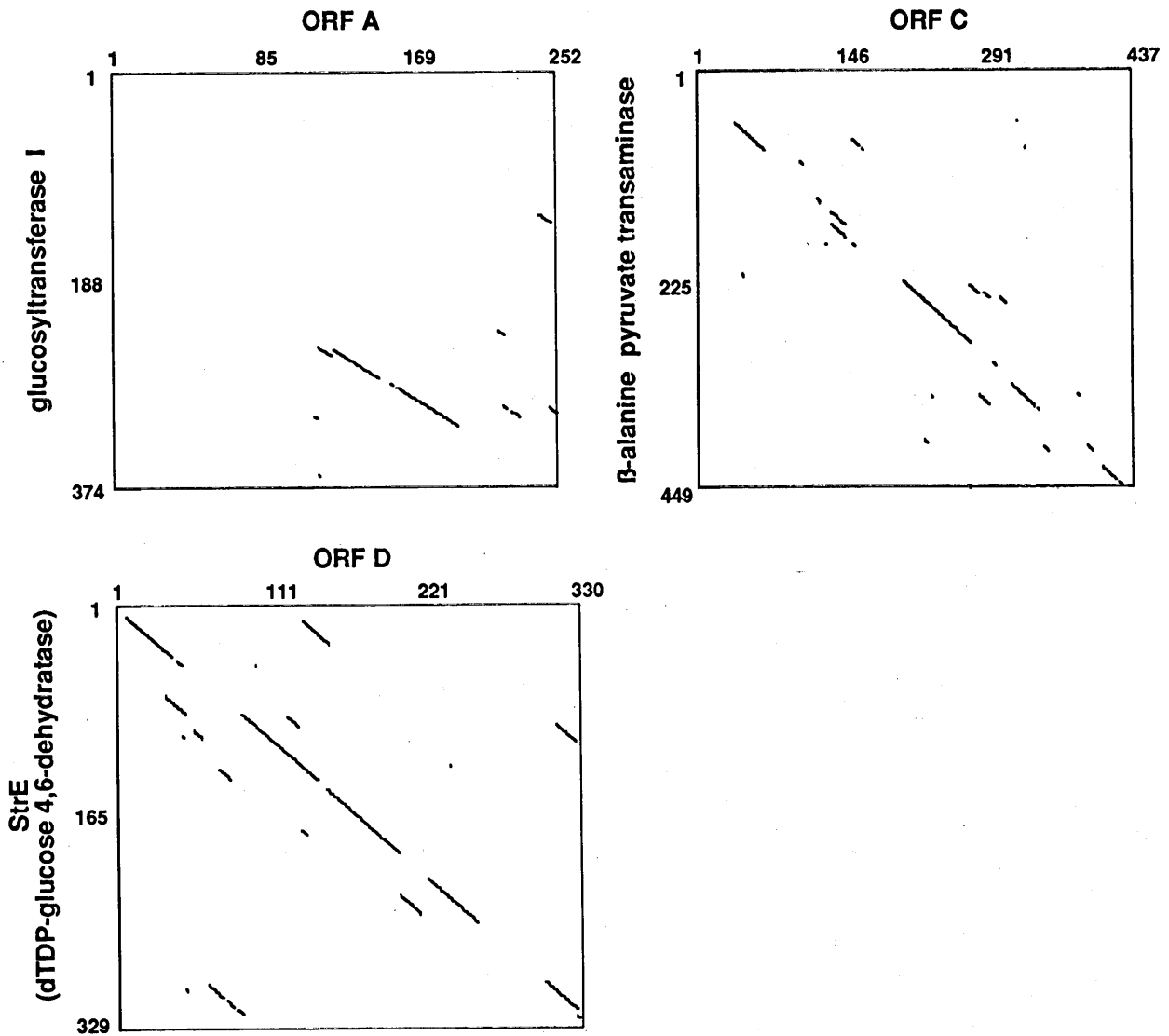
added to the 4'-amino of the kasugamine moiety. These results suggested that UDP-GlcNAc was an early precursor of KSM biosynthesis and, together with other results, led FUKAGAWA *et al.* to propose a tentative biosynthetic route of KSM (Fig. 8, from the thesis of Y. FUKAGAWA, partly modified by S. IKENO).

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.

KasD	1	MSPITTHWAG	RQVLVT-GADG	FIGSHLTETL	VSRGARVTAV	VRRVSAAQVT	50
RfbB	1	M--AT-W----	LVTRGA-G	FIGANFVLEA	VSRGIRVVN-	---LDALTYA	37
StrE	1	M--TTH-----	LLVT-GAAG	FIGSQYVRTL	LGPGGPPDW	VTALDALTYA	42
		*	.*..	*** ** *	***.	*
KasD	51	HRLRNLSAAT					60
RfbB	38	GNLNILASLE					47
StrE	43	GNPDNLA AVR					52
		..*					

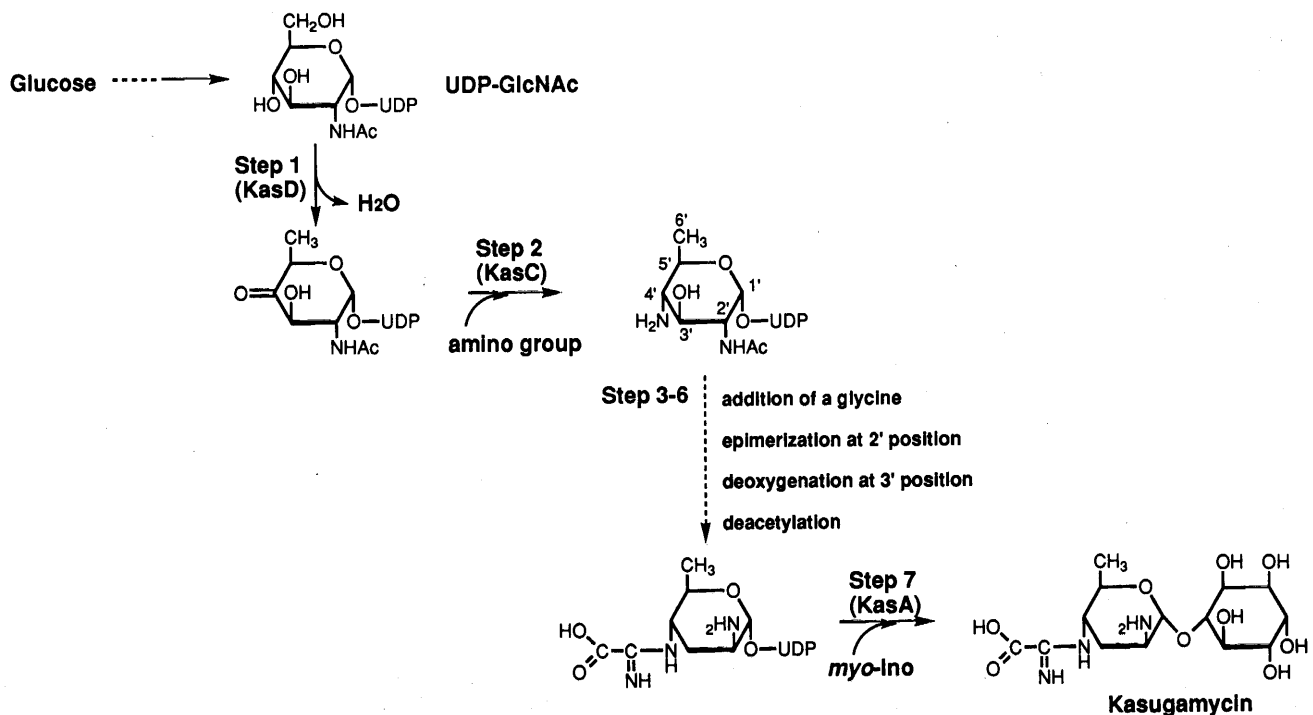
Highly conserved amino acid residues among NAD(P) binding proteins were indicated asterisks (*).

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

		**	*	**	*	*	*	*
1)	237	VIHDEVLITGL	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	GRTGIMFACQ	RDGVT---PD	ILTLSKTLGA	G	276	
5)	237	LIVDEIQTGI	GRTGELICYD	HYK-AFAKPD	IVLLGKALSG	G	276	
Consensus		LLXDEVXXGL	GRXG-----X(12-16)----D		IXXXSKXXXX	G		
		VV	I	PI	S	A		
		II	L	V	A	S		
		MM	M	M		D		
		FF	A	F				
		YY		Y				
		WW		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 operons²⁴). 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 TTA²⁵), 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.

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