REVIEW ARTICLE

Control of Avermectin Biosynthesis in *Streptomyces avermitilis* for the Selective Production of a Useful Component

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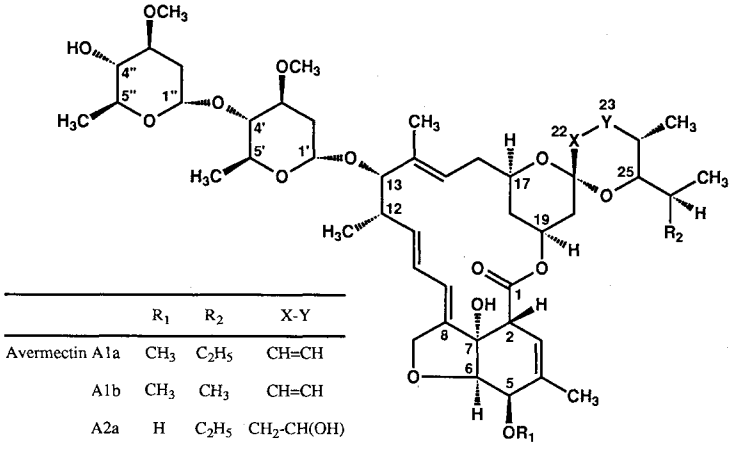
(Received for publication March 1, 1995)

Of the several thousand fermentation products which had been described until the discovery of avermectins, several compounds, anthelmycin[1], anthelvencins[2], aspiculomycin[3], axenomycins[4], G-418[5], hygromycin B[6], destomycin A[7], paromomycin[8], and thaimycins[9], were reported to possess anthelmintic activity. Only aminoglycoside antibiotics, hygromycin B, destomycin A and paromomycin, have been used as antiparasitic drugs in actual, whereas, synthetic compounds dominated the anthelmintic market. The discovery of the avermectins changed the market dramatically because the compounds are potent antiparasitic compounds with a broad spectrum activity against nematode and arthropod parasites in spite of lacking antibacterial and antifungal activities. Its dosage for animal and human is extremely low as well.

"Ivermectin", a semisynthetic avermectin, which is a hydrogenated product of avermectin B1 has been introduced to the market as an antiparasitic agent in 1981, and as an agricultural pesticide and an antiparasitic agent in 1985. The efficacy of ivermectin against nematode and arthropod parasites is unprecedented in potency and breadth of spectrum. Ivermectin is used for livestock production and the health care of companion animals. The efficacy of ivermectin in human onchocerciasis has made it a promising candidate for the control of one of the most insidious and intractable of tropical diseases[10]. Recently, ivermectin has been found to be effective for the treatment of human disease strongyloidiasis in Okinawa Prefecture, Japan[11].

Avermectins produced by *Streptomyces avermitilis* which was isolated from a soil sample collected at Kawana, Ito City, Shizuoka Prefecture, Japan are a complex of eight of sixteen-membered macrocyclic lactones substituted with a disaccharide of L-oleandrose, resulting from structural differences at three positions, C-5, C-22~23, and C-26 (Fig. 1)[12~14]. Among these components, B1 fraction has the most effective antiparasitic activity[15]. Since the industrial separation of B1a and B1b in the B1 fraction is quite diffi-

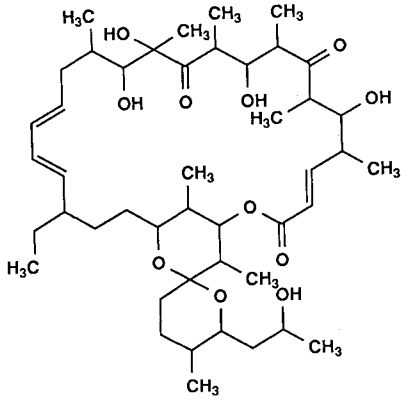
Fig. 1. Structural formulae for avermectins and ivermectins.



	R_1	R_2	X-Y
Avermectin A1a	CH_3	C_2H_5	CH=CH
A1b	CH_3	CH_3	CH=CH
A2a	H	C_2H_5	CH_2-CH(OH)
A2b	H	CH_3	CH_2-CH(OH)
B1a	CH_3	C_2H_5	CH=CH
B1b	CH_3	CH_3	CH=CH
B2a	H	C_2H_5	CH_2-CH(OH)
B2b	H	CH_3	CH_2-CH(OH)
Ivermectin B1a	H	C_2H_5	CH_2-CH_2
B1b	H	CH_3	CH_2-CH_2

Both sugars are α-L-oleandrose. Ivermectin is chemically synthesized from avermectin B1 components (B1a and B1b) by selective hydrogenation and commercial product of ivermectin consists of more than 80% of ivermectin B1a and less than 20% of ivermectin B1b.

Fig. 2. Structure of oligomycin A.



cult, the commercial product contains more than 80% of B1a and less than 20% of B1b components. Although *S. avermitilis* produces eight components of avermectins, only two components, B1a and B1b, are available for the medical, veterinary and agricultural fields. Furthermore, the microorganism produces not only avermectins but also oligomycin (Fig. 2), a polyketide antibiotic, that is a specific inhibitor for the oxidative-phosphorylation system in mammalian cells.

In general, most of microbial secondary metabolites, including bioactive compounds, are produced as closely related complex by their producing microorganisms. Macrolide compounds, including avermectins and their related compounds[17~20], are typical examples produced in such a manner. As a reason that such producing microorganisms produce many components, it is considered that the biosynthesis of these compounds is more complex and/or the enzyme(s) involving in their biosynthetic step(s) possesses lower substrate specificity and, therefore, has an ability to catalyze related substrates.

Thus, there are some problems for the efficient production of useful component(s) of avermectins. To solve such problems it is necessary to elucidate the biosynthetic pathway to avermectin and genes involving in the biosynthesis. In this review, the authors describe overview of avermectin biosynthesis and the control of the biosynthetic pathway for the selective production of specific component(s) of avermectins.

I. Biosynthesis of Avermectins

Several attempts for elucidation of the biosynthetic pathway of avermectin have been independently performed by us and Merck group. Now, the pathway for avermectin biosynthesis has been understood almost completely[21]. The proposed pathway is based on evidence obtained from several studies, including

incorporation of labeled precursors into the avermectins, identification of key intermediates produced by biosynthetically blocked mutants, the conversion of proposed intermediates to avermectins by biosynthetically blocked mutants or wild type producing strain, and the *in vitro* measurement of enzymes involved in the biosynthesis of avermectins.

A. Incorporation of Labeled Precursors

As mentioned above, avermectins are a family of oleandrose disaccharide derivatives of macrocyclic lactones. In the beginning, the studies of biosynthesis was performed by the incorporation of labeled precursors into the avermectin molecules.

CANE *et. al.*[22] clarified the lactone to be formed by a head to tail condensation of seven acetates, five propionates and one branched-chain fatty acid by using corresponding ^{13}C-labeled compounds. The 2-methylbutyryl residue (C25-C28) of the "a" components and the isobutyryl residue (C25-C27) of the "b" components were not labeled by either acetate or propionate. They were derived from L-isoleucine and L-valine, respectively[23,24]. This finding was supported by the efficient incorporation of L-isoleucine into the "a" components and L-valine into the "b" components. Furthermore, C25 of avermectin B1a was highly enriched by feeding of [1-^{13}C] 2-methylbutyrate and that of avermectin B1b by [1-^{13}C] isobutyrate[24].

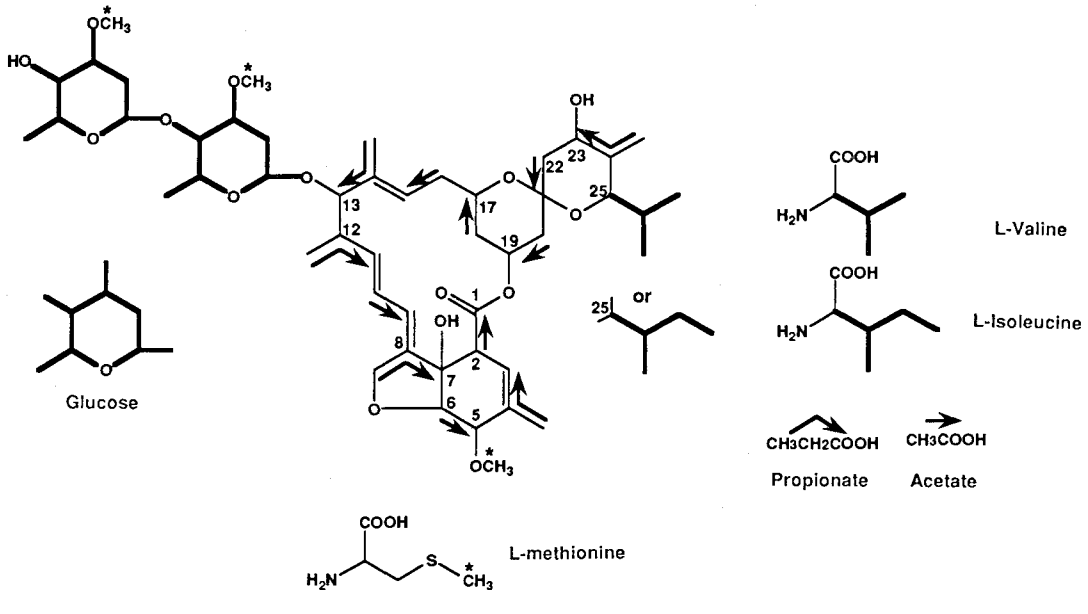
The origin of the oxygen atoms in the lactone was investigated by measuring the incorporation of ^{18}O-labeled-acetate and -propionate[22]. The oxygens at C1, C5, C7, C13, C17, C19, and C23 (for the "2" components) retained their isotope content. The oxygen at C21 would be probably derived from the 2-methylbutyryl or isobutyryl side chain, but the oxygen in the benzofuran between C6 and C8a was not. Although the origin of the oxygen of furan ring in other metabolites has not been investigated, that in the avermectins is seemed to be derived from "molecular oxygen".

The studies with labeled glucose indicated that glucose converted directly to oleandrose, and this finding was supported by studies with [1-^{13}C] glucose and [U-^{13}C] glucose, showing high enrichment of the oleandrose units[25]. Three methoxy groups at C5, C3′ and C3″ in avermectin have been confirmed to be derived from methyl residue of L-methionine by labeling experiments[24]. Figure 3 summarizes the results obtained from the incorporation studies with labeled precursors.

B. Biosynthetically Blocked Mutants and Characteristics of Each Step in Avermectin Biosynthetic Pathway

Several kinds of biosynthetically blocked mutants were isolated by us[21] and Merck group[24], independently. These mutants were classified into two types as follows; mutation in the early stage of biosynthesis (formation of 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycone) and mutation in the late stage of biosynthesis (after formation of aglycone; modifying aglycone). By the analyses of products accumulated by the mutants and conversions of intermediates, the pathway for avermectin biosynthesis

Fig. 3. Incorporation of labeled precursors in the avermectins.



* *aveAI* and *aveAII*.

Table 1. Characteristics of mutants affecting avermectin production.

Mutant class	Fermentation products	Mutation in
*aveA**	none	PKS (polyketide synthase)
aveB	aglycones	glycosylation
aveC	A2 and B2 components	C22,23-dehydration
aveD	"B" components	C5 *O*-methylation
aveE	6,8a-seco-6,8a-deoxy derivatives	C6,8a furan ring closure
aveF	5-oxoavermectins	C5 keto reduction
aveR	none	regulatory region
X	"a" components	?

after formation of avermectin aglycones was elucidated. Table 1 summarizes the characteristics of mutants. Although details about the formation of 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycones have not been clarified, the biosynthetic pathway to avermectins after formation of the aglycones is proposed as shown in Fig. 4.

1. *aveA* (*aveAI* and *aveAII*: step for polyketide synthase)

This class of mutation is in the step(s) of formation of aglycone (polyketide synthase) and the mutants did not yield any avermectin compounds[26], however, the mutants have ability to convert 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycones or avermectin aglycones to natural avermectins[26,27].

2. *aveB* (step for glycosylation)

The *aveB* mutants produce avermectin aglycones, indicating that the mutation affected in the step of glycosylation[26]. Avermectin aglycone glycosyltransferase, which catalyzes the stepwise addition of oleandrose units from the nucleotide sugar thymidine diphosphate oleandrose[28] (dTDP-oleandrose) to the avermectin aglycone, is demonstrated in cell-free extract[24].

3. *aveC* (step for C22-23-dehydration)

This type of mutants produce "2" components of avermectins[21,29]. The chemical structures of "1" components of avermectins differs from those of "2" only in the bond between C22 and C23: "1" components have a double bond, while "2" components have a single bond and additional hydroxyl group at C23. Avermectins A1a and B1a were not labeled by ^{14}C-labeled avermectin B2a aglycone, indicating that neither compound is the precursor of the "1" components[25]. On the other hand, avermectin B2a and avermectin A2a aglycone were converted to avermectin A2a by a *aveA* mutants[25,26] or the wild type strain fed cerulenin, which inhibits the formation of aglycones (unpublished result). These results indicate that dehydration at C22~23 occurs before formation of the aglycones.

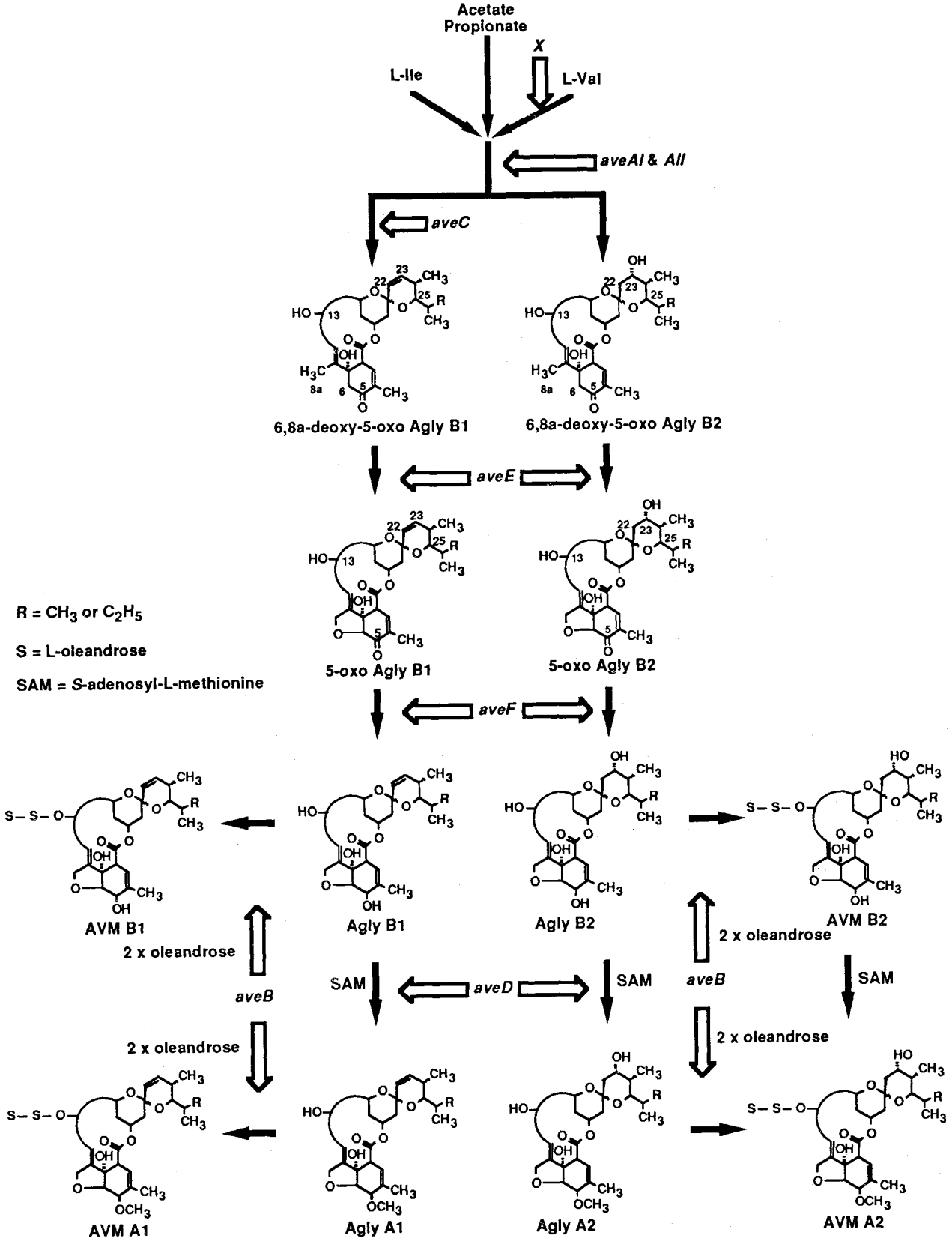
4. *aveD* (step for C5 *O*-methylation)

The mutants produce "B" components of avermectins[30]. The mutants lack the enzyme activity of avermectin B *O*-methyltransferase, which catalyzes the conversion of "B" components to "A" components by transferring the methyl of *S*-adenosyl-L-methionine to the C5 hydroxyl of the "B" components, yielding the "A" components and *S*-adenosyl-L-homocysteine[31]. This enzyme with the molecular weight of 70,000 was purified and characterized the substrate specificity for the avermectins, in order of decreasing activity, is as follows: B2a aglycone > B2a monosaccharide > B1a aglycone > B1a monosaccharide > B2a > B1a. The "2" components react faster than "1" components and there was virtually no activity detected with avermectin B1a as substrate[24].

5. *aveE* (step for furan ring formation at C6 to C8a)

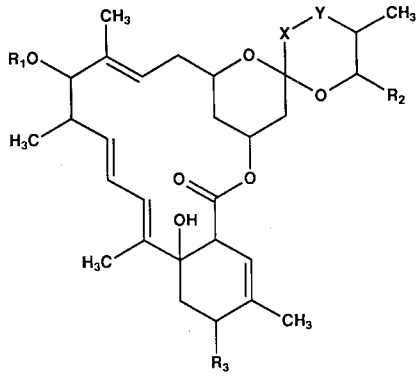
The mutants produce 6,8a-seco-6,8a-deoxyavermectin derivatives causing the mutation in the step of furan ring formation at C6 to C8a[27,32]. *AveE* mutants isolated by us and Merck group, independently, produced several kinds of compounds lacking furan ring consisted of avermectin aglycones, monosaccharides, and disaccharides (Fig. 5). Furthermore, some of compounds had keto group at C5 position. Probably, 6,8a-seco-6,8a-

Fig. 4. Proposed pathway for biosynthesis of the avermectins in *S. avermitilis*.

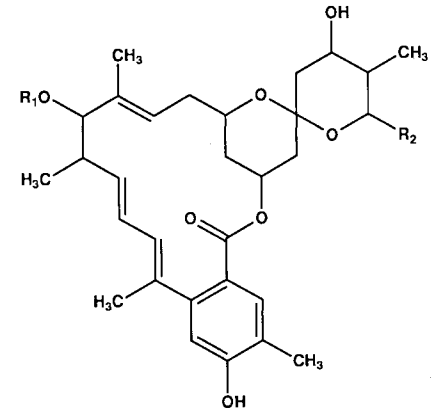


The enzymes involved in *aveB* step catalyze the stepwise addition of oleandrose units from dTDP-oleandrose to the avermectin aglycones.

Fig. 5. Structures of 6,8a-seco-6,8a-deoxy derivatives produced by *aveE* mutants.



R_1	R_2	R_3	X-Y
α-L-Oleandrosyl	Sec. butyl	OCH_3	CH_2–CH(OH)
α-L-Oleandrosyl	Sec. butyl	OH	CH_2–CH(OH)
α-L-Oleandrosyl-α-L-oleandrosyl	Sec. butyl	OH	CH_2–CH(OH)
H	*iso*-Propyl	OCH_3	CH=CH
H	Sec. butyl	=O	CH=CH
H	*iso*-Propyl	=O	CH=CH
α-L-Oleandrosyl-α-L-oleandrosyl	Sec. butyl	OH	CH=CH
α-L-Oleandrosyl	Sec. butyl	OH	CH=CH
α-L-Oleandrosyl	Sec. butyl	=O	CH_2–CH(OH)
α-L-Oleandrosyl	Sec. butyl	=O	CH=CH
α-L-Oleandrosyl	Sec. butyl	OCH_3	CH=CH



R_1	R_2
H	*iso*-Propyl
H	Sec. butyl
α-L-Oleandrosyl-α-L-oleandrosyl	Sec. butyl

deoxyavermectin derivatives produced by the mutants would not be suitable substrates for glycosylation and C5 keto reduction. Feeding of 6,8a-seco-6,8a-deoxyavermectin derivatives to *aveA* mutant culture[27] or the wild type culture supplemented with cerulenin revealed that these aglycones are converted to monosaccharide and disaccharide lacking furan ring at C6 to C8, but not to natural avermectins, although other aglycones such as, 6,8a-seco-6,8a-deoxy-5-oxo derivatives are converted to natural avermectins.

6. *aveF* (step for C5 keto reduction)

Although many attempts for the isolation of this type of mutants by mutagenesis have been done by us and Merck group, the mutants affecting C5 keto reduction have not been isolated. Furthermore, the C5 keto reductase which catalyzes the NADPH specific reduction of 5-oxoavermectins to "B" components has been demonstrated in cell-free extract of *S. avermitilis*[24]. Recently, we isolated *aveF* mutants by genetic engineering[33]. The mutants produce 5-oxoavermectins, but not either aglycones or monosaccharides. From the results of conversion of the derivatives lacking furan ring at C6 to C8a[27], the reduction of C5 keto residue must occur after furan ring formation at C6 to C8a in ordinary biosynthesis.

7. *aveR* (step for regulation of biosynthesis)

The *aveR* mutants showed the same phenotype as that of *aveA* mutants which do not produce any avermectins, however, *aveR* mutants did not have the ability to convert any intermediates which were generated after the formation of aglycones to the natural avermectins[34,35]. This means that the mutation would cause to suppress expression of the formation of aglycones and the followed steps, including furan ring formation at C6 to C8a, C5 keto reduction, stepwise glycosylation at C13 and C4′, and C5 *O*-methylation. From these results the *aveR* mutant was defined as regulatory mutant in avermectin biosynthesis.

8. *X* (step concerning selective incorporation of branched-chain fatty acid into avermectin aglycones)

It has been shown that the substituent groups of the "a" and "b" components at C25 are derived from L-isoleucine and L-valine, respectively[23,24]. The mutants

accumulate avermectins "a" components but not "b" components[21,23]. Since the biochemical and genetic characteristics of the mutant were unclear, the genotype was designed as *X*. It seemed that the mutant could incorporated L-isoleucine into the avermectin aglycone but not L-valine. Consequently the incorporation of L-isoleucine, L-valine and corresponding keto acids, 3-methyl-2-oxovalerate and 2-oxoisovalerate provided through the deamination or transamination of L-isoleucine and L-valine, respectively, into the avermectin aglycone was examined in both parent and mutant strains[21,23]. The both branched-chain amino and keto acids were efficiently incorporated into the avermectin aglycones in the wild type strain. In the case of mutants, the incorporation of L-isoleucine and its keto acid was efficient, but that of L-valine and its keto acid was scarcely incorporated into avermectin aglycones, suggesting that since the incorporation of L-valine or its keto acid into the avermectin aglycone was markedly suppressed in the mutants, the mutants accumulated avermectin "a" components.

Inhibition of enzyme reaction involving biosynthesis by specific inhibitors has made it possible to block specific step in the biosynthetic pathway as well as a biosynthetically blocked mutants. Cerulenin is a potent inhibitor of fatty acid and polyketide syntheses and has been shown to inhibit formation of avermectin aglycones *in vivo* without affecting to biosynthetic steps after it, which is the same manner as that of a biosynthetically blocked mutant *aveA*. It has been confirmed that the conversion of intermediates was efficiently proceeded by the culture of wild-type producer supplemented with cerulenin as described above.

On the other hand, sinefungin, an analogue of *S*-adenosyl-L-methionine, is a potent inhibitor of methyltransferases and has been shown to inhibit C5 *O*-methylation *in vitro*[36]. Feeding to a culture of *aveB* mutant, which produces only the avermectin aglycone of the "A" components, sinefungin inhibited the formation of the "A" components and caused a concominant accumulation of the avermectin aglycone of the "B" components[30]. Furthermore, sinefungin inhibited not only C5 *O*-methylation but also both C3′ and C3″ *O*-methylations. New components lacking methoxy groups on the C3′ and C3″ of the oleandrose were accumulated by adding sinefungin to the wild type culture[30]. The result indicates that both C3′ and C3″ *O*-methylations are also involved in an *S*-adenosyl-L-methionine-dependent methyltransferase. Furthermore, SCHULMAN *et al.*[37] isolated mutants defective in C3′ and C3″ *O*-methylation of avermectins which produce demethylavermectin A or B component.

C. Genetic Background of Avermectin Biosynthesis

Avermectin producer *S. avermitilis* belongs to Gram-positive filamentous bacteria. LIN *et al.* have reported that *Streptomyces* strains have linear chromosome[38]. Recently, *S. avermitilis* has also been shown to possess a linear chromosome with about 8 Mbp by measurement of the sum of *Ase*I-fragments of the chromosome. The first attempt for the genetic information of avermectin biosynthesis was performed with the genetic mapping of several markers including avermectin biosynthesis by us[26,39]. The resulting genetic map of the producer revealed that genes involving avermectin biosynthesis formed a cluster on the chromosome (Fig. 6). The cloning of the gene cluster for avermectin biosynthesis has been reported independently by Merck group[35,40] and us[21,34] as shown in Fig. 7. Within the 95 kbp gene cluster for avermectin biosynthesis, the central 70 kbp segment is required for aglycone biosynthesis. Two 30 kbp segments, *aveAI* and *aveAII*, encode the avermectin polyketide synthase (PKS). The open reading frames in the 70 kbp segment would be the genes encoding multifunctional proteins[40] with strong similarity to erythromycin PKS[41]. The high similarity within the functionally equivalent PKS domains of erythromycin[42] and avermectin PKS[40] was demonstrated by their sequences. The erythromycin PKS is encoded by six similar modules, in which each module encodes a synthase unit with domains similar to those in type I fatty acid synthase. A comparison of putative peptides encoded by these sequences with the complete erythromycin PKS and partial avermectin PKS resulted in the identification of twelve modules[40]. Twelve acyl

Fig. 6. Chromosome map of *S. avermitilis*.

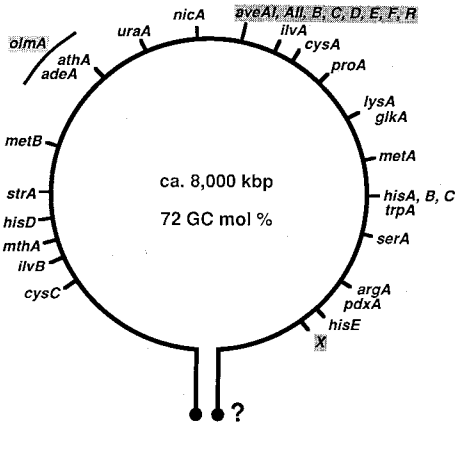
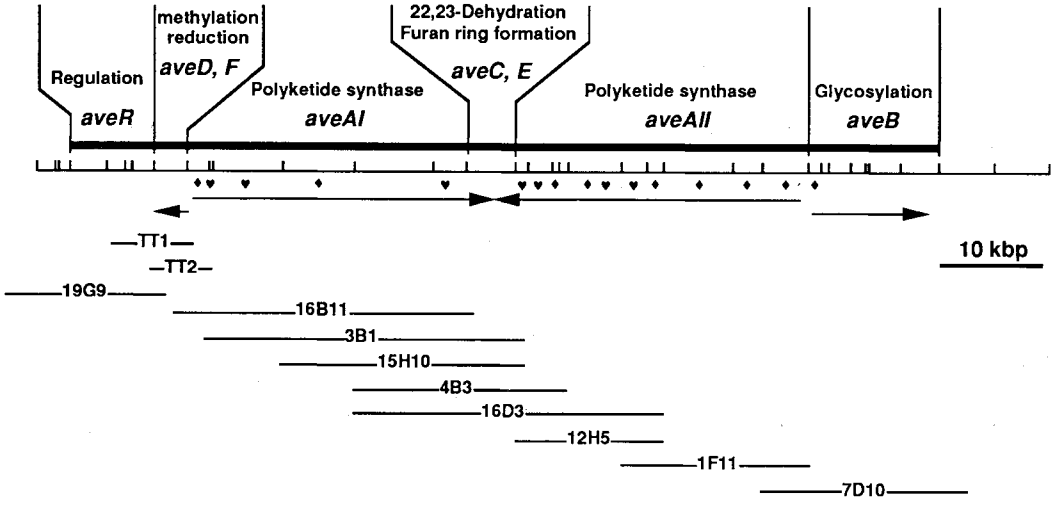


Fig. 7. Physical map of gene cluster for avermectin biosynthesis in *S. avermitilis*.



The map of the *ave* gene cluster was determined by gene complementation with mutants, by gene transreplacement and by analysis overlapping cosmids. *Bam*HI sites are indicated by vertical lines above the map. TT1 and TT2 were clones obtained by shotgun cloning with *aveD* mutant and others were cosmid clones.

condensations are required to incorporate seven acetate, five propionate and one branched-chain fatty acid units into avermectin aglycone[22,24]. It is likely that each of the twelve modules encodes a synthase unit specific for each of twelve condensation steps.

An 11 kbp subclone from the right end of the gene cluster for avermectin biosynthesis restored normal avermectin biosynthesis for all mutants defective in glycosylation of avermectin[35]. A 9 kbp region of the 11 kbp subclone was responsible for both synthesis and attachment of oleandrose disaccharide. There are eight putative open reading frames in the 9 kbp region. Potential functions of several frames of these were identified to be consistent with a proposed pathway for the conversion of glucose to dTDP-oleandrose, including steps for both C3′ and C3″ *O*-methylations.

A 4.82 kbp subclone from the central region in the gene cluster restored partially avermectin biosynthesis in two types of mutants defective C22-23 dehydration (*aveC*) and C6-8a furan ring formation (*aveE*)[32]. DNA sequencing of this 4.82 kbp segment indicated that *aveC* and *aveE* form operons with *aveAI* and *aveAII*, respectively.

About 14 kbp region at the left end of the gene cluster was encoded both functions for C5 keto reduction and C5 *O*-methylation[40] and for positive regulation of avermectin biosynthesis. The strains with deletion and insertion in about 10 kbp region at the left end of the cluster did not methylate or glycosylate the substrates of avermectin even though the structural genes for these functions did not have mutation. The pleiotropic phenotype of these mutants suggested that they lack a positive regulatory function necessary to turn on expression of all the genes involved in avermectin biosynthesis.

A 3.4 kbp-*Bam*HI segment encoded domains of C5 keto reduction and C5 *O*-methylation, and these two genes would be transcribed polycistronically[33,43]. The time courses of expression of the both enzyme activities for C5 *O*-methylation and C5 keto reduction and avermectin production were measured in a number of high-yielding strains of *S. avermitilis*. The maximum specific activities of these enzymes increased in direct proportion to the quantity of avermectin formed. The results support the hypothesis that genes coding for enzymes involved in the biosynthesis of the secondary metabolites would be coordinately expressed and regulated.

II. Application to the Selective Production of Useful Component(s)

Although *S. avermitilis* produces eight related components of avermectins, only two components (Fig. 8), B1a and B1b, are used in medical, veterinary and agricultural field, in which the industrial separation is difficult. Furthermore, the organism produces also an unwanted toxic compound, oligomycin. Thus, some problems remain unsolved for the industrial production of avermectin B1a, the most effective component.

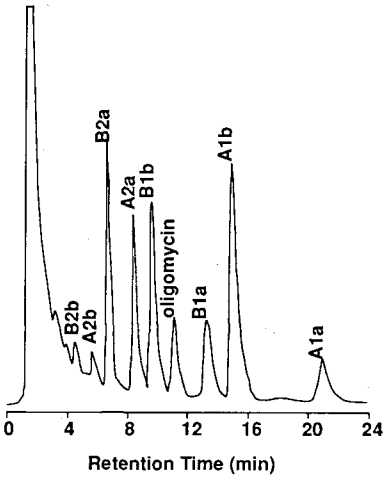
A. Production of Specific Component(s)

The knowledge of biosynthetic pathway of avermectins makes it possible to construct the producer of specific component(s), who produces them by suppression of some steps without affecting other steps in avermectin biosynthesis.

1. Selective Production of Avermectins B1a and B2a

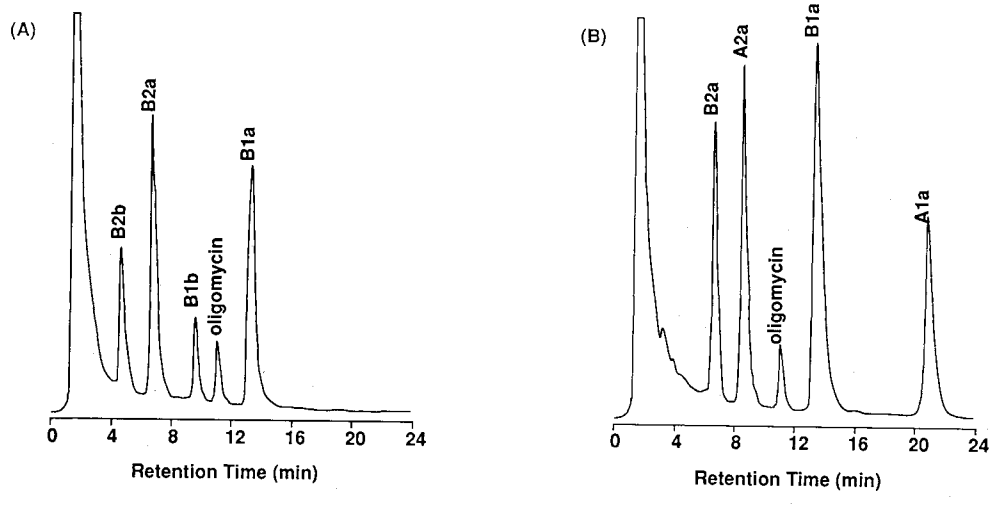
We isolated several types of mutants affecting the production of avermectins. Among of them, two mutants, K2021 and K2034[23] produce only the specific components (Fig. 9). The strain K2034 produces "B" components alone, namely components B1a, B1b, B2a, and B2b. Accumulation of the "B" components in the mutant K2034 is due to lack of the ability of conversion of the components "B" to the "A", whose genotype is defined as *aveD*. Another mutant K2021 produces four "a" components, A1a, A2a, B1a, and B2a, but not the "b" components, of which genotype is defined as *X*. This mutant accumulates the "a" components because the incorporation of L-valine into the avermectin aglycone is markedly suppressed. Therefore, if a strain possessing both phenotypes of the two mutants, K2021 and K2034, can be designed, it should produce only the components B1a and B2a. In the studies of the genetic crossing and mapping of *S. avermitilis*, the recombination frequency was about 10^{-5} to 10^{-7} by mixed culture[26]. But the frequency was dramatically increased to about 10^{-2} by a protoplast fusion in the presence of polyethylene glycol. Moreover, although the multiple crossover in recombinants derived from the mixed culture of spores was scarcely observed, the quadruple or more multiple crossover was detected in the recombinants derived from the protoplast fusion. Therefore, recombinants derived from crossing between closely related two loci on the chromosome would be recovered by the protoplast fusion. In consideration of this view, we tried to isolate the recombinants possessing both phenotypes by protoplast fusion[23]. Protoplasts of the two mutants were prepared and fused in the presence of 40% (w/v) polyethylene glycol MW, 1,000. The protoplasts fused were regenerated to mycelial form with growing on a regeneration medium and the expected recombinants were obtained efficiently without any selection. Eight recombinants out of 192 colonies screened produced

Fig. 8. Chromatogram of analytical HPLC of the mycelial extract from the wild-type strain K139.



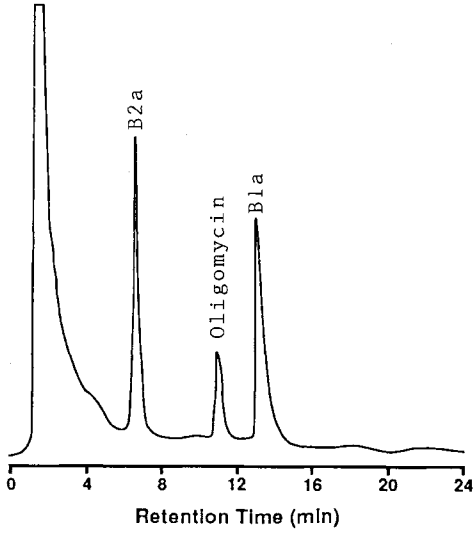
The mycelia from 10 ml-culture were extracted with equal volume of methanol for 15 minutes. A portion of the extract was directly applied to reversed phase silica gel HPLC. The separation conditions were described in ref. 23.

Fig. 9. Chromatogram of analytical HPLC of the mycelial extracts from the mutant strains, (A) K2034 (*aveD*) and (B) K2021 (*X*).



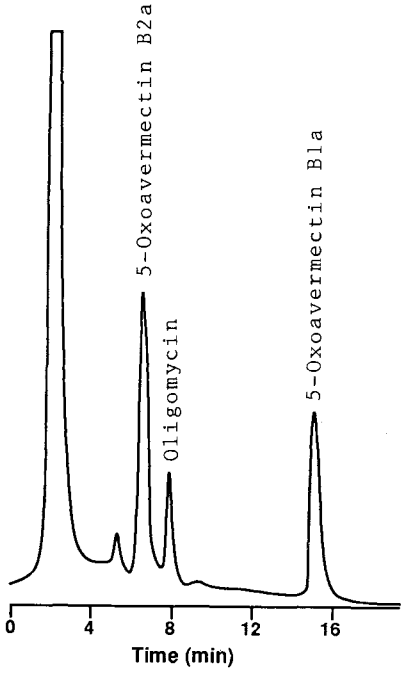
The extraction of products and separation conditions were described in Fig. 8.

Fig. 10. Chromatogram of analytical HPLC of the mycelial extract from the recombinant strain K2038 (*aveD X*).



The extraction of products and separation conditions were described in Fig. 8.

Fig. 11. Chromatogram of analytical HPLC of the mycelial extract from the recombinant strain (*aveF X*) derived from K2021 (*X*).



The extraction of products and separation conditions were described in Fig. 8.

avermectins B1a and B2a (Fig. 10). Since the recombination frequency of crossing between auxotrophic markers was about 10^{-2} by the protoplast fusion, the recombinants possessing both selective producing phenotypes (*aveD* and *X*) were isolated efficiently . We have determined the locus of *X* mutation on the genome. From the result of genetic mapping, the locus of *X* mutation was distant enough from that of the gene cluster for avermectin biosynthesis on the genome[29]. This fact suggests that one of the genes involving in the selective production of the specific components of avermectins might concern the catabolism of branched-chain amino acids, rather than involved directly in avermectin biosynthesis.

2. Selective Production of 5-Oxoavermectins B1a and B2a

A large number of avermectin derivatives have been made by variety of techniques, including mutagenesis[44], biotransformation[45], mutational biosynthesis[46] and chemical modification[47]. The main purpose for the construction of such derivatives is to enhance potency and spectrum and to lower toxicity of the compound(s). Of these derivatives, 5-oxime derivatives enhanced their potency in both avermectins and milbemycins[48]. The 5-oxime derivatives are synthesized by two steps of chemical reactions[49]. Although oximation of keto group of the 5-oxoavermectins is quantitative reaction, the yield of the selective oxidation of hydroxyl group of avermectin "B" components is low. If the mutant strain produces 5-oxoavermectins directly, the 5-oxime derivatives would be easily and efficiently synthesized. In consideration of proposed biosynthetic pathway after formation of avermectin aglycones, if the step for C5 keto reduction is affected, the 5-oxoavermectin aglycones would be converted to the 5-oxoavermectins by stepwise glycosylation at C13 and C4′ hydroxyl groups. During analysis of the region of *aveD* involving in C5 *O*-methylation, we identified the gene for C5 keto reduction step. Both genes for C5 *O*-methylation (*aveD*) and C5 keto reduction (*aveF*) were in the same operon. We modified the operon by the 8 bp-insertion, and the modified DNA segment was introduced into the corresponding region on the chromosome by a gene transreplacement technique using a temperature-sensitive replication plasmid vector. After double crossover recombination event occurs homologous region between the region of gene for C5 keto reduction on the chromosome and that on the recombinant plasmid, the recombinant plasmid was removed by growing at high temperature because the plasmid possessed phenotype of the temperature-sensitive replication. The products from mycelial extracts of transreplacement clones containing insertion mutation derived from wild-type strain were different from that of

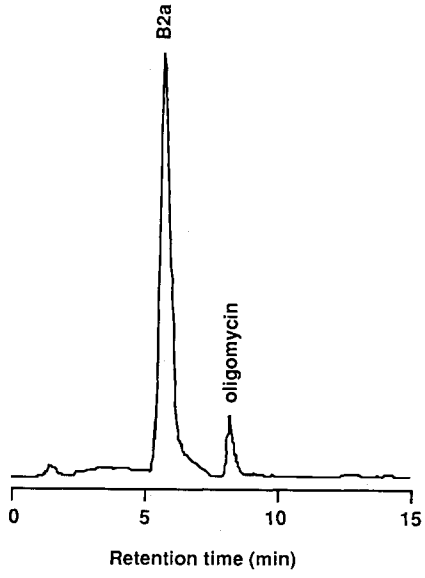
the wild-type strain and were identified as 5-oxo-avermectins B1a, B1b, B2a, and B2b. Furthermore, we applied this technique for the construction of the selective producer K2021 (*X*) which possesses the mutation affecting the selectivity of the incorporation of branched-chain fatty acids into avermectin aglycones (accumulating "a" components). The expected transreplacement clones containing insertion mutation in the operon of *aveD* and *aveF* derived from the selective producer K2021 produced two components of 5-oxoavermectins B1a and B2a (Fig. 11).

3. Selective Production of Avermectin B2a

In consideration of the biosynthetic pathway, we estimated that introduction of at least three mutations required to construct the single component producer into the avermectin biosynthetic pathway of the wild type strain. If three mutations, affecting to the *O*-methylation at C5 hydroxyl group (*aveD*), the selectivity of the incorporation of branched-chain fatty acid into the avermectin aglycones (*X*), and the dehydration between C22 and 23 residues (*aveC*), are introduced to the wild type strain, the resulting mutant let produce only avermectin B2a. Since avermectin B2a can be chemically transformed to 22,23-dihydroavermectin B1a, "Ivermectin B1a"[50], the most potent anthelmintic compound[51], to design a single component producer of avermectin B2a is assumed to be more useful for industrial production of ivermectin B1a.

Since we had already constructed a recombinant strain K2038[23], a producer of avermectins B1a and B2a, by the protoplast fusion of two mutants, *aveD* and *X* construction of avermectin B2a producer might be accomplished by introduction of the mutation phenotype of dehydration between C22 and 23 (*aveC*) into the recombinant strain. As the entire gene cluster for avermectin biosynthesis had been already cloned, and also the region involving in C22,23-dehydration step (*aveC*) had been defined in a 4.82 kbp-*Bam*HI segment at the center of the cluster by us, we attempted to introduce point mutation(s) in *aveC* region on the chromosome of the recombinant strain K2038. There are several procedures for the introduction of the random point mutation(s) into the cloned DNA segment *in vitro*. We tried to introduce point mutation(s) into *aveC* region by using polymerase chain reaction (PCR). A part of the region of structural gene for *aveC* was subcloned, and then the segment was amplified by PCR in which concentrations of dCTP, dGTP and dTTP were increased to four-fold to be misincorporated deoxynucleotides into synthesized chains by *Taq* DNA polymerase during elongation reaction. The amplified segments, in which some of segments contain point mutation(s) by misincorporation of deoxynucleotides, were exchanged with the corresponding region in the chromosome by gene transreplacement technique. As shown in Fig. 12, the resulting transreplacement clone did produce avermectin B2a alone[52].

Fig. 12. Chromatogram of analytical HPLC of the mycelial extract from the recombinant strain K2099 (*aveC aveD X*).



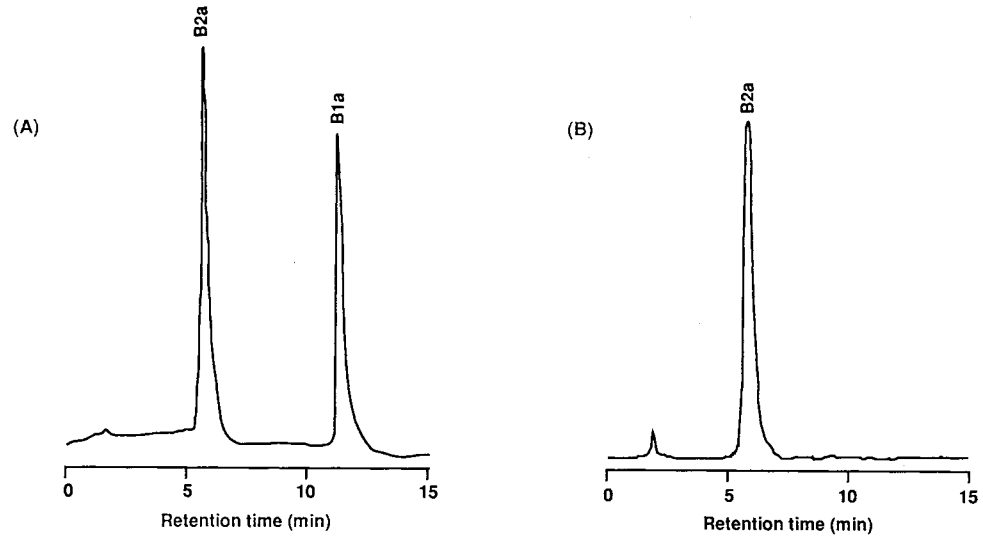
The mycelia from 10 ml-culture were extracted with equal volume of acetone and after removal of acetone by evaporation, products were extracted with methylenechloride. Methylenechloride extract was evaporated to dryness, the residue was dissolved in methanol and a portion of extract was directly applied to reversed phase silica gel HPLC. The separation conditions were described in ref. 23.

4. Suppression of the Accumulation of Useless Toxic Compound, Oligomycin

The wild-type strain of *S. avermitilis* produces not only avermectins but also oligomycin. The selective producers of specific component(s) of avermectins, derived from the wild-type strain still produced useless compound, oligomycin. Being a toxic compound specifically inhibiting oxidative-phosphorylation in mammalian cells, oligomycin should be removed from mycelial extracts for the preparation of the commercial products.

In general, the desired mutant have been isolated from the wild-type strain by induction of mutation with chemical mutagens, radiation and so on. In this case, it is not easy to transfer the mutated phenotype to other strains. We attempted to isolate the mutant by transposon-induced mutagenesis. Because the corre-

Fig. 13. Chromatogram of analytical HPLC of the mycelial extracts from the recombinant strains, (A) *aveD X olmA*::Tn*4560* derived from K2038 and (B) *aveC aveD X olmA*::Tn*4560* derived from K2099.



The extraction of products and separation conditions were described in Fig. 12.

sponding region in the genome is easily cloned from transposon-inserted mutants by the selection of antibiotic resistance of a marker in the transposon as well as the transfer the mutation phenotype to other strain is performed by gene transreplacement assisted homologous recombination using transposon-inserted DNA segment. We used transposon Tn*4560*, one of derivatives of Tn*4556* of neomycin-producing *S. fradiae*, because transpositions of Tn*4560* are randomly distributed on the genome of *S. avermitilis*[34]. About 0.1% of the transposon inserts caused auxotrophy or abolished antibiotic production. Among 2,400 independent clones containing transpositions, two oligomycin-nonproducing mutants in which Tn*4560* was transposed in the gene cluster for oligomycin biosynthesis were detected. These two mutants still produced avermectins. Chromosomal DNA fragments of oligomycin-nonproducing mutants carrying Tn*4560* were easily cloned by selection of viomycin-resistance because Tn*4560* contains viomycin-resistant determinant (viomycin phosphotransferase). Chromosomal fragments containing Tn*4560* insertion in oligomycin biosynthetic genes were subcloned into a plasmid with temperature-sensitive replication and were used to carry transposon mutation to other strains, using the homologous recombination. The resulting transreplacement clones did not produce any oligomycin without affecting production of avermectins[34].

This technique can be used to construct an avermectin-producing strain that no longer makes the toxic oligomycin. For example, the transfer of oligomycin-nonproducing phenotype was applied to two selective producers K2038 (*aveD X*) producing avermectins B1a and B2a, and K2099 (*aveC aveD X*) producing avermectin B2a (Fig. 13). The resulting transreplacement clones produced avermectins B1a and B2a, and only B2a, respectively[34,52]. This obviates the need for separation of avermectin and oligomycin.

III. Conclusion

Now we have elucidated the pathway for avermectin biosynthesis and cloned all genes for avermectin biosynthesis. Furthermore, we are able to introduce mutation(s) in each step of avermectin biosynthesis *in vitro* from the knowledge of avermectin biosynthesis. This means that it is possible to control the biosynthetic pathway and to design the producer which produces specific component(s) by the combination of mutations in avermectin biosynthesis. The attempt of these techniques might be applied to not only avermectin producer but also other metabolite-producing microorganisms, and it might be useful for the studies on biosynthesis of secondary metabolites and the construction of selective producer of valuable component in the industrial field.

Acknowledgments

We thank H. TAKESHIMA for critical reading of this manuscript and comments.

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