SELECTIVE PRODUCTION OF SPECIFIC COMPONENTS OF AVERMECTINS IN *Streptomyces avermitilis*

Sir:

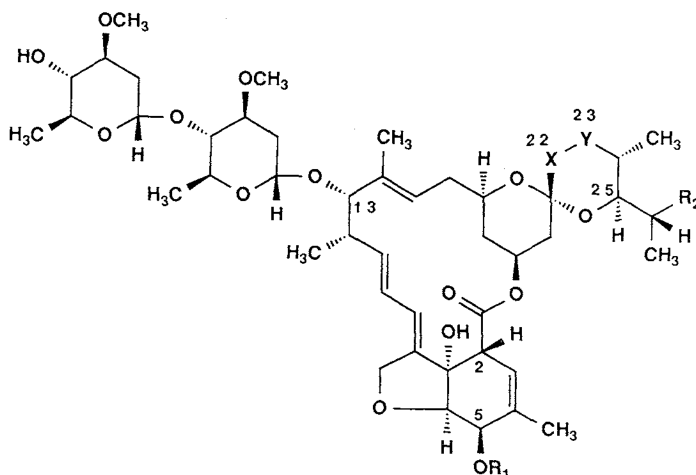
The anthelmintic antibiotic avermectin complex produced by *Streptomyces avermitilis* is a family of four closely related major components, A1a, A2a, B1a, and B2a, and four minor components, A1b, A2b, B1b, and B2b, which are lower homologs of the corresponding major components as shown in Fig. 1[1]. Each component is considered to be biosynthesized as follows[2]. "A" components are derived from "B" components through the methylation at C-5. The group "1" is derived from the precursor group "2" through the dehydration at C-22 and C-23. The aglycon moiety of avermectins has been proved to be synthesized *via* a hypothetical intermediate "polyketide" derived from seven acetate units, five propionate units and a branched-chain fatty acid unit[3,4]. The C-25 atom and its *sec*-butyl substituent of "a" components is derived from L-isoleucine, while the 25-isopropyl substituent of "b" components is from L-valine.

It has been shown that a hydroxyl group at C-5 position and the disaccharide moiety are essential for good activity. The hydrogenated product of B1 component, 22,23-dihydroavermectin B1 (ivermectin) is used as an important anthelmintic in veterinary fields and for the control of onchocerciasis in human.

In the present communication, the authors describe designing the selective producer from *S. avermitilis* which produces only two components, the most effective compounds B1a and B2a.

Mutagenesis of *S. avermitilis* K139[2] was performed by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described by the authors[2]. NTG-treated spores were spread onto a YMS plate[2] and the plate was incubated at 30°C for 5 days. Each colony was transferred patchily onto a YMS plate with 1 cm^2 square. After incubating at 30°C for 5 days, the plate was stored at 12°C and used as a master plate. The replicated plate was incubated at 30°C for 8 days. Each patch-like shape colony on the medium was cut out, and put into a 1.5-ml Eppendorf tube. Avermectins produced in the mycelia were extracted with 0.5 ml of acetone at

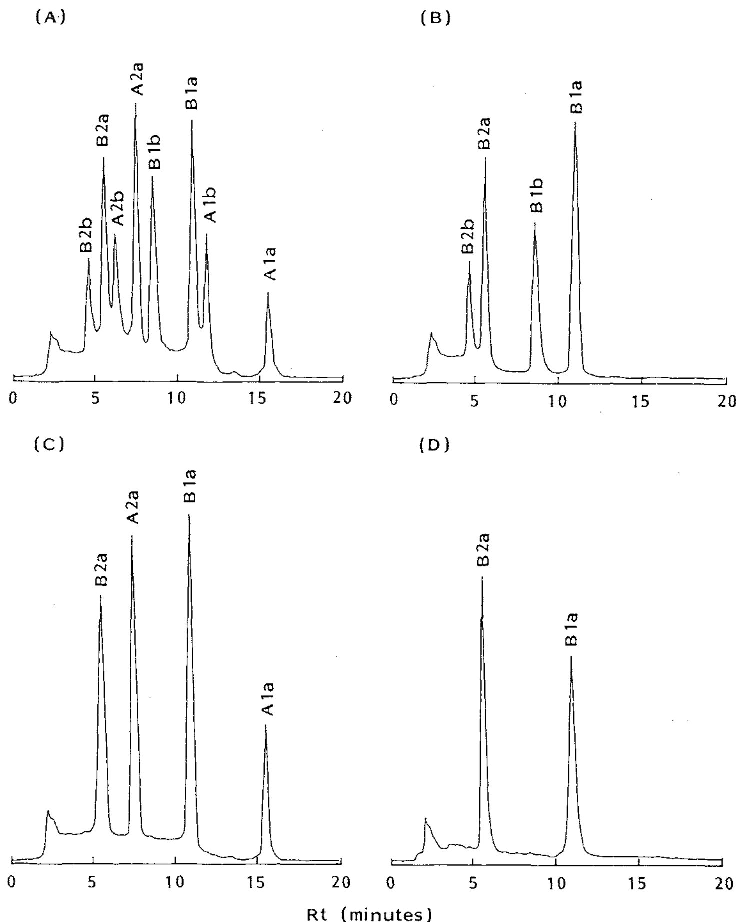
Fig. 1. Structures of avermectins.



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

Fig. 2. Chromatograms of analytical HPLC of the mycelial extracts.

(A) Parent strain K139, (B) mutant strain K2034 (*aveD*), (C) mutant strain K2021 (*X*), (D) recombinant strain K2038 (*aveD X*).



Each strain was cultured in the production medium[2] at 28°C for 7 days. After the mycelia were harvested by centrifugation at 3,000 rpm for 5 minutes, the products were extracted with equal volume of methanol. After the removal of methanol by evaporation, the concentrate was extracted with equal volume of dichloromethane. The extract was directly applied to HPLC. The column which packed with Hypersil (ODS, 3 μm; 4.6 i.d. mm × 75 mm) was developed with acetonitrile - methanol - water (62 : 18 : 20) and the flow rate was 0.6 ml/minute. Avermectins was detected by UV absorption at 246 nm.

room temperature for 15 minutes. The concentrated extracts were applied to normal or reversed phase silica gel TLC using 15% 2-propanol in *n*-hexane or 70% acetonitrile in water, respectively, as developing solvent. Avermectins were detected by irradiation of UV rays at 254 nm.

As shown in Fig. 2(A), the parent strain *S. avermitilis* K139 produced four major components, avermectins A1a, A2a, B1a and B2a, and four minor components, A1b, A2b, B1b and B2b. After the mutagenesis of the parent strain, mutant strains, K2021 and K2034, which produced only the specific components, were obtained. Strain K2034 produced "B" components alone, namely two major components, B1a and B2a, and two minor components, B1b and B2b (Fig. 2B). The genotype of K2034 was defined as *aveD*. SCHULMAN *et al.*[5] has already reported such type of mutants that lack avermectin B2 5-*O*-methyltransferase activity. Hence, the accumulation of "B" components alone by the mutant K2034 is due to the lack of the ability of the conversion of "B" components to "A".

The other mutant K2021 produced four "a" components, A1a, A2a, B1a and B2a (Fig. 2C), but

Table 1. Incorporation of branched-chain amino and keto acids into avermectins.

Strain	Incorporation of labeled precursors into avermectins (cpm)[a]			
	L-[U-^{14}C]Isoleucine	L-[3,4-^{3}H]Valine	[U-^{14}C]-3-Methyl-2-oxovalerate	[3,4-^{3}H]-2-Oxoisovalerate
K139 (parent)	398±22	423±20	655±35	575±43
K2021	512±26	22±3	927±34	25±5

[a] Each strain was cultivated in 10 ml of the chemically defined production medium[7] at 28°C with shaking. The labeled precursor (*ca.* 100,000 cpm) was added to a 4-day culture. After incubating for 6 hours at 28°C, the mycelia were harvested by centrifugation at 3,000 rpm for 5 minutes. The products were extracted from the mycelia with equal volume of methanol. The extract was evaporated under the reduced pressure to remove methanol and the concentrate was extracted with equal volume of dichloromethane. The dichloromethane layer was collected and evaporated to dryness. A portion of the crude residue was applied to a silica gel TLC using 15% 2-propanol in *n*-hexane as developing solvent. Avermectins were detected by UV irradiation at 254 nm. The area on the chromatogram corresponding to avermectins was scraped off and put into a scintillation vial. Avermectins in the silica gel were extracted with 0.5 ml of methanol. Then 5 ml of scintillation cocktail was added to the extract and the radioactivity was counted by a scintillation spectrometer.

did not produce "b" components. Since the biochemical and genetic characteristics of the mutation were unclear, the genotype was disignated as *X*. It has been shown that the substituted group of "a" components at C-25 is derived from L-isoleucine and that of "b" components is from L-valine[3]. It seemed that the mutant K2021 could incorporate L-isoleucine into the avermectin aglycon but not L-valine. So, the incorporation of L-isoleucine, L-valine and corresponding keto acids, 3-methyl-2-oxovalerate and 2-oxoisovalerate, which were provided through the transamination or deamination of L-isoleucine and L-valine, respectively, into the avermectin skeleton was examined in both parent and mutant strains. As shown in Table 1, the both branched-chain amino acids and keto acids were efficiently incorporated into the avermectin skeleton in the parent strain. In the case of the mutant strain K2021, the incorporation of L-isoleucine and its keto acid, 3-methyl-2-oxovalerate, was efficient, but L-valine and its keto acid, 2-oxoisovalerate, were scarcely incorporated into avermectins (Table 1), suggesting that since the incorporation of L-valine or its keto acid into the avermectin skeleton was markedly suppressed in this mutant, the mutant accumulated "a" components alone.

If a new strain which possesses both phenotypes of the above two mutants, *i.e.* lacking avermectin B2 5-*O*-methyltransferase activity (accumulating "B" components) and showing efficient incorporation of L-isoleucine or 3-methyl-2-oxovalerate alone into the avermectin skeleton (accumulating "a" components), can be designed, it should produce the components B1a and B2a alone. Then, we tried to isolate the recombinant strains which possess both phenotypes. Protoplasts of the two mutants K2021 and K2034 were prepared and then fused in the presence of 40% w/v polyethylene glycol MW 1000[6]. The protoplasts fused were regenerated to mycelial form by growing on the regeneration medium[6]. Two types of colonies appeared on the regeneration medium. One of them was dark brown and resembled to the mutant K2021 producing "a" components. The other one was pale brown and resembled to the mutant K2034 producing "B" components. The fermentation products of regenerated colonies were checked by reversed phase silica gel TLC. As a result, the expected recombinants were obtained efficiently at the frequency of about 10^{-2}. As shown in Fig. 2D, for example, the recombinant K2038 produced the components B1a and B2a alone. The productivities of avermectins in "B" components producer (K2034) and "a" components producer (K2021) were 0.2 and 1.0 g/liter, respectively. All recombinants obtained produced about 0.2 g/liter of avermectins. Since the recombinants possessing both phenotypes were isolated efficiently by protoplast fusion, the locus of the mutation affecting the avermectin B2 5-*O*-methyltransferase might be enough distant from that of the mutation affecting the selectivity of the incorporation of branched-chain keto acids into the avermectin skeleton on the chromosome.

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