COMMUNICATION TO THE EDITOR

Construction of a Single Component Producer from the Wild Type Avermectin Producer *Streptomyces avermitilis*

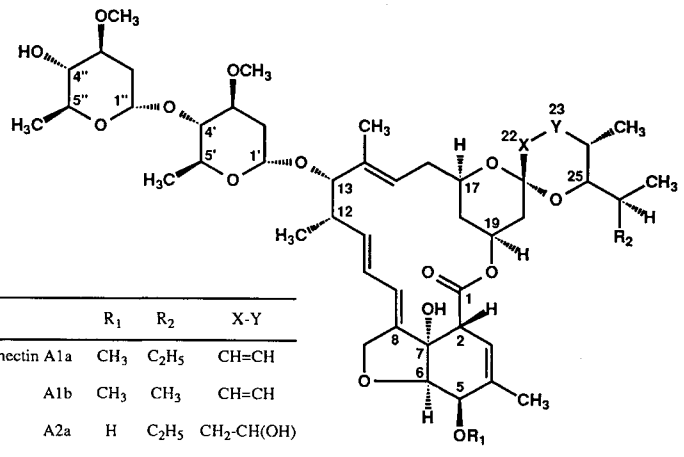
Sir:

Most microbial secondary metabolites are produced as complexes of closely related compounds by their producing organisms. The avermectins[1], milbemycins[2] and nemadectins[3~5] are typical examples produced in such a manner. The milbemycin complex is known to consist of more than twenty components[6~8], however, only a few are commercial products. A rationale for this phenomenon may be related to the complexity of their biosyntheses and/or lower degrees of substrate specificities of the biosynthetic enzymes. The avermectin-complex is a family of eight closely related components as shown in Fig. 1. Knowledge of the avermectin biosynthetic pathway made it possible to construct a producer of specific component(s). In consideration of the avermectin biosynthetic pathway[9], we estimated that introduction of at least three mutations would be required to construct a single component producer from the wild type strain. If three mutations, affecting *O*-methylation at the C-5 hydroxyl residue (*aveD*), the selectivity of the incorporation of branched-chain keto acids into the avermectin skeleton (*X*), and the dehydration between the C-22 and 23 residues (*aveC*), were introduced into the wild type strain, the resulting mutant would produce only avermectin B2a. MROZIK *et al.*[10] have established 22,23-dihydroavermectin B1a (ivermectin B1a), which is produced chemically from avermectin B2a, to be the most potent anthelmintic compound[11]. Therefore, a single component producer of avermectin B2a would be assumed to be extremely useful for industrial production of ivermectin B1a. As we had already constructed a recombinant strain K2038 (*aveD X*), a producer of avermectins B1a and B2a[12] (Fig. 2-A), our goal was expected to be accomplished by introducing only one mutation, *aveC*, into the recombinant strain.

In the present communication, we describe a single component (avermectin B2a) producer derived from *Streptomyces avermitilis* by two approaches, *in vivo* and *in vitro* mutagenesis of the *aveC* region. Mutagenesis and culture conditions of *S. avermitilis* were performed as described by us[13,14]. Mycelial products were analyzed by silica gel thin layer chromatography or HPLC as described previously[12~14].

In the first approach, we treated recombinant strain K2038 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and isolated a single component avermectin B2a producer (mutant H48), from several thousand clones. Since the

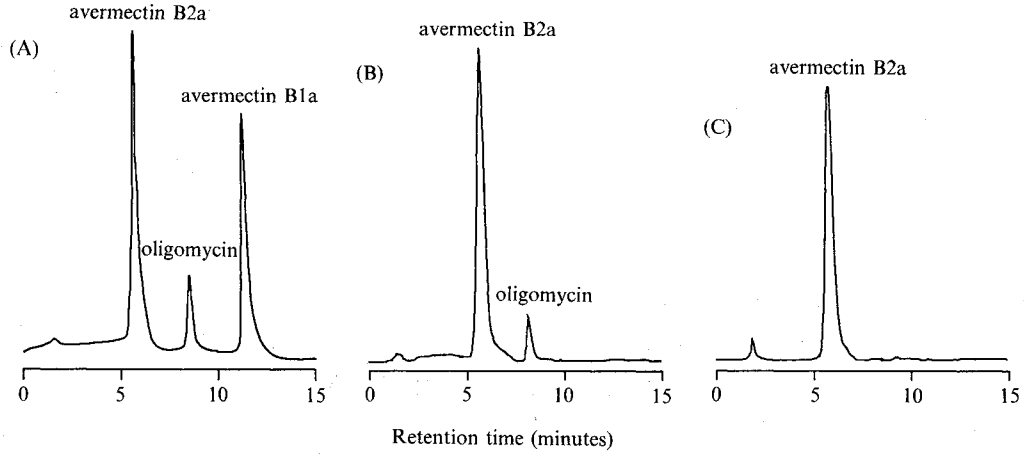
Fig. 1. Structural formulae for avermectins and ivermectins.



	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[11] and commercial ivermectin consists of more than 80% ivermectin B1a and less than 20% ivermectin B1b.

Fig. 2. Analytical HPLC chromatograms of the mycelial extracts from the parent strain K2038 (A), its transreplacement clones K2099 (*aveC aveD X*; B) and K2101 (*aveC aveD X olmA*::Tn*4560*; C).



The mycelia from a 10 ml-culture were extracted with an equal volume of acetone and after removal of acetone by evaporation, products were extracted with methylenechloride. The methylenechloride extract was evaporated to dryness, the residue was dissolved in methanol and a portion of the extract was directly applied to reversed phase silica gel HPLC. The separation conditions are described in ref. 12.

productivity of the antibiotic by mutant H48 was very low in comparison with that of the parent strain K2038, we considered the mutant to contain other mutation(s) in the genes involving avermectin production.

We tried *in vitro* mutagenesis as the second approach. As the entire gene cluster for avermectin biosynthesis had been already cloned[9,15], and the region involving the C-22,23-dehydration step (*aveC*) had been defined in a 4.82 kbp-*Bam*HI fragment by us, we attempted transition or transversion mutation in the *aveC* region on the chromosome of the recombinant strain K2038. As a result, we subcloned a 640 bp-*Pst*I/*Sph*I fragment in the left side of the *Bam*HI fragment[9] containing the *aveC* region. The resulting fragment was amplified by the polymerase chain reaction (PCR) in which concentrations of three nucleotides (dCTP, dGTP and dTTP) were increased 4-fold to cause deoxynucleotide misincorporation into synthesized chains by *Taq* DNA polymerase during the elongation reaction. The amplified fragments of which some would contain transition or transversion mutation due to misincorporation of deoxynucleotides, were exchanged with the corresponding region in the chromosome by gene transreplacement technique. The resulting transreplacement clone did produce avermectin B2a as the sole avermectin component (Fig. 2-B).

To confirm the structures of the products from both single component producers, the mutant H48 and the transreplacement clone (K2099) were cultivated in a liquid production medium as described previously[13,14]. Mycelia from each cultured broth were extracted with acetone, and the extracts from which mycelia were removed by filtration, were evaporated under the reduced pressure. Each concentrate was suspended in deionized water and then extracted twice with methylenechloride. Ultimately, the main products of each strain were separated by a preparative HPLC to obtain purified material. Physico-chemical properties of purified materials derived from both mutant strains were identical to those of authentic avermectin B2 purified from the wild type strain. Furthermore, ^{1}H and ^{13}C NMR data for purified materials from both strains were identical to avermectin B2a. These producers, however, produced not only avermectin B2a but also oligomycin, which is an unwanted toxic compound. We have already established the disruption of oligomycin production without affecting avermectin production by transposon mutagenesis[16]. That is, a part of the oligomycin biosynthetic region carrying transposon Tn*4560* was exchanged with the corresponding region in chromosome of K2099 by gene transreplacement technique. The resulting transreplacement clone (K2101) produced avermectin B2a but not oligomycin (Fig. 2-C).

Thus, a producer (K2101) of avermectin B2a was constructed successfully by specific mutation of the gene for the C-22, 23-dehydration step and by disruption of oligomycin biosynthesis without affecting the productivity of avermectin by the producer, *S. avermitilis*. As avermectin B2a could be recovered without the separation from other related components, it would become easier to convert avermectin B2a to ivermectin B1a by chemical transformation.

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