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Avermectin B_2 *O*-Methyltransferase Activity in "*Streptomyces avermitilis*" Mutants That Produce Increased Amounts of the Avermectins

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The level of activity of avermectin B *O*-methyltransferase, the enzyme which catalyzes the conversion of avermectin B components to avermectin A components, was analyzed in a series of "*Streptomyces avermitilis*" mutants selected for increased production of the avermectins. In all of the mutants, increased avermectin production was accompanied by increased avermectin B *O*-methyltransferase activity. Both the average specific activity and the maximum observed specific activity of avermectin B *O*-methyltransferase increased in direct proportion to avermectin production. The level of avermectin B *O*-methyltransferase alone did not determine the extent of conversion of avermectin B components to avermectin A components, since a constant ratio of B components to A components was maintained throughout the fermentation even though avermectin B *O*-methyltransferase specific activity varied three- to fivefold. These results indicate that avermectin B *O*-methyltransferase is not rate limiting. The correlation between avermectin B *O*-methyltransferase specific activity and avermectin production is compatible with the hypothesis that genes coding for successive steps in the same secondary metabolite biosynthetic pathway are coordinately regulated.

"*Streptomyces avermitilis*" produces a number of potent anthelmintic and insecticidal agents known as avermectins (4). These are a group of structurally related oleandrose disaccharide derivatives of pentacyclic 16-membered lactones (1, 2, 6, 10). "*S. avermitilis*" normally produces eight major avermectin components (Fig. 1). Approximately 60% of the avermectins have a hydroxyl group attached to C_5 and are termed B components, while the remaining 40% have a methoxyl group attached to C_5 and are termed A components. The conversion of avermectin B components to avermectin A components is catalyzed by the enzyme avermectin B *O*-methyltransferase, which transfers the methyl of *S*-adenosylmethionine (SAM) to the C_5 hydroxyl of an avermectin B component to yield an avermectin A component and *S*-adenosylhomocysteine (Fig. 2) (M. D. Schulman, D. Valentino, and C. Ruby, Fed. Proc. **44**:931, 1985).

Avermectin B *O*-methyltransferase is the only enzyme involved in avermectin biosynthesis that has been described to date. Recent reports have demonstrated the existence of true operons in S. coelicolor (9) and have indicated a strong tendency for the genes coding for successive steps in the same antibiotic biosynthetic pathway to be contiguous with each other (5) (D. Hopwood, Proc. 6th Int. Symp. Biol. Actinomyces, 1985). These findings suggest that the enzymes of a biosynthetic pathway may also be coordinately expressed.

We have consequently determined avermectin B *O*-methyltransferase activity in a number of strains of "*S. avermitilis*" with diverse yields of the avermectins to determine if there is any correlation between enzyme activity and avermectin titer. In this paper we report the results of these studies, which demonstrate a direct correlation between increased avermectin production and increased avermectin B *O*-methyltransferase specific activity in these strains.

MATERIALS AND METHODS

Bacterial strains. "*S. avermitilis*" A is a stable spontaneous mutant of "*S. avermitilis*" MA4848. Mutants B, C, and D were derived from "*S. avermitilis*" A by a series of mutagenic steps involving UV light and *N*-methyl-*N*-nitrosourethan treatment. For UV light mutagenesis, spores were suspended in sterile dionized water and irradiated at 24 ergs/min per cm^2 for 1 min. This resulted in a 95% loss of viability. For mutagenesis with *N*-methyl-*N*-nitrosourethan, spores suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) were treated with 1% *N*-methyl-*N*-nitrosourethan dissolved in the same buffer for 90 min; a 99% loss of viability resulted. Spore suspensions were agitated during mutagenesis to prevent clumping.

After the mutagenic treatment, spores were plated on agar medium which contained the following: yeast extract (Difco Laboratories), 4 g/liter; malt extract (Difco), 10 g/liter; glucose, 4 g/liter; agar (Difco), 20 g/liter; and a 200× trace element solution (5.0 ml/liter). The 200× trace element solution in 0.6 N HCl contained the following (in grams per liter): $MgSO_4 \cdot 7H_2O$, 61.1; $CaCO_3$, 2.0; $FeCl_3 \cdot 6H_2O$, 5.4; $ZnSO_4 \cdot 7H_2O$, 1.44; $MnSO_4 \cdot H_2O$, 1.11; $CuSO_4 \cdot 5H_2O$, 0.25; $CoCl_2 \cdot 6H_2O$, 0.28; H_3BO_3, 0.062; and $Na_2MoO_4 \cdot 2H_2O$, 0.49. The plates were incubated at 27°C for 5 to 7 days. Single-colony isolates were tested in fermentation. Those which produced increased quantities of avermectins were reisolated and retested. Stable reisolates were designated as mutants and stored.

Media and fermentation. "*S. avermitilis*" was grown in modified medium B as described by Burg et al. (2).

Preparation of cell-free extracts. Fermentation broths from replicate fermentation flasks were pooled (total, approxi-

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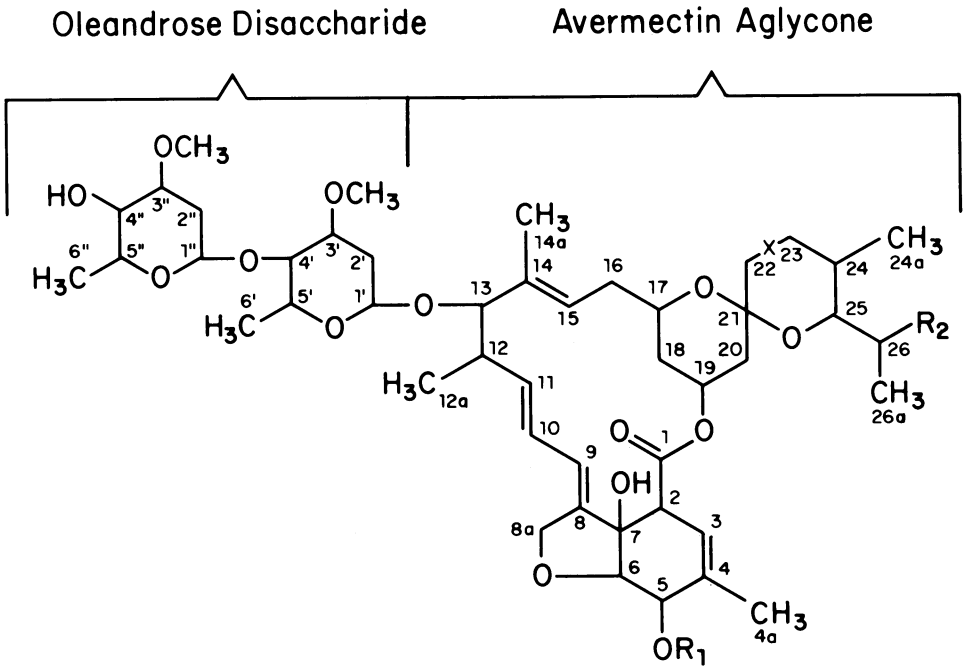

FIG. 1. General structure of avermectins. Avermectin terminology is as follows: R_1 = H in B components; R_1 = CH_3 in A components; X = CH = CH in l components; X = CH_2CHOH in 2 components; R_2 = CH_2–CH_3 in a components; R_2 = CH_3 in b components.

mately 40 ml) in 50-ml polypropylene tubes, and the cells were harvested by centrifugation at 1,000 × *g* for 10 min in a Beckman model TJ-6 centrifuge at 5°C and washed twice in cold distilled water. The mycelial pellet was weighed, suspended in 0.05 mM Tris hydrochloride (pH 7.6) containing 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonylfluoride (1 g of cells per 5 ml), and ruptured by two passes through a French pressure cell at 12,000 lb/in^2. The homogenate from the French pressure cell was centrifuged at 5,000 × *g* at 5°C in a Sorvall RC-5B centrifuge for 10 min; the pellet was discarded, and the supernatant was centrifuged at 102,000 × *g* for 1 h at 5°C in a Beckman L8-80 centrifuge. The clear supernatant was designated the cell-free homogenate.

Avermectin B *O*-methyltransferase assay. The reaction mixture (0.5 ml) contained the following: 100 mM Tris hydrochloride (pH 7.6), 1.0 mM dithiothreitol, 1.0 mM $MgCl_2$, 44 μM avermectin B_{2a} aglycone, 0.5 mM *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([*methyl*-^{14}C]SAM) (5 × 10^6 dpm/μmol), and enzyme (1 to 2 mg of protein).

A saturated solution of avermectin B_{2a} aglycone (approximately 0.5 μM) in 0.05 mM Tris hydrochloride (pH 7.6) containing 0.1% CHAPSO {3-[(cholamidopropyl)dimethylamimonio]-2-hydroxyl-1-propanesulfonate} was made by stirring the mixture vigorously at room temperature for 2 h. Excess solids were removed by centrifugation at 132,000 × *g* for 1 h at 5°C in a Beckiman L8-80 centrifuge.

Assays were conducted at room temperature (25°C) in 15-ml conical polypropylene tubes. The reaction was initiated by the addition of [*methyl*-^{14}C]SAM and terminated by the addition of 2.0 ml of ethyl acetate. Carrier avermectin A_{1a} aglycone and A_{2a} aglycone (40 μl of a 1-mg/μl solution of each in methanol) were added, and the components were

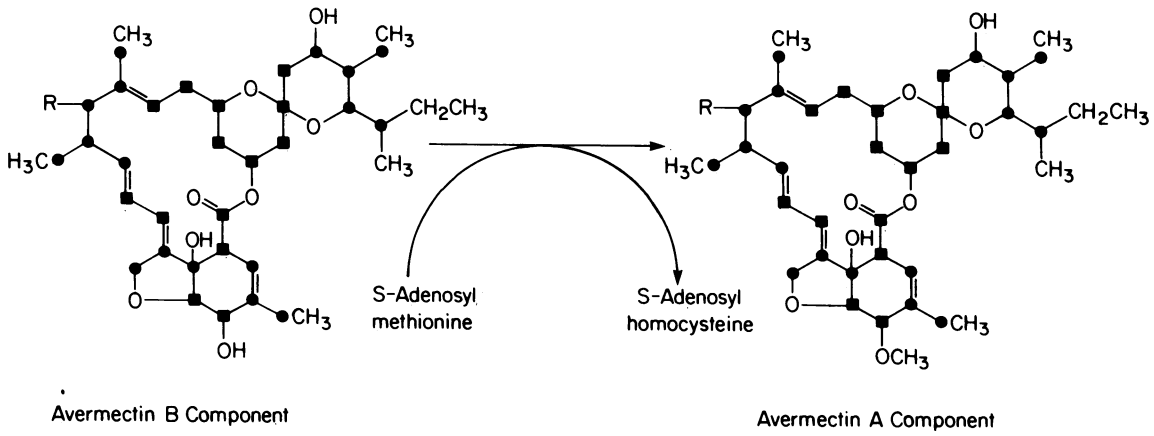

FIG. 2. Reaction catalyzed by avermectin B *O*-methyltransferase. R = oleandrose disaccharide.

extracted by shaking the mixture vigorously in an Eberbach reciprocal shaker. After phase separation, 1.5 ml of ethyl acetate was removed and evaporated to dryness. The residue was dissolved in 0.1 ml of methanol, and 20- to 40-ml samples were applied to silica gel-60 F-254-precoated thin-layer chromatography plates (20 by 20 cm, 0.25 mm thick; E. M. Laboratories). The plates were developed in methylene chloride-ethyl acetate (85:15, vol/vol) for 1 to 1.5 h to separate the substrates SAM (R_f = 0) and avermectin B_{2a} aglycone (R_f = 0.29) from the product avermectin A_{2a} aglycone (R_f = 0.48). ^{14}C incorporated in avermectin A_{2a} aglycone was detected by using a Bioscan system 200 (Bioscan, Inc.) coupled to a Hewlett-Packard 85B computer. The assay can detect as little as 20 pmol of product. If increased sensitivity is needed, [*methyl*-^{14}C]SAM with a higher specific activity can be used.

Analysis and determination of avermectins. Samples of the fermentation broth (2 ml) were brought to 80% saturation with methanol (vol/vol) and shaken vigorously for 15 min in an Eberbach reciprocal shaker. The solids were removed by centrifugation, and avermectin in the supernatant was determined by high-pressure liquid chromatography on a Du Pont Zorbax C-18 column (4.6 by 25 cm) at 60°C with methanol-water (85:15, vol/vol) as the mobile phase (6). The elution volumes (in milliliters) of the eight major components in order of elution were as follows: B_{2b}, 6.51; B_{2a}a, 7.43; A_{2b}, 8.50; A_{2a}, 9.47; B_{1b}, 10.26; B_{1a}, 12.26; A_{1b}, 13.36; and A_{1a}, 16.20. The A_{247} was monitored. The sensitivity was 1 μg/ml when a 10-μm titer flow cell was used.

Other assays. Protein was determined by the microbiuret method described by Zamenhoff (11).

Chemicals. Dithiothreitol was obtained from Sigma Chemical Co., CHAPSO was obtained from Calbiochem-Behring, and [*methyl*-^{14}C]SAM was obtained from Amersham Corp.

RESULTS

Assay of avermectin B *O*-methyltransferase. Figure 3 summarizes the results obtained in a typical assay of avermectin B *O*-methyltransferase. The amount of radioactive avermectin A components formed was linear with time from 0 to 90 min and with enzyme from 0 to 4 mg of protein per ml. Avermectin B_{2a} aglycone was the most reactive (Schulman et al., Fed. Proc., 1985) and consequently was routinely used as the substrate.

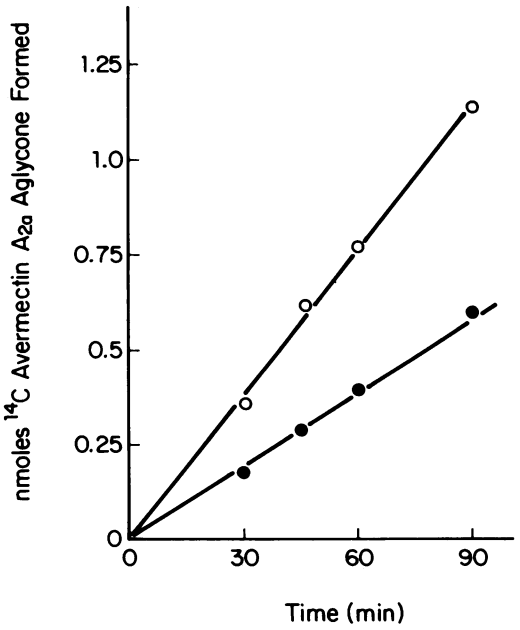

FIG. 3. Assay of avermectin B *O*-methyltransferase. Symbols: ●, 25 μl of cell-free extract containing 0.6 mg of protein; ○, 50 μl of cell-free extract containing 1.2 mg of protein.

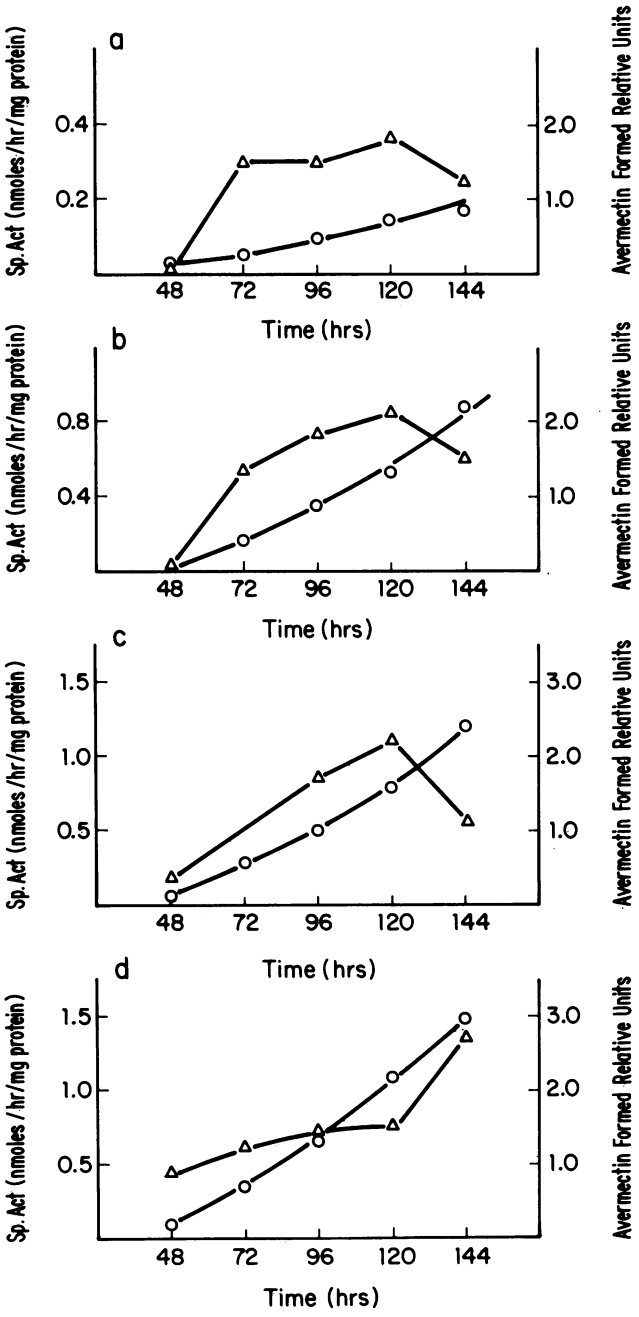

FIG. 4. Avermectin production and avermectin B *O*-methyltransferase specific activity (Sp. Act) in "*S. avermitilis*" strains. "*S. avermitilis*" A (a), B (b), C (c), and D (d) were grown under fermentation conditions, and avermectin (○) was determined in whole broth samples by high-pressure liquid chromatography at the times indicated. Each point represents the average of four replicates. Cell-free extracts were prepared at the times indicated, and avermectin B *O*-methyltransferase specific activity (Δ) was determined.

TABLE 1. Avermectin B O-methyltransferase activity and avermectin production in strains A, B, C, and D

Strain	Sp act of O-methyltransferase (nmol/h per mg of protein)		Total avermectin at 144 h (relative units)	Fold increase in:		
				Sp act of O-methyltransferase		Avermectin
	Maximum observed	Avg (48 to 144 h)		Maximum	Avg	
A	0.37	0.26	0.85	1	1	1
B	0.86	0.54	2.10	2.3	2.1	2.5
C	1.06	0.63	2.4	2.9	2.5	2.8
D	1.35	0.85	3.0	3.6	3.3	3.5

Avermectin B O-methyltransferase activity in avermectin-producing strains of "*S. avermitilis*." The time course of avermectin production and the activity of avermectin B O-methyltransferase in cell-free extracts of several mutants of "*S. avermitilis*" are shown in Fig. 4. In all mutants, avermectin synthesis began at approximately 48 h and continued through 144 h. Avermectin B O-methyltransferase activity in strains A, B, and C appeared between 48 and 72 h, peaked at 120 h, and then decreased. Enzyme activity in strain D followed a different time course; substantial activity was evident at 48 h and increased continuously throughout the fermentation. In other experiments, peak activity for strain D was observed at 144 to 168 h (data not shown).

Comparisons of the average specific activity of avermectin B O-methyltransferase (48 to 144 h), the maximum observed specific activity of the O-methyltransferase, and the avermectin titers of the four strains are shown in Table 1. The results demonstrated that both the average specific activity and the maximum specific activity of avermectin B O-methyltransferase increased in direct proportion to the amount of avermectin formed.

DISCUSSION

Avermectin B O-methyltransferase catalyzes the transfer of the methyl of SAM to the C_5 hydroxyl of avermectin B components to yield avermectin A components. Previous studies have shown that this enzyme is specific for the C_5 hydroxyl and does not catalyze methyl transfer to either the C_3' or C_3'' hydroxyl of oleandrose disaccharide (Schulman et al., Fed. Proc., 1985). The enzyme thus differs from macrocin O-methyltransferase (7) and erythromycin C O-methyltransferase (3), which catalyze the transfer of the methyl of SAM to the hexose moieties of these macrolide antibiotics. The requirement for avermectin B O-methyltransferase for the formation of avermectin A components has been demonstrated by the following: (i) the isolation of mutants which lacked enzyme activity and produced only avermectin B components (C. L. Ruby, M. D. Schulman, D. Zink, and S. Streicher, Proc. 6th Int. Symp. Biol. Actinomyces, 1985); and (ii) the inhibition of avermectin B O-methyltransferase by sinefungin in vivo, resulting in a decrease in the formation of avermectin A components and a concomitant accumulation of avermectin B components (6a).

The results of this study show that avermectin B O-methyltransferase activity can be measured in crude cell-free extracts of "*S. avermitilis*" and that increased avermectin production is accompanied by increased specific activity of avermectin B O-methyltransferase. Both the maximum observed specific activity of avermectin B O-methyltransferase and the average specific activity of avermectin B O-methyltransferase (48 to 144 h) increased in direct proportion to avermectin production. Although avermectin B O-methyltransferase is required for the formation of avermectin A components, it does not determine the amounts of the A components formed. The ratio of B components to A components (60:40) was similar in all four mutants and was constant throughout each fermentation, even though O-methyltransferase activity varied three- to fivefold. This result suggests that O-methyltransferase was always in excess and that other factors, such as the availability of SAM and inhibition by the reaction products, determined the rate of reaction and the B component/A component ratio. The observation that avermectin B O-methyltransferase is inhibited by S-adenosylhomocysteine, a reaction product (Schulman et al., Fed. Proc., 1985), supports this hypothesis. A similar type of control was found with macrocin O-methyltransferase (8).

In conclusion, avermectin B O-methyltransferase specific activity has been shown to increase in direct proportion to avermectin production. The close correlation between the specific activity of this enzyme, which is not rate limiting, and avermectin production is compatible with the hypothesis that genes coding for enzymes involved in the biosynthesis of secondary metabolites may be coordinately expressed.

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