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"*Streptomyces avermitilis*" Mutants Defective in Methylation of Avermectins

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"*Streptomyces avermitilis*" mutants defective in the methylation of the avermectins have been isolated and characterized. Four mutant strains, CR-1, CR-2, CR-3, and CR-4, were unable to methylate the oxygen at C_5 of the macrolide moiety and produced essentially only the avermectin B components. These four strains lack avermectin B_2 *O*-methyltransferase (B_2OMT) activity. Two mutant strains were unable to methylate the oleandrose moiety at the oxygens at C_3' and C_3'' and produced essentially only demethylavermectin components. One of these mutants, strain CR-5 (derived from wild-type "*S. avermitilis*"), produced demethylavermectin A and B components and possessed normal B_2OMT levels. The other mutant, strain CR-6 (derived from strain CR-1, which lacks B_2OMT activity), produced only demethylavermectin B components. Reaction of 3''-*O*-demethylavermectin B_2a and *S*-adenosylmethionine with either cell extracts or purified B_2OMT resulted in the methylation of the oxygen at C_5 of the macrolide moiety and yielded only 3''-*O*-demethylavermectin A_2a as the product. These experiments indicate that different enzymes are required for methylation of the macrolide (the oxygen at C_5) and the oleandrose (oxygen at C_3) and that methylation of the oleandrose occurs before attachment to the macrolide ring.

"*Streptomyces avermitilis*" produces a complex of highly potent anthelmintic and insecticidal agents known as avermectins (1, 2, 4, 5). These compounds are a group of structurally related oleandrose disaccharide derivatives of pentacyclic 16-membered lactones. The wild-type "*S. avermitilis*" strain normally produces eight components (Fig. 1). The A components, which have a methoxyl group at C_5, constitute approximately 35% of the avermectins, and the B components, which have a hydroxyl group at C_5, constitute the remaining 65%. The B components are converted to the A components via the *S*-adenosylmethionine-dependent enzyme, avermectin B_2 *O*-methyltransferase (B_2OMT) (7). There is no evidence for a demethylation reaction converting A components into B components. The methoxyl groups on the C_3' and C_3'' of the oleandrose disaccharide are also derived from the methyl group of methionine (7). Sinefungin, an analog of *S*-adenosylmethionine, inhibited methylation at all three sites, C_3', C_3'', and C_5, resulting in the accumulation of demethylavermectins (8).

A large collection of "*S. avermitilis*" mutants defective in avermectin biosynthesis has been generated. Among these mutants are strains that lack the ability to perform the methylation reactions. Two types of methylation-deficient mutants were observed. The first type was unable to methylate the C_5 hydroxyl oxygen of the macrolide moiety but was able to methylate the oxygens at the C_3' and C_3'' positions of the oleandrose disaccharide. These strains produced essentially only the avermectin B components and lacked B_2OMT activity. The second mutant type was able to methylate the C_5 hydroxyl oxygen but was unable to methylate the oxygens at the C_3' or C_3'' of the oleandrose disaccharide. These mutant strains had normal levels of B_2OMT activity, and produced demethylavermectin A and B components. A mutant unable to methylate all three positions was derived from an B_2OMT-deficient strain. This strain produced only demethylavermectin B components. Cell extracts of "*S. avermitilis*" and purified *O*-methyltransferase catalyzed the methylation of demethylavermectin B_2a only at the oxygen at the C_5 position, forming demethylavermectin A_2a exclusively. These data indicate that there are two distinct *O*-methyltransferases involved with avermectin biosynthesis, one which methylates the hydroxyl at C_5 and another which methylates the oleandrose, and that the oleandrosyl moieties are methylated before attachment to the macrolide ring.

MATERIALS AND METHODS

Bacterial strains. "*S. avermitilis*" WT produces the normal eight avermectin components and was derived from the original soil isolate MA4680 (2) by a series of mutagenic steps including UV light, and *N*-methyl-*N*-nitrosourethane treatments (7). Mutant strains CR-1, CR-2, CR-3, CR-4, and CR-5 were derived from strain WT via *N*-methyl-*N*-nitrosourethane mutagenesis, and strain CR-6 was derived from strain CR-1 by the same type of treatment. Spores derived from survivors of the mutagenic treatment were plated on solid media, and isolated single colonies were picked and fermented in liquid. At the end of the fermentations, broths were brought to 80% (vol/vol) saturation with methanol and shaken vigorously to extract the avermectins from the cells (5, 8). A sample of each was spotted on a thin-layer chromatography plate and developed (5, 8). Isolates with altered avermectin compositions were purified and retested and stable ones were saved for further analysis. All strains were stored at −70°C as spore suspensions in 50% glycerol–0.85% NaCl.

Chemicals. L-[*methyl*-^{14}C]methionine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine were from Amersham Corp. (Arlington Heights, Ill.), and sinefungin was from Calbiochem-Behring (La Jolla, Calif.).

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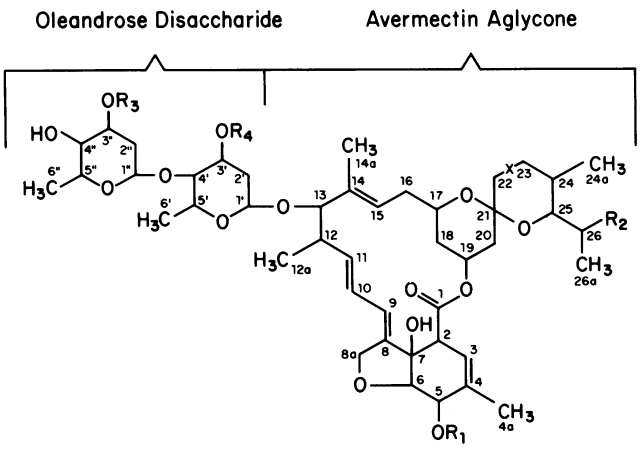

FIG. 1. General structures of avermectins. Avermectin terminology is as follows: R_1 = H in B components; R_1 = CH_3 in A components; R_2 = CH_2CH_3 in a components; R_2 = CH_3 in b components; R_3 = CH_3 in avermectin; R_3 = H in 3''-*O*-demethylavermectin; R_4 = CH_3 in avermectin; R_4 = H in 3'-*O*-demethylavermectin; x = CH=CH in 1 components; x = CH_2CHOH in 2 components.

Media and fermentations. "*S. avermitilis*" was grown in a modified medium B as described by Burg et al. (2). For fermentations in the presence of sinefungin or [*methyl*-^{14}C]methionine or both, 0.05 ml of 53 mM sinefungin or 40 μl of radiolabeled substrate containing 4.0 μCi (specific activity, 59.2 μCi/μmol) was added 72 h after the beginning of fermentation. The quantities of avermectins in the whole broth were monitored at 24-h intervals. Samples of the fermentation broth were brought to 80% (vol/vol) saturation with methanol, shaken vigorously, and centrifuged, and the avermectins in the supernatant were determined by high-pressure liquid chromatography (7).

Isolation of avermectins. Avermectins were isolated from fermentation broth as previously described (6–8), and their radioactivity was determined with a liquid scintillation spectrometer (model LS 8100; Beckman Instruments, Inc., Fullerton, Calif.). All samples were corrected for quench by using an external standard. Identity of the demethylavermectins was verified by mass spectrometry (8).

Preparation of cell extracts and assay of avermectin B *O*-methyltransf Cell extracts from cultures WT, CR-1, CR-2, CR-3, (CR-5, and CR-6 were prepared and assayed for ave tin B *O*-methyltransferase (7).

TABLE 1. Methyltransferase-defective mutants of "*S. avermitilis*"

Strain	Major products	Total avermectin (relative units)	% A components	% B components
WT	A_1a, A_2a, A_1b, A_2b, B_1a, B_1b, B_2a, B_2b	1.0	35	65
CR-1	B_1a, B_1b, B_2a, B_2b	0.71	5	95
CR-2	B_1a, B_1b, B_2a, B_2b	0.80	3	97
CR-3	B_1a, B_1b, B_2a, B_2b	0.72	3	97
CR-4	A_1a, A_2a, A_1b, A_2b, B_1a, B_1b, B_2a, B_2b	0.67	12	88
CR-5	A_2a bisdemet, A_1a bisdemet, B_1a bisdemet, B_2a bisdemet, 3''-B_2a demet	0.14	24	76
CR-6	B_2a bisdemet, B_1a bisdemet, 3'-B_2a demet	0.78	6	94

TABLE 2. Avermectin B_2 *O*-methyltransferase activity in mutants of "*S. avermitilis*"

Strain	*O*-Methyl groups present			Avg sp act of *O*-methyltransferase (nmol/h per mg)	B:A ratio
	C_5	C_3'	C_3''		
WT	+	+	+	0.57	65:35
CR-1	−	+	+	0.06	97:03
CR-2	−	+	+	0	97:03
CR-3	−	+	+	0	97:03
CR-4	−	+	+	0.18	88:12
CR-5	+	−	−	0.29	24:76
CR-6	−	−	−	0	94:06

RESULTS

Classification of "*S. avermitilis*" methylation-deficient mutants. The major avermectin components produced by "*S. avermitilis*" WT and mutants CR-1 through CR-6 are summarized in Table 1. Strain WT produced eight avermectin components with a ratio of A components to B components of 35:65. Strains CR-1, CR-2, and CR-3 produced avermectin B components almost exclusively and were unable to methylate the oxygen at C_5 of the macrolide moiety. Strain CR-4 produced reduced amounts of avermectin A components and appears to be partially defective in methylation of the C_5 oxygen. Strain CR-5 produced demethylavermectins of the A and B series and appears to be unable to methylate the oleandrose disaccharide. Strain CR-6, derived from strain CR-1, is deficient in methylation at all three positions and produced predominantly demethylavermectin B components.

Avermectin B *O*-methyltransferase activity. B_2OMT activity was not detected in strains CR-2, CR-3, or CR-6, which produced avermectin B components almost exclusively (Table 2). Strain CR-1 had a trace of the wild-type B_2OMT activity but had the same avermectin B:A ratio as strains devoid of B_2OMT activity. Strain CR-4 had about 30% of the wild-type B_2OMT level and a corresponding decrease in the proportion of A components synthesized. Strain CR-5, which lacks the ability to methylate the oleandrose disaccharide, had about 50% of the wild-type B_2OMT level.

Incorporation of [*methyl*-^{14}C]methionine. The methyl of methionine is incorporated into the avermectins only at the C_3', C_3'', and C_5 positions (7). The incorporation of [*methyl*-^{14}C]methionine into the demethylavermectins by strain CR-5 is presented in Table 3. ^{14}C was detected in the A components, which contain a methoxyl group at C_5, but not in the B components, which have a hydroxyl at C_5. The specific radioactivity of the "2" components was 1.5-fold greater

TABLE 3. Incorporation of [*methyl*-^{14}C]methionine into demethylavermectins by CR-5

Avermectin component	Sp act (dpm/μg)
3' -*O*-demethyl A_1a monosaccharide	266
3' -*O*-demethyl A_2a monosaccharide	370
3' -*O*,3''-*O* bisdemethyl A_1a	219
3' -*O*,3''-*O* bisdemethyl A_2a	350
3' -*O*,3''-*O* bisdemethyl B_1a	4
3' -*O*,3''-*O* bisdemethyl B_2a	10

than that of the "1" components. This probably reflects the higher activity of B_2OMT with 2 components versus 1 components (M. D. Schulman, D. Valentino, and C. Ruby, Fed. Proc. **44**:931, 1985).

Effect of sinefungin. Sinefungin, an analog of *S*-adenosylmethionine, has previously been shown to inhibit the incorporation of the methyl of methionine into the avermectins at the C_3', C_3'', and C_5 positions (8). The addition of sinefungin to a fermentation of strain CR-5 which produces demethylavermectin A components resulted in the inhibition of the synthesis of A components and a concomitant increase in the amount of B components (Table 4). The overall avermectin yield in the presence of sinefungin was 88 to 105% of the control values, and the ratio of B components to A components rose approximately fivefold. These results indicate that the demethylavermectin B components are converted to the A components via an *S*-adenosylmethionine-dependent methylation.

In vitro methylation of demethylavermectins. 3''-*O*-demethylavermectin B_2a has two sites available for methylation, the oxygen at C_5 of the macrolide and the oxygen at C_3'' of the oleandrose disaccharide. This compound was used as the substrate acceptor to determine the specificity of *S*-adenosylmethionine-dependent *O*-methyltransferases. Methylation of only the macrolide at C_5 would yield 3''-*O*-demethylavermectin A_2a; methylation of only the C_3'' of the oleandrose disaccharide moiety would yield B_2a; and methylation at both sites would yield A_2a. Both crude cell extracts of strain WT and purified B_2OMT were used as enzyme sources. The results obtained with the crude cell extract (Fig. 2), represent a trace of the radioactivity found on a thin-layer chromatography plate. 3''-*O*-demethylavermectin A_2a was the only radioactive product detected demonstrating that methylation occurred only at the oxygen at the C_5 position of the macrolide. Identical results were obtained by using purified B_2OMT.

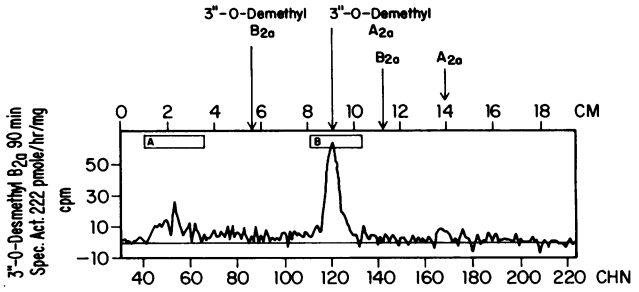

FIG. 2. *S*-Adenosylmethionine-dependent methylation of 3''-*O*-demethylavermectin by crude extracts of "*S. avermitilis*" D. Arrows indicate locations of unlabeled standards. Precoated silica gel 60 thin-layer chromatography plates (E. M. Industries, Inc., Cherry Hill, N.J.) were developed in methylene chloride:ethylacetate:methanol (9:9:1, vol/vol/vol). Radioactivity was measured for 10 min with a Bioscan System 200 (Bioscan Inc., Washington, D.C.) and integrated (7). CHN, Channels.

DISCUSSION

The avermectins can contain three methoxyl groups, one on the macrolide at C_5 and two on the oleandrose disaccharide at C_3' and C_3'', all of which are derived from the methyl of methionine (7). Methylation at the oxygen at C_5 occurs via the *S*-adenosylmethionine-dependent enzyme B_2OMT (7). The involvement of *S*-adenosylmethionine in methylation of the oleandrose moiety, although not yet demonstrated, appears highly probable since these methylations are inhibited by the *S*-adenosylmethionine analog sinefungin and since methylations of hexose moieties of other macrolide antibiotics have been shown to require *S*-adenosylmethionine (3, 9, 10). B_2OMT has been shown to increase in direct proportion with avermectin production, but the level of enzyme did not determine the extent of conversion of B components to A components (7).

TABLE 4. Effect of sinefungin on the formation of demethylavermectins by CR-5

Time (h) after addition	Addition	Demethylavermectins (relative U/ml)		B/A ratio
		Total A components	Total B components	
96	None	86	14	0.16
	Sinefungin[a]	62	44	0.71
120	None	108	16	0.15
	Sinefungin	67	57	0.75
144	None	124	19	0.15
	Sinefungin	71	55	0.77

[a] Concentration was 0.13 mM in the fermentation.

The results of this study show that B_2OMT is required for formation of avermectin A components and that methylation of the oleandrose moieties is catalyzed by an enzyme(s) other than B_2OMT. Mutants CR-1, CR-2, CR-3, and CR-6 produce avermectin B components almost exclusively and have virtually no B_2OMT activity. In mutant CR-4, the production of A components and the B_2OMT activity are approximately one-third those of its parent. The presence of B_2OMT thus correlates with the ability of cultures to produce A components.

Strain CR-5 produces only demethylavermectin A and B components. Radiolabeling experiments demonstrated the incorporation of [^{14}C-*methyl*]methionine into the demethylavermectin A components but not into the demethylavermectin B components. In addition, sinefungin inhibited the formation of the demethylavermectin A components with a concomitant rise in the level of the demethylavermectin B components. These results indicate that this strain contains B_2OMT activity but apparently lacks the enzyme responsible for methylation of the oleandrose moiety (glycosyl *O*-methyltransferase). The low level of B_2OMT activity in this strain probably is a reflection of the low levels of avermectins produced during the fermentation. It was previously found that the B_2OMT levels are in direct proportion to the production of the avermectins (7).

Strain CR-6 produces only demethylavermectin B. This strain was derived from strain CR-1 and therefore lacks B_2OMT activity. In addition, the strain lacks either glycosyl *O*-methyltransferase or the ability to synthesize adequate amounts of *S*-adenosylmethionine.

The isolation of these methyltransferase mutants indicates that different enzymes are required for methylation of the macrolide at the C_5 oxygen and the oleandrose moiety at C_3' and C_3'' oxygen. This was supported by the observation that purified B_2OMT catalyzes methylation of the oxygen at C_5 of the demethylavermectins but not of C_3'' oxygen. The finding that extracts of "*S. avermitilis*" also catalyze methyl transfer only at the C_5 oxygen of the demethylavermectins suggests that methylation of the oleandrose moiety occurs as a nucleotide sugar before attachment to the macrolide ring.

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