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STREPTOMYCES AVERMITILIS GENE DIRECTING THE RATIO OF B2:B1 AVERMECTINS

1. FIELD OF THE INVENTION

The present invention is directed to compositions and methods for the efficient production of avermectins such as "doramectin", which are primarily useful in the field of animal health. More particularly, the present invention relates to polynucleotide molecules comprising nucleotide sequences encoding an AveC gene product, which can be used to modulate the ratio of class 2:1 avermectins produced by fermentation cultures of *Streptomyces avermitilis*. The present invention further relates to vectors, transformed host cells, and novel mutant strains of *S. avermitilis* in which the *aveC* gene has been mutated so as to modulate the ratio of class 2:1 avermectins produced.

2. BACKGROUND OF THE INVENTION

2.1. Avermectins

Streptomyces species produce a wide variety of secondary metabolites, including the avermectins, which comprise a series of eight related sixteen-membered macrocyclic lactones having potent anthelmintic and insecticidal activity. The eight distinct but closely related compounds are referred to as A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b. The "a" series of compounds refers to the natural avermectin where the substituent at the C25 position is (S)-sec-butyl, and the "b" series refers to those compounds where the substituent at the C25 position is isopropyl. The designations "A" and "B" refer to avermectins where the substituent at the C5 position is methoxy and hydroxy, respectively. The numeral "1" refers to avermectins where a double bond is present at the C22,23 position, and the numeral "2" refers to avermectins having a hydrogen at the C22 position and a hydroxy at the C23 position. Among the related avermectins, the B1 type of avermectin, such as doramectin, is recognized as having the most effective antiparasitic and pesticidal activity, and is therefore the most commercially desirable avermectin.

The avermectins and their production by aerobic fermentation of strains of *S. avermitilis* are described in United States Patent Nos. 4,310,519 and 4,429,042. The biosynthesis of natural avermectins is believed to be initiated endogenously from the CoA thioester analogs of isobutyric acid and S-(+)-2-methyl butyric acid.

A combination of both strain improvement through random mutagenesis and the use of exogenously supplied fatty acids has led to the efficient production of avermectin analogs. Mutants of *S. avermitilis* that are deficient in branched-chain 2-oxo acid dehydrogenase (bkd deficient mutants) can only produce avermectins when fermentations are supplemented with fatty acids. Screening and isolation of mutants deficient in branched-chain dehydrogenase activity (*e.g.*, *S. avermitilis*, ATCC 53567) are described in European Patent (EP) 276103. Fermentation of such mutants in the presence of exogenously supplied fatty acids results in

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production of only the four avermectins corresponding to the fatty acid employed. Thus, supplementing fermentations of *S. avermitilis* (ATCC 53567) with S-(+)-2-methylbutyric acid results in production of the natural avermectins A1a, A2a, B1a and B2a; supplementing fermentations with isobutyric acid results in production of the natural avermectins A1b, A2b, B1b, and B2b; and supplementing fermentations with cyclopentanecarboxylic acid results in the production of the four novel cyclopentylavermectins A1, A2, B1, and B2.

If supplemented with other fatty acids, novel avermectins are produced. By screening over 800 potential precursors, more than 60 other novel avermectins have been identified. (See, *e.g.*, Dutton *et al.*, 1991, J. Antibiot. 44:357-365; and Banks *et al.*, 1994, Roy. Soc. Chem. 147:16-26). In addition, mutants of *S. avermitilis* deficient in 5-O-methyltransferase activity produce essentially only the B analog avermectins. Consequently, *S. avermitilis* mutants lacking both branched-chain 2-oxo acid dehydrogenase and 5-O-methyltransferase activity produce only the B avermectins corresponding to the fatty acid employed to supplement the fermentation. Thus, supplementing such double mutants with S-(+)-2-methylbutyric acid results in production of only the natural avermectins B1a and B2a, while supplementing with isobutyric acid or cyclopentanecarboxylic acid results in production of the natural avermectins B1b and B2b or the novel cyclopentyl B1 and B2 avermectins, respectively. Supplementation of the double mutant strain with cyclohexane carboxylic acid is a preferred method for producing the commercially important novel avermectin, cyclohexylavermectin B1 (doramectin). The isolation and characteristics of such double mutants, *e.g.*, *S. avermitilis* (ATCC 53692), is described in EP 276103.

2.2. Genes Involved In Avermectin Biosynthesis

In many cases, genes involved in production of secondary metabolites and genes encoding a particular antibiotic are found clustered together on the chromosome. Such is the case with the *Streptomyces* polyketide synthase gene cluster (PKS) (see Hopwood and Sherman, 1990, Ann. Rev. Genet. 24:37-66). Thus, one strategy for cloning genes in a biosynthetic pathway has been to isolate a drug resistance gene and then test adjacent regions of the chromosome for other genes related to the biosynthesis of that particular antibiotic. Another strategy for cloning genes involved in the biosynthesis of important metabolites has been complementation of mutants. For example, portions of a DNA library from an organism capable of producing a particular metabolite are introduced into a non-producing mutant and transformants screened for production of the metabolite. Additionally, hybridization of a library using probes derived from other *Streptomyces* species has been used to identify and clone genes in biosynthetic pathways.

Genes involved in avermectin biosynthesis (*ave* genes), like the genes required for biosynthesis of other *Streptomyces* secondary metabolites (*e.g.*, PKS), are found clustered on

the chromosome. A number of *ave* genes have been successfully cloned using vectors to complement *S. avermitilis* mutants blocked in avermectin biosynthesis. The cloning of such genes is described in U.S. Patent No. 5,252,474. In addition, Ikeda *et al.*, 1995, *J. Antibiot.* 48:532-534, describes the localization of a chromosomal region involving the C22,23 dehydration step (*aveC*) to a 4.82 Kb *Bam*HI fragment of *S. avermitilis*, as well as mutations in the *aveC* gene that result in the production of a single component B2a producer. Since ivermectin, a potent anthelmintic compound, can be produced chemically from avermectin B2a, such a single component producer of avermectin B2a is considered particularly useful for commercial production of ivermectin.

U.S. Patent No. 6,248,579 to Stutzman-Engwall et al., issued June 19, 2001, describes certain mutations to the *aveC* gene of *Streptomyces avermitilis* leading to a reduction in the ratio of cyclohexyl B2:cyclohexyl B1 ratio to about 0.75:1.

PCT Publication WO 01/12821 by Pfizer Products Inc., published February 22, 2001, describes certain additional mutations to the *aveC* gene of *Streptomyces avermitilis* leading to further reductions in the ratio of cyclohexyl B2:cyclohexyl B1 ratio down to 0.40:1.

Identification of additional mutations or combinations of mutations in the *aveC* gene that further minimize the complexity of avermectin production, such as, *e.g.*, mutations that further decrease the B2:B1 ratio of avermectins, would simplify production and purification of commercially important avermectins.

3. SUMMARY OF THE INVENTION

The present invention provides a polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the *Streptomyces avermitilis aveC* allele, the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the *aveC* ORF of *S. avermitilis* as presented in FIGURE 1 (SEQ ID NO:1), or a degenerate variant thereof, but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO:2, such that cells of *S. avermitilis* strain ATCC 53692 in which the wild-type *aveC* allele has been inactivated and that express the polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2:1 ratio of avermectins that is reduced compared to the ratio produced by cells of *S. avermitilis* strain ATCC 53692 that instead express only the wild-type *aveC* allele, wherein when the class 2:1 avermectins are cyclohexyl B2:cyclohexyl B1 avermectins, the ratio of class 2:1 avermectins is 0.35:1 or less. In a more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a

more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less.

In a particular embodiment thereof, the combination of amino acid substitutions comprises a combination selected from the group consisting of:

(a) D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L;

(b) G40S, D48E, L136P, G179S, E238D;

(c) D48E, L136P, R163Q, G179S;

(d) D48E, L136P, R163Q, G179S, E238D;

(e) D48E, L136P, R163Q, G179S, A200G, E238D;

(f) D48E, L136P, G179S, E238D;

(g) D48E, A61T, L136P, G179S, E238D;

(h) D48E, A61T, L136P, G179S;

(i) D48E, A89T, S138T, A139T, G179S;

(j) D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L;

(k) D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L;

(l) D48E, L136P, G179S, A198G, E238D, P289L;

(m) D48E, A61T, S138T, A139F, G179S, A198G, P289L;

(n) D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L;

(o) Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D;

(p) D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L;

(q) D48E, L136P, G179S, R250W;

(r) D48E, A89T, S138T, A139T, R163Q, G179S;

(s) D48E, L136P, G179S, A198G, P289L;

(t) D48E, F78L, A89T, L136P, G179S;

(u) D48E, A89T, S138T, A139T, G179S, E238D, F278L;

(v) D48E, A89T, L136P, R163Q, G179S;

(w) D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L;

(x) D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S;

(y) D48E, A89T, S90G, L136P, R163Q, G179S, E238D;

(z) D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L;

(aa) D48E, A89T, S138T, A139T, G179S;

(ab) D48E, L136P, R163Q, G179S, S231L;

(ac) D48E, L136P, S138T, A139F, G179S, V196A, E238D;

(ad) D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D;

(ae) G35S, D48E, A89T, S138T, A139T, G179S, P289L;

(af) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D;

(ag) D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D;
(ah) S41G, D48E, A89T, L136P, G179S;
(ai) D48E, A89T, L136P, R163Q, G179S, P252S;
(aj) D48E, A89T, L136P, G179S, F234S;
(ak) D48E, A89T, L136P, R163Q, G179S, E238D;
(al) Q36R, D48E, A89T, L136P, G179S, E238D;
(am) D48E, A89T, L136P, R163Q, G179S;
(an) D48E, A89T, S138T, G179S;
(ao) D48E, A89T, L136P, G179S, E238D;
(ap) D48E, A89T, L136P, K154E, G179S, E238D;
(aq) D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L;
(ar) D48E, A89T, S138T, A139F, G179S, V196A, E238D;
(as) D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L;
(at) D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L;
(au) D48E, A89T, L136P, G179S;
(av) D48E, A89T, V120A, L136P, G179S;
(aw) D48E, A61T, A89T, S138T, A139F,G179S, V196A, A198G, E238D;
(ax) D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D;
(ay) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L;
(az) D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D;
(ba) D48E, A89T, S138T, A139F, G179S, A198G, V220A;
(bb) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L;
(bc) D48E, A61T, A89T, L136P, G179S, P289L;
(bd) D48E, A89T, S138T, A139T, G179S, V196A, E238D, P289L; and
(be) D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D.

The present invention further provides a polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the *Streptomyces avermitilis aveC* allele, the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the *aveC* ORF of *S. avermitilis* as presented in FIGURE 1 (SEQ ID NO:1), or a degenerate variant thereof, but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO:2, such that cells of *S. avermitilis* strain ATCC 53692 in which the wild-type *aveC* allele has been inactivated and that express a polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2:1 ratio of avermectins that is reduced compared to the ratio produced by cells of *S. avermitilis* strain ATCC 53692 that instead express only the wild-type *aveC* allele,

wherein when the class 2:1 avermectins are cyclohexyl B2:cyclohexyl B1 avermectins, the ratio of class 2:1 avermectins is reduced to about 0.40:1 or less, and wherein the combination of amino acid substitutions comprises a combination selected from the group consisting of:

(bf) D48E, S138T, A139T, G179S, E238D; and

(bg) Y28C, Q38R, D48E, L136P, G179S, E238D.

The present invention further provides a recombinant vector comprising a polynucleotide molecule of the present invention.

The present invention further provides a host cell comprising a polynucleotide molecule or a recombinant vector of the present invention. In a preferred embodiment, the host cell is a *Streptomyces* cell. In a more preferred embodiment, the host cell is a cell of *Streptomyces avermitilis*.

The present invention further provides a method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions is selected from (a) through (be) listed above.

The present invention further provides a method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein cells comprising the mutated *aveC* allele or degenerate variant are capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less. In a non-limiting embodiment thereof, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a non-limiting embodiment, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a non-limiting embodiment, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less. In a non-limiting embodiment, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions is selected from the group consisting of (bf) and (bg) listed above. In a preferred embodiment thereof, cells of *S. avermitilis* comprising such a mutated *aveC* allele or degenerate variant are capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.40:1 or less.

The present invention further provides a cell of a *Streptomyces* species that comprises a mutated *S. avermitilis aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from (a) through (be) listed above. In a preferred embodiment thereof, the species of *Streptomyces* is *S. avermitilis*.

The present invention further provides a cell of *Streptomyces avermitilis* capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less. In a non-limiting embodiment thereof, the cell comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a non-limiting embodiment thereof, the cells comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a non-limiting embodiment thereof, the cells comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less. In a non-limiting embodiment thereof, the cells comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product

comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a cell of a *Streptomyces* species, comprising a mutated *S. avermitilis aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (bf) and (bg) listed above. In a preferred embodiment thereof, the species of *Streptomyces* is *S. avermitilis*. In a more preferred embodiment, the cell is a cell of *S. avermitilis* capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.40:1 or less.

The present invention further provides a process for producing avermectins, comprising culturing a strain of *Streptomyces avermitilis* cells of the present invention in culture media under conditions that permit or induce the production of avermectins therefrom, and recovering said avermectins from the culture.

The present invention further provides a composition of cyclohexyl B2:cyclohexyl B1 avermectins produced by cells of *Streptomyces avermitilis*, comprising the cyclohexyl B2:cyclohexyl B1 avermectins present in a culture medium in which the cells have been cultured, wherein the ratio of the cyclohexyl B2:cyclohexyl B1 avermectins present in the culture medium is 0.35:1 or less, preferably about 0.30:1 or less, more preferably about 0.25:1 or less, and more preferably about 0.20:1 or less. In a particular embodiment, the composition of cyclohexyl B2:cyclohexyl B1 avermectins is produced by cells of a strain of *S. avermitilis* that express a mutated *aveC* allele or degenerate variant thereof which encodes a gene product that results in the reduction of the class 2:1 ratio of cyclohexyl B2:cyclohexyl B1 avermectins produced by the cells compared to cells of the same strain of *S. avermitilis* that do not express the mutated aveC allele but instead express only the wild-type aveC allele.

In a preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.30:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.25:1 or less, the composition is produced

by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.20:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a composition of cyclohexyl B2:cyclohexyl B1 avermectins produced by cells of *Streptomyces avermitilis*, comprising the cyclohexyl B2:cyclohexyl B1 avermectins present in a culture medium in which the cells have been cultured, wherein the ratio of the cyclohexyl B2:cyclohexyl B1 avermectins present in the culture medium is about 0.40:1 or less, and produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (bf) and (bg) listed above.

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. DNA sequence (SEQ ID NO:1) comprising the *S. avermitilis aveC* ORF, and deduced amino acid sequence (SEQ ID NO:2).

FIGURE 2. Plasmid vector pSE186 (ATCC 209604) comprising the entire ORF of the *aveC* gene of *S. avermitilis*.

FIGURE 3. Gene replacement vector pSE180 (ATCC 209605) comprising the *ermE* gene of *Sacc. erythraea* inserted into the *aveC* ORF of *S. avermitilis*.

FIGURE 4. *Bam*HI restriction map of the avermectin polyketide synthase gene cluster from *S. avermitilis* with five overlapping cosmid clones identified (*i.e.*, pSE65, pSE66, pSE67, pSE68, pSE69). The relationship of pSE118 and pSE119 is also indicated.

FIGURE 5. HPLC analysis of fermentation products produced by *S. avermitilis* strains. Peak quantitation was performed by comparison to standard quantities of cyclohexyl B1. Cyclohexyl B2 retention time was 7.4-7.7 min; cyclohexyl B1 retention time was 11.9-12.3 min. FIG. 5A. *S. avermitilis* strain SE180-11 with an inactivated *aveC* ORF. FIG. 5B. *S. avermitilis* strain SE180-11 transformed with pSE186 (ATCC 209604). FIG. 5C. *S. avermitilis* strain SE180-11 transformed with pSE187. FIG. 5D. *S. avermitilis* strain SE180-11 transformed with pSE188.

FIGURE 6A-M. Compiled list of combinations of amino acid substitutions encoded by mutations to the *aveC* allele as identified by a second round of "gene shuffling", and their effects on the ratio of cyclohexyl B2:cyclohexyl B1 production. For each plasmid, in the column entitled "Mutations", the upper box lists the amino acid substitutions, and the lower

box lists the nucleotide base changes resulting in those amino acid substitutions. Nucleotide base changes in parentheses are silent changes, i.e., they do not result in changes to the amino acid sequence.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification and characterization of polynucleotide molecules having nucleotide sequences that encode the AveC gene product from *Streptomyces avermitilis*, the construction of novel strains of *S. avermitilis* that can be used to screen mutated AveC gene products for their effect on avermectin production, and the discovery that certain mutated AveC gene products can reduce the ratio of B2:B1 avermectins produced by *S. avermitilis*. By way of example, the invention is described in the sections below for a polynucleotide molecule having either a nucleotide sequence that is the same as the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or the nucleotide sequence of the ORF of FIGURE 1 (SEQ ID NO:1), and for polynucleotides molecules having mutated nucleotide sequences derived therefrom and degenerate variants thereof. However, the principles set forth in the present invention can be analogously applied to other polynucleotide molecules, including *aveC* homolog genes from other *Streptomyces* species including, *e.g.*, *S. hygroscopicus* and *S. griseochromogenes*, among others.

5.1. Polynucleotide Molecules Encoding The *S. avermitilis* AveC Gene Product

The present invention provides an isolated polynucleotide molecule comprising the complete *aveC* ORF of *S. avermitilis* or a substantial portion thereof, which isolated polynucleotide molecule lacks the next complete ORF that is located downstream from the aveC ORF *in situ* in the *S. avermitilis* chromosome.

The isolated polynucleotide molecule of the present invention preferably comprises a nucleotide sequence that is the same as the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or that is the same as the nucleotide sequence of the ORF of FIGURE 1 (SEQ ID NO:1) or substantial portion thereof. As used herein, a "substantial portion" of an isolated polynucleotide molecule comprising a nucleotide sequence encoding the *S. avermitilis* AveC gene product means an isolated polynucleotide molecule comprising at least about 70% of the complete *aveC* ORF sequence shown in FIGURE 1 (SEQ ID NO:1), and that encodes a functionally equivalent AveC gene product. In this regard, a "functionally equivalent" AveC gene product is defined as a gene product that, when expressed in *S. avermitilis* strain ATCC 53692 in which the native *aveC* allele has been inactivated, results in the production of substantially the same ratio and amount of

avermectins as produced by *S. avermitilis* strain ATCC 53692 which instead expresses only the wild-type, functional *aveC* allele native to *S. avermitilis* strain ATCC 53692.

In addition to the nucleotide sequence of the *aveC* ORF, the isolated polynucleotide molecule of the present invention can further comprise nucleotide sequences that naturally flank the *aveC* gene *in situ* in *S. avermitilis*, such as those flanking nucleotide sequences shown in FIGURE 1 (SEQ ID NO:1).

The present invention further provides an isolated polynucleotide molecule comprising the nucleotide sequence of SEQ ID NO:1 or a degenerate variant thereof, as based on the known degeneracy of the genetic code.

As used herein, the terms "polynucleotide molecule," "polynucleotide sequence," "coding sequence," "open-reading frame," and "ORF" are intended to refer to both DNA and RNA molecules, which can either be single-stranded or double-stranded, and that can be transcribed and translated (DNA), or translated (RNA), into an AveC gene product, or into a polypeptide that is homologous to an AveC gene product in an appropriate host cell expression system when placed under the control of appropriate regulatory elements. A coding sequence can include but is not limited to prokaryotic sequences, cDNA sequences, genomic DNA sequences, and chemically synthesized DNA and RNA sequences.

The nucleotide sequence shown in FIGURE 1 (SEQ ID NO:1) comprises four different GTG codons at bp positions 42, 174, 177 and 180. As previously described in U.S. Patent No. 6,248,579, multiple deletions of the 5' region of the *aveC* ORF (FIGURE 1; SEQ ID NO:1) were constructed to help define which of these codons could function in the *aveC* ORF as start sites for protein expression. Deletion of the first GTG site at bp 42 did not eliminate AveC activity. Additional deletion of all of the GTG codons at bp positions 174, 177 and 180 together eliminated AveC activity, indicating that this region is necessary for protein expression. The present invention thus encompasses variable length *aveC* ORFs.

The present invention further provides a polynucleotide molecule having a nucleotide sequence that is homologous to the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or to the nucleotide sequence of the *aveC* ORF presented in FIGURE 1 (SEQ ID NO:1) or substantial portion thereof. The term "homologous" when used to refer to a polynucleotide molecule that is homologous to an *S. avermitilis* AveC gene product-encoding sequence means a polynucleotide molecule having a nucleotide sequence: (a) that encodes the same AveC gene product as the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or that encodes the same AveC gene product as the nucleotide sequence of the *aveC* ORF presented in FIGURE 1 (SEQ ID NO:1), but that includes one or more silent changes to the nucleotide sequence according to the degeneracy of the genetic code (*i.e.*, a degenerate variant); or (b) that hybridizes to the

complement of a polynucleotide molecule having a nucleotide sequence that encodes the amino acid sequence encoded by the AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or that encodes the amino acid sequence shown in FIGURE 1 (SEQ ID NO:2) under moderately stringent conditions, *i.e.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2xSSC/0.1% SDS at 42°C (see Ausubel *et al.* (eds.), 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3), and encodes a functionally equivalent AveC gene product as defined above. In a preferred embodiment, the homologous polynucleotide molecule hybridizes to the complement of the AveC gene product-encoding nucleotide sequence of plasmid pSE186 (ATCC 209604) or to the complement of the nucleotide sequence of the *aveC* ORF presented in FIGURE 1 (SEQ ID NO:1) or substantial portion thereof under highly stringent conditions, *i.e.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel *et al.*, 1989, above), and encodes a functionally equivalent AveC gene product as defined above.

The activity of an AveC gene product and potential functional equivalents thereof can be determined through HPLC analysis of fermentation products, as described in the examples below. Polynucleotide molecules having nucleotide sequences that encode functional equivalents of the *S. avermitilis* AveC gene product may include naturally occurring *aveC* genes present in other strains of *S. avermitilis*, *aveC* homolog genes present in other species of *Streptomyces*, and mutated *aveC* alleles, whether naturally occurring or engineered.

The present invention further provides a polynucleotide molecule comprising a nucleotide sequence that encodes a polypeptide having an amino acid sequence that is homologous to the amino acid sequence encoded by the AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or the amino acid sequence of FIGURE 1 (SEQ ID NO:2) or substantial portion thereof. As used herein, a "substantial portion" of the amino acid sequence of FIGURE 1 (SEQ ID NO:2) means a polypeptide comprising at least about 70% of the amino acid sequence shown in FIGURE 1 (SEQ ID NO:2), and that constitutes a functionally equivalent AveC gene product, as defined above.

As used herein to refer to amino acid sequences that are homologous to the amino acid sequence of an AveC gene product from *S. avermitilis*, the term "homologous" refers to a polypeptide which otherwise has the amino acid sequence of FIGURE 1 (SEQ ID NO:2), but in which one or more amino acid residues has been conservatively substituted with a different amino acid residue, wherein said amino acid sequence has at least about 70%, more preferably at least about 80%, and most preferably at least about 90% amino acid sequence identity to the polypeptide encoded by the AveC gene product-encoding sequence of plasmid

pSE186 (ATCC 209604) or the amino acid sequence of FIGURE 1 (SEQ ID NO:2) as determined by any standard amino acid sequence identity algorithm, such as the BLASTP algorithm (GENBANK, NCBI), and where such conservative substitution results in a functionally equivalent gene product, as defined above. Conservative amino acid substitutions are well known in the art. Rules for making such substitutions include those described by Dayhof, M.D., 1978, Nat. Biomed. Res. Found., Washington, D.C., Vol. 5, Sup. 3, among others. More specifically, conservative amino acid substitutions are those that generally take place within a family of amino acids that are related in the acidity or polarity. Genetically encoded amino acids are generally divided into four groups: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) non-polar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan and tyrosine are also jointly classified as aromatic amino acids. One or more replacements within any particular group, *e.g.*, of a leucine with an isoleucine or valine, or of an aspartate with a glutamate, or of a threonine with a serine, or of any other amino acid residue with a structurally related amino acid residue, *e.g.*, an amino acid residue with similar acidity or polarity, or with similarity in some combination thereof, will generally have an insignificant effect on the function of the polypeptide.

Production and manipulation of the polynucleotide molecules disclosed herein are within the skill in the art and can be carried out according to recombinant techniques described, *e.g.*, in Maniatis, *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel, et al., 1989, Current Protocols In Molecular Biology, Greene Publishing Associates & Wiley Interscience, NY; Sambrook, *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Innis *et al.* (eds), 1995, PCR Strategies, Academic Press, Inc., San Diego; and Erlich (ed), 1992, PCR Technology, Oxford University Press, New York, all of which are incorporated herein by reference. Polynucleotide clones encoding AveC gene products or AveC homolog gene products can be identified using any method known in the art, including but not limited to the methods set forth in Section 7, below. Genomic DNA libraries can be screened for *aveC* and *aveC* homolog coding sequences using techniques such as the methods set forth in Benton and Davis, 1977, Science 196:180, for bacteriophage libraries, and in Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. USA, 72:3961-3965, for plasmid libraries. Polynucleotide molecules having nucleotide sequences known to include the *aveC* ORF, as present, *e.g.*, in plasmid pSE186 (ATCC 209604), or in plasmid pSE119 (described in Section 7, below), can be used as probes in these screening experiments. Alternatively, oligonucleotide probes can be synthesized that correspond to nucleotide

sequences deduced from partial or complete amino acid sequences of the purified AveC homolog gene product.

5.2. Recombinant Systems

5.2.1. Cloning And Expression Vectors

The present invention further provides recombinant cloning vectors and expression vectors which are useful in cloning or expressing polynucleotide molecules of the present invention comprising, *e.g.*, the *aveC* ORF of *S. avermitilis* or any *aveC* homolog ORFs. In a non-limiting embodiment, the present invention provides plasmid pSE186 (ATCC 209604), which comprises the complete ORF of the *aveC* gene of *S. avermitilis*.

All of the following description regarding the *aveC* ORF from *S. avermitilis*, or a polynucleotide molecule comprising the *aveC* ORF from *S. avermitilis* or portion thereof, or an *S. avermitilis* AveC gene product, also refers to mutated *aveC* alleles as described below, unless indicated explicitly or by context.

A variety of different vectors have been developed for specific use in *Streptomyces*, including phage, high copy number plasmids, low copy number plasmids, and *E.coli-Streptomyces* shuttle vectors, among others, and any of these can be used to practice the present invention. A number of drug resistance genes have also been cloned from *Streptomyces*, and several of these genes have been incorporated into vectors as selectable markers. Examples of current vectors for use in *Streptomyces* are presented, among other places, in Hutchinson, 1980, Applied Biochem. Biotech. 16:169-190.

Recombinant vectors of the present invention, particularly expression vectors, are preferably constructed so that the coding sequence for the polynucleotide molecule of the invention is in operative association with one or more regulatory elements necessary for transcription and translation of the coding sequence to produce a polypeptide. As used herein, the term "regulatory element" includes but is not limited to nucleotide sequences that encode inducible and non-inducible promoters, enhancers, operators and other elements known in the art that serve to drive and/or regulate expression of polynucleotide coding sequences. Also, as used herein, the coding sequence is in "operative association" with one or more regulatory elements where the regulatory elements effectively regulate and allow for the transcription of the coding sequence or the translation of its mRNA, or both.

Typical plasmid vectors that can be engineered to contain a polynucleotide molecule of the present invention include pCR-Blunt, pCR2.1 (Invitrogen), pGEM3Zf (Promega), and the shuttle vector pWHM3 (Vara *et al.*, 1989, J. Bact. 171:5872-5881), among many others.

Methods are well-known in the art for constructing recombinant vectors containing particular coding sequences in operative association with appropriate regulatory elements, and these can be used to practice the present invention. These methods include *in vitro*

recombinant techniques, synthetic techniques, and *in vivo* genetic recombination. See, *e.g.*, the techniques described in Maniatis *et al.*, 1989, above; Ausubel *et al.*, 1989, above; Sambrook *et al.*, 1989, above; Innis et al., 1995, above; and Erlich, 1992, above.

The regulatory elements of these vectors can vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements can be used. Non-limiting examples of transcriptional regulatory regions or promoters for bacteria include the β-gal promoter, the T7 promoter, the TAC promoter, λ left and right promoters, trp and lac promoters, trp-lac fusion promoters and, more specifically for *Streptomyces*, the promoters *ermE*, *melC*, and *tipA*, *etc.* In a specific embodiment, an expression vector can be generated that contains the *aveC* ORF or mutated ORF thereof cloned adjacent to a strong constitutive promoter, such as the *ermE* promoter from *Saccharopolyspora erythraea.* As described in U.S. Patent No. 6,248,579, a vector comprising the *ermE* promoter was transformed into *S. avermitilis*, and subsequent HPLC analysis of fermentation products indicated an increased titer of avermectins produced compared to production by the same strain which instead expresses only the wild-type *aveC* allele.

Fusion protein expression vectors can be used to express an AveC gene product-fusion protein. The purified fusion protein can be used to raise antisera against the AveC gene product, to study the biochemical properties of the AveC gene product, to engineer AveC fusion proteins with different biochemical activities, or to aid in the identification or purification of the expressed AveC gene product. Possible fusion protein expression vectors include but are not limited to vectors incorporating sequences that encode β-galactosidase and trpE fusions, maltose-binding protein fusions, glutathione-S-transferase fusions and polyhistidine fusions (carrier regions). In an alternative embodiment, an AveC gene product or a portion thereof can be fused to an AveC homolog gene product, or portion thereof, derived from another species or strain of *Streptomyces*, such as, *e.g.*, *S. hygroscopicus* or *S. griseochromogenes*. Such hybrid vectors can be transformed into S. *avermitilis* cells and tested to determine their effect, *e.g.*, on the ratio of class 2:1 avermectin produced.

AveC fusion proteins can be engineered to comprise a region useful for purification. For example, AveC-maltose-binding protein fusions can be purified using amylose resin; AveC-glutathione-S-transferase fusion proteins can be purified using glutathione-agarose beads; and AveC-polyhistidine fusions can be purified using divalent nickel resin. Alternatively, antibodies against a carrier protein or peptide can be used for affinity chromatography purification of the fusion protein. For example, a nucleotide sequence coding for the target epitope of a monoclonal antibody can be engineered into the expression vector in operative association with the regulatory elements and situated so that the expressed

epitope is fused to the AveC polypeptide. For example, a nucleotide sequence coding for the FLAG™ epitope tag (International Biotechnologies Inc.), which is a hydrophilic marker peptide, can be inserted by standard techniques into the expression vector at a point corresponding, *e.g.*, to the carboxyl terminus of the AveC polypeptide. The expressed AveC polypeptide-FLAG™ epitope fusion product can then be detected and affinity-purified using commercially available anti-FLAG™ antibodies.

The expression vector encoding the AveC fusion protein can also be engineered to contain polylinker sequences that encode specific protease cleavage sites so that the expressed AveC polypeptide can be released from the carrier region or fusion partner by treatment with a specific protease. For example, the fusion protein vector can include DNA sequences encoding thrombin or factor Xa cleavage sites, among others.

A signal sequence upstream from, and in reading frame with, the *aveC* ORF can be engineered into the expression vector by known methods to direct the trafficking and secretion of the expressed gene product. Non-limiting examples of signal sequences include those from α-factor, immunoglobulins, outer membrane proteins, penicillinase, and T-cell receptors, among others.

To aid in the selection of host cells transformed or transfected with cloning or expression vectors of the present invention, the vector can be engineered to further comprise a coding sequence for a reporter gene product or other selectable marker. Such a coding sequence is preferably in operative association with the regulatory element coding sequences, as described above. Reporter genes that are useful in the invention are well-known in the art and include those encoding green fluorescent protein, luciferase, *xylE*, and tyrosinase, among others. Nucleotide sequences encoding selectable markers are well known in the art, and include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include those that encode resistance to erythromycin, thiostrepton or kanamycin, among many others.

5.2.2. Transformation Of Host Cells

The present invention further provides transformed host cells comprising a polynucleotide molecule or recombinant vector of the invention, and novel strains or cell lines derived therefrom. Host cells useful in the practice of the invention are preferably *Streptomyces* cells, although other prokaryotic cells or eukaryotic cells can also be used. Such transformed host cells typically include but are not limited to microorganisms, such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA vectors, or yeast transformed with recombinant vectors, among others.

The polynucleotide molecules of the present invention are intended to function in *Streptomyces* cells, but can also be transformed into other bacterial or eukaryotic cells, *e.g.*,

for cloning or expression purposes. A strain of *E. coli* can typically be used, such as, *e.g.*, the DH5α strain, available from the American Type Culture Collection (ATCC), Rockville, MD, USA (Accession No. 31343), and from commercial sources (Stratagene). Preferred eukaryotic host cells include yeast cells, although mammalian cells or insect cells can also be utilized effectively.

The recombinant expression vector of the invention is preferably introduced, e.g., transformed or transfected, into one or more host cells of a substantially homogeneous culture of cells. The expression vector is generally introduced into host cells in accordance with known techniques, such as, *e.g.*, by protoplast transformation, calcium phosphate precipitation, calcium chloride treatment, microinjection, electroporation, transfection by contact with a recombined virus, liposome-mediated transfection, DEAE-dextran transfection, transduction, conjugation, or microprojectile bombardment. Selection of transformants can be conducted by standard procedures, such as by selecting for cells expressing a selectable marker, *e.g.*, antibiotic resistance, associated with the recombinant vector, as described above.

Once the expression vector is introduced into the host cell, the integration and maintenance of the *aveC* coding sequence either in the host cell chromosome or episomally can be confirmed by standard techniques, *e.g.*, by Southern hybridization analysis, restriction enzyme analysis, PCR analysis, including reverse transcriptase PCR (rt-PCR), or by immunological assay to detect the expected gene product. Host cells containing and/or expressing the recombinant *aveC* coding sequence can be identified by any of at least four general approaches which are well-known in the art, including: (i) DNA-DNA, DNA-RNA, or RNA-antisense RNA hybridization; (ii) detecting the presence of "marker" gene functions; (iii) assessing the level of transcription as measured by the expression of *aveC*-specific mRNA transcripts in the host cell; and (iv) detecting the presence of mature polypeptide product as measured, *e.g.*, by immunoassay or by the presence of AveC biological activity (*e.g.*, the production of specific ratios and amounts of avermectins indicative of AveC activity in, *e.g.*, *S. avermitilis* host cells).

5.2.3. Expression And Characterization Of A Recombinant AveC Gene Product

Once the native or mutated *aveC* coding sequence has been stably introduced into an appropriate host cell, the transformed host cell is clonally propagated, and the resulting cells can be grown under conditions conducive to the maximum production of the native or mutated AveC gene product. Such conditions typically include growing cells to high density. Where the expression vector comprises an inducible promoter, appropriate induction conditions such as, *e.g.*, temperature shift, exhaustion of nutrients, addition of gratuitous inducers (*e.g.*,

analogs of carbohydrates, such as isopropyl-β-D-thiogalactopyranoside (IPTG)), accumulation of excess metabolic by-products, or the like, are employed as needed to induce expression.

Where the expressed AveC gene product is retained inside the host cells, the cells are harvested and lysed, and the product isolated and purified from the lysate under extraction conditions known in the art to minimize protein degradation such as, *e.g.*, at 4°C, or in the presence of protease inhibitors, or both. Where the expressed AveC gene product is secreted from the host cells, the exhausted nutrient medium can simply be collected and the product isolated therefrom.

The expressed AveC gene product can be isolated or substantially purified from cell lysates or culture medium, as appropriate, using standard methods, including but not limited to any combination of the following methods: ammonium sulfate precipitation, size fractionation, ion exchange chromatography, HPLC, density centrifugation, and affinity chromatography. Where the expressed AveC gene product exhibits biological activity, increasing purity of the preparation can be monitored at each step of the purification procedure by use of an appropriate assay. Whether or not the expressed AveC gene product exhibits biological activity, it can be detected as based, *e.g.*, on size, or reactivity with an antibody otherwise specific for AveC, or by the presence of a fusion tag. As used herein, an AveC gene product is "substantially purified" where the product constitutes more than about 20 wt% of the protein in a particular preparation. Also, as used herein, an AveC gene product is "isolated" where the product constitutes at least about 80 wt% of the protein in a particular preparation.

The present invention thus provides a recombinantly-expressed isolated or substantially purified *S. avermitilis* AveC gene product comprising the amino acid sequence encoded by the AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604), or the amino acid sequence of FIGURE 1 (SEQ ID NO:2) or a substantial portion thereof, and mutated versions and degenerate variants thereof.

The present invention further provides a method for producing an AveC gene product, comprising culturing a host cell transformed with a recombinant expression vector, said vector comprising a polynucleotide molecule having a nucleotide sequence encoding the AveC gene product, which polynucleotide molecule is in operative association with one or more regulatory elements that control expression of the polynucleotide molecule in the host cell, under conditions conducive to the production of the recombinant AveC gene product, and recovering the AveC gene product from the cell culture.

The recombinantly expressed *S. avermitilis* AveC gene product is useful for a variety of purposes, including for screening compounds that alter AveC gene product function and thereby modulate avermectin biosynthesis, and for raising antibodies directed against the AveC gene product.

Once an AveC gene product of sufficient purity has been obtained, it can be characterized by standard methods, including by SDS-PAGE, size exclusion chromatography, amino acid sequence analysis, biological activity in producing appropriate products in the avermectin biosynthetic pathway, *etc.* For example, the amino acid sequence of the AveC gene product can be determined using standard peptide sequencing techniques. The AveC gene product can be further characterized using hydrophilicity analysis (see, *e.g.*, Hopp and Woods, 1981, Proc. Natl. Acad. Sci. *USA* 78:3824), or analogous software algorithms, to identify hydrophobic and hydrophilic regions of the AveC gene product. Structural analysis can be carried out to identify regions of the AveC gene product that assume specific secondary structures. Biophysical methods such as X-ray crystallography (Engstrom, 1974, Biochem. Exp. Biol. 11: 7-13), computer modelling (Fletterick and Zoller (eds), 1986, in: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and nuclear magnetic resonance (NMR) can be used to map and study sites of interaction between the AveC gene product and its substrate. Information obtained from these studies can be used to select new sites for mutation in the *aveC* ORF to help develop new strains of *S. avermitilis* having more desirable avermectin production characteristics .

5.3. Construction And Use Of AveC Mutants

A primary objective of the present invention is to identify novel mutations in the *aveC* allele of *S. avermitilis* that result in a change, and most preferably a reduction, in the ratio of B2:B1 avermectins. The present invention thus provides polynucleotide molecules useful to produce novel strains of *S. avermitilis* cells that exhibit a detectable change in avermectin production compared to cells of the same strain but which instead express only the wild-type *aveC* allele. In a preferred embodiment, such polynucleotide molecules are useful to produce novel strains of *S. avermitilis* cells that produce avermectins in a reduced class 2:1 ratio compared to cells of the same strain which instead express only the wild-type *aveC* allele. The cells of such strains can also comprise additional mutations to produce an increased amount of avermectins compared to cells of the same strain that instead express only a single wild-type *aveC* allele.

Mutations to the *aveC* allele or coding sequence include any mutations that introduce one or more amino acid substitutions, deletions and/or additions into the AveC gene product, or that result in truncation of the AveC gene product, or any combination thereof, and that produce the desired result. Such mutated *aveC* allele sequences are intended to include any degenerate variants thereof. For example, the present invention provides a polynucleotide molecule comprising the nucleotide sequence of the *aveC* allele or a degenerate variant thereof, or the AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or a degenerate variant thereof, or the nucleotide sequence of the *aveC* ORF of *S. avermitilis* as

present in FIGURE 1 (SEQ ID NO:1) or a degenerate variant thereof, but that further comprises mutations that encode a combination of amino acid substitutions at selected positions in the AveC gene product. In a non-limiting embodiment, such substitutions occur at one or more amino acid positions of the AveC gene product corresponding to amino acid positions 25, 28, 35, 36, 38, 40, 41, 48, 55, 61, 78, 84, 89, 90, 99, 107, 108, 111, 120, 123, 136, 138, 139, 141, 154, 159, 163, 179, 192, 196, 198, 200, 202, 220, 228, 229, 230, 231, 234, 238, 239, 250, 252, 266, 275, 278, 289 or 298 of SEQ ID NO:2. Preferred combinations of amino acid positions to be substituted comprise one or more of amino acid residues D48, A61, A89, L136, S138, A139, R163, G179, V196, A198, E238 and P289. Specifically preferred combinations of amino acid substitutions comprise substitutions at both D48 and G179, and more specifically D48E and G179S. Specific examples of combinations of amino acid substitutions that result in a reduction in cyclohexylB2;cyclohexyl B1 ratios are listed in FIGURE 6A-J.

The present invention thus provides a polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the *Streptomyces avermitilis aveC* allele, the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the *aveC* ORF of *S. avermitilis* as presented in FIGURE 1 (SEQ ID NO:1), or a degenerate variant thereof, but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO:2, such that cells of *S. avermitilis* strain ATCC 53692 in which the wild-type *aveC* allele has been inactivated and that express the polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2:1 ratio of avermectins that is reduced compared to the ratio produced by cells of *S. avermitilis* strain ATCC 53692 that instead express only the wild-type *aveC* allele, wherein when the class 2:1 avermectins are cyclohexyl B2:cyclohexyl B1 avermectins, the ratio of class 2:1 avermectins is 0.35:1 or less. In a more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a more preferred embodiment, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less.

In a particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (a): D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE538 (see FIGURE 6).

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (b): G40S, D48E, L136P, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE559.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (c): D48E, L136P, R163Q, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE567.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (d): D48E, L136P, R163Q, G179S, E238D. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE570 and pSE572.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (e): D48E, L136P, R163Q, G179S, A200G, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE571.

In a particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (f): D48E, L136P, G179S, E238D. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE501 and pSE546.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (g): D48E, A61T, L136P, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE510.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (h): D48E, A61T, L136P, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE512.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (i): D48E, A89T, S138T, A139T, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE519.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (j): D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE526.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (k): D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE528.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (l): D48E, L136P, G179S, A198G, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE530.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (m): D48E, A61T, S138T, A139F, G179S, A198G, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE531.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (n): D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE534.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (o): Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE535.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (p): D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE542.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (q): D48E, L136P, G179S, R250W. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE545.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (r): D48E, A89T, S138T, A139T, R163Q, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE548.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (s): D48E, L136P, G179S, A198G, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE552.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (t): D48E, F78L, A89T, L136P, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE557.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (u): D48E, A89T, S138T, A139T, G179S, E238D, F278L. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE564 and pSE565.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (v): D48E, A89T, L136P, R163Q, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE568.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (w): D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L . A non-limiting example of a plasmid encoding these amino acid substitutions is pSE543.

In a particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (x): D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE504.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (y): D48E, A89T, S90G, L136P, R163Q, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE508.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (z): D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE511.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (aa): D48E, A89T, S138T, A139T, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE520.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ab): D48E, L136P, R163Q, G179S, S231L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE523.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ac): D48E, L136P, S138T, A139F, G179S, V196A, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE527.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ad): D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE539.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ae): G35S, D48E, A89T, S138T, A139T, G179S, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE540.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (af): D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE547.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ag): D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE550.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ah): S41G, D48E, A89T, L136P, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE558.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ai): D48E, A89T, L136P, R163Q, G179S, P252S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE563.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (aj): D48E, A89T, L136P, G179S, F234S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE566.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ak): D48E, A89T, L136P, R163Q, G179S, E238D. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE573 and pSE578.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (al): Q36R, D48E, A89T, L136P, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE574.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (am): D48E, A89T, L136P, R163Q, G179S. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE575 and pSE576.

In another particular embodiment, the combination of amino acid substitutions comprises the combination of group (an): D48E, A89T, S138T, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE577.

In a particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ao): D48E, A89T, L136P, G179S, E238D. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE502 and pSE524.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ap): D48E, A89T, L136P, K154E, G179S, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE503.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (aq): D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE505.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ar): D48E, A89T, S138T, A139F, G179S, V196A, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE506.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (as): D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE507.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (at): D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE509.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (au): D48E, A89T, L136P, G179S. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE514 and pSE525.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (av): D48E, A89T, V120A, L136P, G179S. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE515.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (aw): D48E, A61T, A89T, S138T, A139F,G179S, V196A, A198G, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE517.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ax): D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE518.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ay): D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE529 and pSE554.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (az): D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE532.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (ba) D48E, A89T, S138T, A139F, G179S, A198G,

V220A. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE536.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (bb): D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE537.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (bc): D48E, A61T, A89T, L136P, G179S, P289L. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE541.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (bd): D48E, A89T, S138T, A139T, G179S, V196A, E238D, P289L. Non-limiting examples of plasmids encoding these amino acid substitutions are pSE549 and pSE553.

In another particular embodiment thereof, the combination of amino acid substitutions comprises the combination of group (be): D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D. A non-limiting example of a plasmid encoding these amino acid substitutions is pSE551.

The present invention further provides a polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the *Streptomyces avermitilis aveC* allele, the *S. avermitilis* AveC gene product-encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the *aveC* ORF of *S. avermitilis* as presented in FIGURE 1 (SEQ ID NO:1), or a degenerate variant thereof, but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO:2, such that cells of *S. avermitilis* strain ATCC 53692 in which the wild-type *aveC* allele has been inactivated and that express a polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2:1 ratio of avermectins that is reduced compared to the ratio produced by cells of *S. avermitilis* strain ATCC 53692 that instead express only the wild-type *aveC* allele, wherein when the class 2:1 avermectins are cyclohexyl B2:cyclohexyl B1 avermectins, the ratio of class 2:1 avermectins is reduced to about 0.40:1 or less, and wherein the combination of amino acid substitutions comprises a combination selected from the group consisting of:

(bf) D48E, S138T, A139T, G179S, E238D; and

(bg) Y28C, Q38R, D48E, L136P, G179S, E238D.

Non-limiting examples of a plasmid encoding the amino acid substitutions of group (bf) are pSE556 and pSE569. A non-limiting example of a plasmid encoding the amino acid substitutions of group (bg) is pSE561.

The present invention contemplates that any of the aforementioned amino acid substitutions can be accomplished by any modification to the nucleotide sequence of the *aveC* allele or a degenerate variant thereof that results in such substitutions. For example, it is possible to effect most of the amino acid substitutions described herein by changing a native codon sequence or a degenerate variant thereof to any one of several alternative codons that encode the same amino acid substitution. The various possible sequences that can encode the aforementioned amino acid substitutions will be readily apparent to a person of skill in the art in view of the present disclosure and the known degeneracy of the genetic code. In a non-limiting embodiment for each particular combination recited above, the amino acid substitutions are achieved by the non-silent nucleotide changes set forth in FIGURE 6.

As used herein, the phrase "the combination of amino acid substitutions comprises the combination of group ... ", and the like, means that the amino acid substitutions in the AveC gene product according to the present invention include at least those substitutions that are specifically recited, and may include other amino acid substitutions, or amino acid deletions, or amino acid additions, or some combination thereof, wherein the expression of the resulting AveC gene product in the *S. avermitilis* cell yields a desirable reduction in the ratio of B2:B1 avermectins.

Mutations to the *aveC* allele or degenerate variant thereof can be carried out by any of a variety of known methods, including by use of error-prone PCR, or by cassette mutagenesis. For example, oligonucleotide-directed mutagenesis can be employed to alter the sequence of the *aveC* allele or ORF in a defined way such as, *e.g.*, to introduce one or more restriction sites, or a termination codon, into specific regions within the *aveC* allele or ORF. Methods such as those described in U.S. Patent 5,605,793, U.S. Patent 5,830,721 and U.S. Patent 5,837,458, which involve random fragmentation, repeated cycles of mutagenesis, and nucleotide shuffling, can also be used to generate large libraries of polynucleotides having nucleotide sequences encoding *aveC* mutations.

Targeted mutations can be useful, particularly where they serve to alter one or more conserved amino acid residues in the AveC gene product. For example, a comparison of the deduced amino acid sequence of the AveC gene product of *S. avermitilis* (SEQ ID NO:2) with AveC homolog gene products from *S. griseochromogenes* (SEQ ID NO:5) and *S. hygroscopicus* (SEQ ID NO:4), as described in U.S. Patent No. 6,248,579, indicates sites of significant conservation of amino acid residues between these species. Targeted mutagenesis that leads to a change in one or more of these conserved amino acid residues may be effective in producing novel mutant strains that exhibit desirable alterations in avermectin production.

Random mutagenesis can also be useful, and can be carried out by exposing cells of *S. avermitilis* to ultraviolet radiation or x-rays, or to chemical mutagens such as N-methyl-N'-nitrosoguanidine, ethyl methane sulfonate, nitrous acid or nitrogen mustards. See, *e.g.*, Ausubel, 1989, above, for a review of mutagenesis techniques.

Once mutated polynucleotide molecules are generated, they are screened to determine whether they can modulate avermectin biosynthesis in *S. avermitilis*. In a preferred embodiment, a polynucleotide molecule having a mutated nucleotide sequence is tested by complementing a strain of *S. avermitilis* in which the *aveC* gene has been inactivated to give an *aveC* negative (*aveC*$^-$) background. In a non-limiting method, the mutated polynucleotide molecule is spliced into an expression plasmid in operative association with one or more regulatory elements, which plasmid also preferably comprises one or more drug resistance genes to allow for selection of transformed cells. This vector is then transformed into *aveC*$^-$ host cells using known techniques, and transformed cells are selected and cultured in appropriate fermentation media under conditions that permit or induce avermectin production, for example, by including appropriate starter subunits in the medium, and culturing under optimal conditions for avermectin production as known in the art. Fermentation products are then analyzed by HPLC to determine the ability of the mutated polynucleotide molecule to complement the host cell. Several plasmid vectors bearing mutated polynucleotide molecules capable of reducing the B2:B1 ratio of avermectins, including pSE188, pSE199, pSE231, pSE239, and pSE290 through pSE297, are exemplified in Section 8.3, below. Other examples of such plasmid vectors are recited in FIGURE 6.

Any of the aforementioned methods of the present invention can be carried out using fermentation culture media preferably supplemented with cyclohexane carboxylic acid, although other appropriate fatty acid precursors, such as any one of the fatty acid precursors listed in TABLE 1, or methylthiolactic acid, can also used.

Once a mutated polynucleotide molecule that modulates avermectin production in a desirable direction has been identified, the location of the mutation in the nucleotide sequence can be determined. For example, a polynucleotide molecule having a nucleotide sequence encoding a mutated AveC gene product can be isolated by PCR and subjected to DNA sequence analysis using known methods. By comparing the DNA sequence of the mutated *aveC* allele to that of the wild-type *aveC* allele, the mutation(s) responsible for the alteration in avermectin production can be determined. For example, *S. avermitilis* AveC gene products comprising either single amino acid substitutions at any of residues 55 (S55F), 138 (S138T), 139 (A139T), or 230 (G230D), or double substitutions at positions 138 (S138T) and 139 (A139T or A139F), yielded changes in AveC gene product function such that the ratio of class 2:1 avermectins produced was altered (see Section 8, below), wherein the recited amino acid

positions correspond to those presented in FIGURE 1 (SEQ ID NO:2). In addition, the following seven combinations of mutations have each been shown to effectively reduce the class 2:1 ratio of avermectins: (1) D48E/A89T; (2) S138T/A139T/G179S; (3) Q38P/L136P/E238D; (4) F99S/S138T/A139T/ G179S; (5) A139T/ M228T; (6) G111V/P289L; (7) A139T/K154E/Q298H. The present invention provides fifty-nine (59) additional combinations of mutations that are shown to reduce the cyclohexyl B2:cyclohexyl B1 ratio of avermectins, and these are presented in FIGURE 6 and recited in the appended claims.

As used herein, the aforementioned designations, such as A139T, indicate the original amino acid residue by single letter designation, which in this example is alanine (A), at the indicated position, which in this example is position 139 (referring to SEQ ID NO:2) of the polypeptide, followed by the amino acid residue which replaces the original amino acid residue, which in this example is threonine (T).

As used herein, where an amino acid residue encoded by an *aveC* allele in the *S. avermitilis* chromosome, or in a vector or isolated polynucleotide molecule of the present invention is referred to as "corresponding to" a particular amino acid residue of SEQ ID NO:2, or where an amino acid substitution is referred to as occurring at a particular position "corresponding to" that of a specific numbered amino acid residue of SEQ ID NO:2, this is intended to refer to the amino acid residue at the same relative location in the AveC gene product, which the skilled artisan can quickly determine by reference to the amino acid sequence presented herein as SEQ ID NO:2.

As used herein, where specific mutations in the *aveC* allele encoding particular mutations are recited as base changes at specific nucleotide positions in the *aveC* allele "corresponding to" particular nucleotide positions as shown in SEQ ID NO:1, or where a nucleotide position in the *aveC* allele is otherwise referred to as "corresponding to" a particular nucleotide position in SEQ ID NO:1, this is intended to refer to the nucleotide at the same relative location in the *aveC* nucleotide sequence or a degenerate variant thereof, which the skilled artisan can quickly determine by reference to the nucleotide sequence presented herein as SEQ ID NO:1.

As used herein to refer to ratios of cyclohexyl B2:cyclohexyl B1 avermectins, the term "about" refers to the specifically stated numerical value plus or minus 10% of that stated value.

A polynucleotide molecule of the present invention may be "isolated", which means either that it is: (i) purified to the extent that it is substantially free of other polynucleotide molecules having different nucleotide sequences, or (ii) present in an environment in which it would not naturally occur, e.g., where an *aveC* allele from *S. avermitilis*, or a mutated version thereof, is present in a cell other than a cell of *S. avermitilis*, or (iii) present in a form in which it would not naturally occur, e.g., as a shorter piece of DNA, such as a restriction fragment

digested out of a bacterial chromosome, comprising predominantly the *aveC* coding region or a mutated version thereof, with or without any associated regulatory sequences thereof, or as subsequently integrated into a heterologous piece of DNA, such as the chromosome of a bacterial cell (other than a cell of *S. avermitilis*) or the DNA of a vector such as a plasmid or phage, or integrated into the *S. avermitilis* chromosome at a locus other than that of the native *aveC* allele.

The present invention further provides a recombinant vector comprising a polynucleotide molecule of the present invention. Such a recombinant vector can be used to target any of the polynucleotide molecules comprising mutated nucleotide sequences of the present invention to the site of the *aveC* allele of the *S. avermitilis* chromosome to either insert into or replace the *aveC* ORF or a portion thereof, e.g., by homologous recombination. According to the present invention, however, a polynucleotide molecule comprising a mutated nucleotide sequence of the present invention provided herewith can also function to modulate avermectin biosynthesis when inserted into the *S. avermitilis* chromosome at a site other than at the *aveC* allele, or when maintained episomally in *S. avermitilis* cells. Thus, the present invention further provides vectors comprising a polynucleotide molecule comprising a mutated nucleotide sequence of the present invention, which vectors can be used to insert the polynucleotide molecule at a site in the *S. avermitilis* chromosome other than at the *aveC* gene, or to be maintained episomally.

In a non-limiting embodiment, the vector is a gene replacement vector that can be used to insert a mutated *aveC* allele or degenerate variant thereof according to the present invention into cells of a strain of *S. avermitilis*, thereby generating novel strains of *S. avermitilis*, the cells of which can produce avermectins in a reduced class 2:1 ratio compared to cells of the same strain which instead express only the wild-type *aveC* allele. Such gene replacement vectors can be constructed using mutated polynucleotide molecules present in expression vectors provided herewith, such as those expression vectors exemplified in Section 8 below.

The present invention further provides vectors that can be used to insert a mutated *aveC* allele or degenerate variant thereof into cells of a strain of *S. avermitilis* to generate novel strains of cells that produce altered amounts of avermectins compared to cells of the same strain which instead express only the wild-type *aveC* allele. In a preferred embodiment, the amount of avermectins produced by the cells is increased. In a specific though non-limiting embodiment, such a vector comprises a strong promoter as known in the art, such as, *e.g.*, the strong constitutive *ermE* promoter from *Saccharopolyspora erythraea*, that is situated upstream from, and in operative association with, the *aveC* ORF. Such vectors can be

constructed using the mutated *aveC* allele of plasmid pSE189, and according to methods described in U.S. Patent No. 6,248,579,

The present invention provides gene replacement vectors that are useful to inactivate the *aveC* gene in a wild-type strain of *S. avermitilis*. In a non-limiting embodiment, such gene replacement vectors can be constructed using the mutated polynucleotide molecule present in plasmid pSE180 (ATCC 209605), which is exemplified in Section 8.1, below (FIGURE 3). The present invention further provides gene replacement vectors that comprise a polynucleotide molecule comprising or consisting of nucleotide sequences that naturally flank the *aveC* gene *in situ* in the *S. avermitilis* chromosome, including, *e.g.*, those flanking nucleotide sequences shown in FIGURE 1 (SEQ ID NO:1), which vectors can be used to delete the *S. avermitilis aveC* ORF.

The present invention further provides a host cell comprising a polynucleotide molecule or recombinant vector of the present invention. The host cell can be any prokaryotic or eukaryotic cell capable of use as a host for the polynucleotide molecule or recombinant vector. In a preferred embodiment, the host cell is a bacterial cell. In a more preferred embodiment, the host cell is a *Streptomyces* cell. In a more preferred embodiment, the host cell is a cell of *Streptomyces avermitilis*.

The present invention further provides a method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions is selected from (a) through (be) listed above.

The present invention further provides a method for making a novel strain of *S. avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein cells comprising the mutated *aveC* allele or degenerate variant are capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less. In a non-limiting embodiment thereof, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a non-limiting embodiment thereof, the mutated *aveC*

allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a non-limiting embodiment thereof, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less. In a non-limiting embodiment thereof, the mutated *aveC* allele or degenerate variant thereof encodes an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions is selected from the group consisting of (bf) and (bg). In a preferred embodiment thereof, cells of *S. avermitilis* comprising such a mutated *aveC* allele or degenerate variant are capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.40:1 or less.

By so mutating the *aveC* allele, or by so introducing a mutated aveC allele or degenerate variant thereof, according to the above-recited steps, a new strain of *S avermitilis* is made.

The present invention further provides a cell of a *Streptomyces* species that comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from (a) through (be) listed above. In a preferred embodiment thereof, the species of *Streptomyces* is *S. avermitilis*.

The present invention further provides a cell of *S. avermitilis* capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less. In a non-limiting embodiment thereof, the cell comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.30:1 or less. In a non-limiting embodiment thereof, the cell comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product

comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.25:1 or less. In a non-limiting embodiment thereof, the cell comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a more preferred embodiment thereof, the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less. In a non-limiting embodiment thereof, the cell comprises a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a cell of a *Streptomyces* species, comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (bf) and (bg) listed above. In a preferred embodiment thereof, the species of *Streptomyces* is *S. avermitilis*. In a more preferred embodiment thereof, the cell is a cell of *S. avermitilis* capable of producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.40:1 or less.

Although any of the recited mutations can be present in cells of the present invention on an extrachromosomal element such as a plasmid, it is preferred that such mutations are present in an *aveC* coding sequence integrated into the *S. avermitilis* chromosome, and preferably, though not necessarily, at the site of the native *aveC* allele.

Such novel strains of cells are useful in the large-scale production of commercially desirable avermectins such as doramectin.

The present invention further provides a process for producing avermectins, comprising culturing the *S. avermitilis* cells of the present invention in culture media under conditions that permit or induce the production of avermectins therefrom, and recovering said avermectins from the culture. In a preferred embodiment, the cells used in the process produce cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less, more preferably in a ratio of about 0.30:1 or less, more preferably in a ratio of about 0.25:1 or less, and more preferably in a ratio of about 0.20:1 or less.

In a preferred embodiment thereof, cells producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a further preferred embodiment thereof, cells producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.30:1 or less comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a further preferred embodiment thereof, cells producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.25:1 or less comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a further preferred embodiment thereof, cells producing cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.20:1 or less comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

In another embodiment, the cells produce cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.40:1 or less and comprise a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (bf) and (bg) listed above.

The process of the invention provides increased efficiency in the production of commercially valuable avermectins such as doramectin.

The present invention further provides a composition of cyclohexyl B2:cyclohexyl B1 avermectins produced by cells of *Streptomyces avermitilis*, comprising the cyclohexyl B2:cyclohexyl B1 avermectins present in a culture medium in which the cells have been cultured, wherein the ratio of the cyclohexyl B2:cyclohexyl B1 avermectins present in the culture medium is 0.35:1 or less, preferably about 0.30:1 or less, more preferably about 0.25:1 or less, and more preferably about 0.20:1 or less. In a particular embodiment, the composition of cyclohexyl B2:cyclohexyl B1 avermectins is produced by cells of a strain of *S. avermitilis* that express a mutated *aveC* allele or degenerate variant thereof which encodes a gene product that results in the reduction in the ratio of cyclohexyl B2:cyclohexyl B1 avermectins produced by the cells compared to cells of the same strain of *S. avermitilis* that do not express the mutated aveC allele but instead express only the wild-type aveC allele.

In a preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of 0.35:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (a) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.30:1 or less, the composition is produced

by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (f) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.25:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (w) through (be) listed above.

In a further preferred embodiment thereof, where the composition is cyclohexyl B2:cyclohexyl B1 avermectins in a ratio of about 0.20:1 or less, the composition is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (ao) through (be) listed above.

The present invention further provides a composition of cyclohexyl B2:cyclohexyl B1 avermectins produced by cells of *Streptomyces avermitilis*, comprising the cyclohexyl B2:cyclohexyl B1 avermectins present in a culture medium in which the cells have been cultured, wherein the ratio of the cyclohexyl B2:cyclohexyl B1 avermectins present in the culture medium is about 0.40:1 or less, and which is produced by cells comprising a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of (bf) and (bg) listed above.

Although it is preferred that the novel avermectin composition is present in a culture medium in which the cells have been cultured, e.g., in partially or totally exhausted fermentation culture fluid, the avermectin composition may alternatively be partially or substantially purified from the culture fluid by known biochemical techniques of purification, such as by ammonium sulfate precipitation, dialysis, size fractionation, ion exchange chromatography, HPLC, *etc.*

In addition to making novel strains of *S. avermitilis* comprising cells that are capable of producing reduced ratios of cyclohexyl B2:cyclohexyl B1 as described above, the present invention contemplates that additional mutations can be incorporated into cells of *S. avermitilis* to further improve characteristics of avermectin production. In a non-limiting embodiment, cells of the present invention can further comprise modifications to increase the production level of avermectins. In one embodiment, such cells can be prepared by (i) mutating the *aveC* allele in a cell of *S. avermitilis*, or (ii) introducing a mutated *aveC* allele or degenerate variant thereof into cells of a strain of *S. avermitilis*, wherein the expression of the mutated allele results in an increase in the amount of avermectins produced by cells of a strain of *S.*

avermitilis expressing the mutated *aveC* allele compared to cells of the same strain that instead express only a single wild-type *aveC* allele, and selecting transformed cells that produce avermectins in an increased amount compared to the amount of avermectins produced by cells of the strain that instead express only the single wild-type *aveC* allele. For example, the *aveC* allele can be modified so that it comprises a strong promoter, such as the strong constitutive *ermE* promoter from *Saccharopolyspora erythraea*, inserted upstream from and in operative association with the *aveC* ORF. In another embodiment, one or more mutations can be introduced into the *aveR1* and/or *aveR2* genes of *S. avermitilis*, thereby increasing the level of avermectin production as described in U.S. Patent No. 6,197,591 to Stutzman-Engwall et al., issued March 6, 2001.

5.4. Uses Of Avermectins

Avermectins are highly active antiparasitic agents having particular utility as anthelmintics, ectoparasiticides, insecticides and acaricides. Avermectin compounds produced according to the methods of the present invention are useful for any of these purposes. For example, avermectin compounds produced according to the present invention are useful to treat various diseases or conditions in humans, particularly where those diseases or conditions are caused by parasitic infections, as known in the art. See, e.g., Ikeda and Omura, 1997, Chem. Rev. 97(7):2591-2609. More particularly, avermectin compounds produced according to the present invention are effective in treating a variety of diseases or conditions caused by endoparasites, such as parasitic nematodes, which can infect humans, domestic animals, swine, sheep, poultry, horses or cattle.

More specifically, avermectin compounds produced according to the present invention are effective against nematodes that infect humans, as well as those that infect various species of animals. Such nematodes include gastrointestinal parasites such as *Ancylostoma*, *Necator*, *Ascaris*, *Strongyloides*, *Trichinella*, *Capillaria*, *Trichuris*, *Enterobius*, *Dirofilaria*, and parasites that are found in the blood or other tissues or organs, such as filarial worms and the extract intestinal states of *Strongyloides* and *Trichinella*.

The avermectin compounds produced according to the present invention are also useful in treating ectoparasitic infections including, e.g., arthropod infestations of mammals and birds, caused by ticks, mites, lice, fleas, blowflies, biting insects, or migrating dipterous larvae that can affect cattle and horses, among others.

The avermectin compounds produced according to the present invention are also useful as insecticides against household pests such as, e.g., the cockroach, clothes moth, carpet beetle and the housefly among others, as well as insect pests of stored grain and of agricultural plants, which pests include spider mites, aphids, caterpillars, and orthopterans such as locusts, among others.

Animals that can be treated with the avermectin compounds produced according to the present invention include sheep, cattle, horses, deer, goats, swine, birds including poultry, and dogs and cats.

An avermectin compound produced according to the present invention is administered in a formulation appropriate to the specific intended use, the particular species of host animal being treated, and the parasite or insect involved. For use as a parasiticide, an avermectin compound produced according to the present invention can be administered orally in the form of a capsule, bolus, tablet or liquid drench or, alternatively, can be administered as a pour-on, or by injection, or as an implant. Such formulations are prepared in a conventional manner in accordance with standard veterinary practice. Thus, capsules, boluses or tablets can be prepared by mixing the active ingredient with a suitable finely divided diluent or carrier additionally containing a disintegrating agent and/or binder such as starch, lactose, talc, magnesium stearate, *etc.* A drench formulation can be prepared by dispersing the active ingredient in an aqueous solution together with a dispersing or wetting agent, *etc.* Injectable formulations can be prepared in the form of a sterile solution, which can contain other substances such as, *e.g.*, sufficient salts and/or glucose to make the solution isotonic with blood.

Such formulations will vary with regard to the weight of active compound depending on the patient, or species of host animal to be treated, the severity and type of infection, and the body weight of the host. Generally, for oral administration a dose of active compound of from about 0.001 to 10 mg per kg of patient or animal body weight given as a single dose or in divided doses for a period of from 1 to 5 days will be satisfactory. However, there can be instances where higher or lower dosage ranges are indicated, as determined, *e.g.*, by a physician or veterinarian, as based on clinical symptoms.

As an alternative, an avermectin compound produced according to the present invention can be administered in combination with animal feedstuff, and for this purpose a concentrated feed additive or premix can be prepared for mixing with the normal animal feed.

For use as an insecticide, and for treating agricultural pests, an avermectin compound produced according to the present invention can be applied as a spray, dust, emulsion and the like in accordance with standard agricultural practice.

6. EXAMPLE: FERMENTATION OF *STREPTOMYCES AVERMITILIS* AND B2:B1 AVERMECTIN ANALYSIS

Strains lacking both branched-chain 2-oxo acid dehydrogenase and 5-O-methyltransferase activities produce no avermectins if the fermentation medium is not supplemented with fatty acids. This example demonstrates that in such mutants a wide range

of B2:B1 ratios of avermectins can be obtained when biosynthesis is initiated in the presence of different fatty acids.

6.1. Materials And Methods

Streptomyces avermitilis ATCC 53692 was stored at -70°C as a whole broth prepared in seed medium consisting of: Starch (Nadex, Laing National) - 20g; Pharmamedia (Trader's Protein, Memphis, TN) - 15 g; Ardamine pH (Yeast Products Inc.) - 5 g; calcium carbonate - 1 g. Final volume was adjusted to 1 liter with tap water, pH was adjusted to 7.2, and the medium was autoclaved at 121°C for 25 min.

Two ml of a thawed suspension of the above preparation was used to inoculate a flask containing 50 ml of the same medium. After 48 hrs incubation at 28°C on a rotary shaker at 180 rpm, 2 ml of the broth was used to inoculate a flask containing 50 ml of a production medium consisting of: Starch - 80 g; calcium carbonate - 7 g; Pharmamedia - 5 g; dipotassium hydrogen phosphate - 1 g; magnesium sulfate - 1 g; glutamic acid - 0.6 g; ferrous sulfate heptahydrate - 0.01 g; zinc sulfate - 0.001 g; manganous sulfate - 0.001 g. Final volume was adjusted to 1 liter with tap water, pH was adjusted to 7.2, and the medium was autoclaved at 121°C for 25 min.

Various carboxylic acid substrates (see TABLE 1) were dissolved in methanol and added to the fermentation broth 24 hrs after inoculation to give a final concentration of 0.2 g/liter. The fermentation broth was incubated for 14 days at 28°C, then the broth was centrifuged (2,500 rpm for 2 min) and the supernatant discarded. The mycelial pellet was extracted with acetone (15 ml), then with dichloromethane (30 ml), and the organic phase separated, filtered, then evaporated to dryness. The residue was taken up in methanol (1 ml) and analyzed by HPLC with a Hewlett-Packard 1090A liquid chromatograph equipped with a scanning diode-array detector set at 240 nm. The column used was a Beckman Ultrasphere C-18, 5 μm, 4.6 mm x 25 cm column maintained at 40°C. Twenty-five μl of the above methanol solution was injected onto the column. Elution was performed with a linear gradient of methanol-water from 80:20 to 95:5 over 40 min at 0.85/ml min. Two standard concentrations of cyclohexyl B1 were used to calibrate the detector response, and the area under the curves for B2 and B1 avermectins was measured.

6.2. Results

The HPLC retention times observed for the B2 and B1 avermectins, and the 2:1 ratios, are shown in TABLE 1.

TABLE 1

	HPLC Retention Time (min)		Ratio
Substrate	B2	B1	B2:B1
4-Tetrahydropyran carboxylic acid	8.1	14.5	0.25
Isobutyric acid	10.8	18.9	0.5
3-Furoic acid	7.6	14.6	0.62
S-(+)-2-methylbutyric acid	12.8	21.6	1.0
Cyclohexanecarboxylic acid	16.9	26.0	1.6
3-Thiophenecarboxylic acid	8.8	16.0	1.8
Cyclopentanecarboxylic acid	14.2	23.0	2.0
3-Trifluoromethylbutyric acid	10.9	18.8	3.9
2-Methylpentanoic acid	14.5	24.9	4.2
Cycloheptanecarboxylic acid	18.6	29.0	15.0

The data presented in TABLE 1 demonstrates an extremely wide range of B2:B1 avermectin product ratios, indicating a considerable difference in the results of dehydrative conversion of class 2 compounds to class 1 compounds, depending on the nature of the fatty acid side chain starter unit supplied. This indicates that changes in B2:B1 ratios resulting from alterations to the AveC protein may be specific to particular substrates. Consequently, screening for mutants exhibiting changes in the B2:B1 ratio obtained with a particular substrate needs to be done in the presence of that substrate. The subsequent examples described below use cyclohexanecarboxylic acid as the screening substrate. However, this substrate is used merely to exemplify the potential, and is not intended to limit the applicability, of the present invention.

7. EXAMPLE: ISOLATION OF THE *aveC* GENE

This example describes the isolation and characterization of a region of the *Streptomyces avermitilis* chromosome that encodes the AveC gene product. As demonstrated below, the *aveC* gene was identified as capable of modifying the ratio of cyclohexyl-B2 to cyclohexyl-B1 (B2:B1) avermectins produced.

7.1. Materials And Methods

7.1.1. Growth Of *Streptomyces* For DNA Isolation

The following method was followed for growing *Streptomyces*. Single colonies of *S. avermitilis* ATCC 31272 (single colony isolate #2) were isolated on 1/2 strength YPD-6 containing: Difco Yeast Extract - 5 g; Difco Bacto-peptone - 5 g; dextrose - 2.5 g; MOPS - 5 g; Difco Bacto agar - 15 g. Final volume was adjusted to 1 liter with dH_20, pH was adjusted to 7.0, and the medium was autoclaved at 121°C for 25 min.

The mycelia grown in the above medium were used to inoculate 10 ml of TSB medium (Difco Tryptic Soy Broth - 30 g, in 1 liter dH_2O, autoclaved at 121°C for 25 min) in a 25 mm x 150 mm tube which was maintained with shaking (300 rpm) at 28°C for 48-72 hrs.

7.1.2. Chromosomal DNA Isolation From *Streptomyces*

Aliquots (0.25 ml or 0.5 ml) of mycelia grown as described above were placed in 1.5 ml microcentrifuge tubes and the cells concentrated by centrifugation at 12,000 x g for 60 sec. The supernatant was discarded and the cells were resuspended in 0.25 ml TSE buffer (20 ml 1.5 M sucrose, 2.5 ml 1 M Tris-HCl, pH 8.0, 2.5 ml 1 M EDTA, pH 8.0, and 75 ml dH_2O) containing 2 mg/ml lysozyme. The samples were incubated at 37°C for 20 min with shaking, loaded into an AutoGen 540™ automated nucleic acid isolation instrument (Integrated Separation Systems, Natick, MA), and genomic DNA isolated using Cycle 159 (equipment software) according to manufacturer's instructions.

Alternatively, 5 ml of mycelia were placed in a 17 mm x 100 mm tube, the cells concentrated by centrifugation at 3,000 rpm for 5 min, and the supernatant removed. Cells were resuspended in 1 ml TSE buffer, concentrated by centrifugation at 3,000 rpm for 5 min, and the supernatant removed. Cells were resuspended in 1 ml TSE buffer containing 2 mg/ml lysozyme, and incubated at 37°C with shaking for 30-60 min. After incubation, 0.5 ml 10% sodium dodecyl sulfate (SDS) was added and the cells incubated at 37°C until lysis was complete. The lysate was incubated at 65°C for 10 min, cooled to rm temp, split into two 1.5 ml Eppendorf tubes, and extracted 1x with 0.5 ml phenol/chloroform (50% phenol previously equilibrated with 0.5 M Tris, pH 8.0; 50% chloroform). The aqueous phase was removed and extracted 2 to 5x with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by adding 1/10 volume 3M sodium acetate, pH 4.8, incubating the mixture on ice for 10 min, centrifuging the mixture at 15,000 rpm at 5°C for 10 min, and removing the supernatant to a clean tube to which 1 volume of isopropanol was added. The supernatant plus isopropanol mixture was then incubated on ice for 20 min, centrifuged at 15,000 rpm for 20 min at 5°C, the supernatant removed, and the DNA pellet washed 1x with 70% ethanol. After the pellet was dry, the DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

7.1.3. Plasmid DNA Isolation From *Streptomyces*

An aliquot (1.0 ml) of mycelia was placed in 1.5 ml microcentrifuge tubes and the cells concentrated by centrifugation at 12,000 x g for 60 sec. The supernatant was discarded, the cells were resuspended in 1.0 ml 10.3% sucrose and concentrated by centrifugation at 12,000 x g for 60 sec, and the supernatant discarded. The cells were then resuspended in 0.25 ml TSE buffer containing 2 mg/ml lysozyme, and incubated at 37°C for 20 min with shaking and loaded into the AutoGen 540™ automated nucleic acid isolation instrument. Plasmid DNA was isolated using Cycle 106 (equipment software) according to manufacturer's instructions.

Alternatively, 1.5 ml of mycelia were placed in 1.5 ml microcentrifuge tubes and the cells concentrated by centrifugation at 12,000 x g for 60 sec. The supernatant was discarded, the cells were resuspended in 1.0 ml 10.3% sucrose and concentrated by centrifugation at 12,000 x g for 60 sec, and the supernatant discarded. The cells were resuspended in 0.5 ml TSE buffer containing 2 mg/ml lysozyme, and incubated at 37°C for 15-30 min. After incubation, 0.25 ml alkaline SDS (0.3N NaOH, 2% SDS) was added and the cells incubated at 55°C for 15-30 min or until the solution was clear. Sodium acetate (0.1 ml, 3M, pH 4.8) was added to the DNA solution, which was then incubated on ice for 10 min. The DNA samples were centrifuged at 14,000 rpm for 10 min at 5°C. The supernatant was removed to a clean tube, and 0.2 ml phenol:chloroform (50% phenol:50% chloroform) was added and gently mixed. The DNA solution was centrifuged at 14,000 rpm for 10 min at 5°C and the upper layer removed to a clean Eppendorf tube. Isopropanol (0.75 ml) was added, and the solution was gently mixed and then incubated at rm temp for 20 min. The DNA solution was centrifuged at 14,000 rpm for 15 min at 5°C, the supernatant removed, and the DNA pellet was washed with 70% ethanol, dried, and resuspended in TE buffer.

7.1.4. Plasmid DNA Isolation From *E. coli*

A single transformed *E. coli* colony was inoculated into 5 ml Luria-Bertani (LB) medium (Bacto-Tryptone - 10 g, Bacto-yeast extract - 5 g, and NaCl - 10 g in 1 liter dH_2O, pH 7.0, autoclaved at 121°C for 25 min, and supplemented with 100 µg/ml ampicillin). The culture was incubated overnight, and a 1 ml aliquot placed in a 1.5 ml microcentrifuge tube. The culture samples were loaded into the AutoGen 540™ automated nucleic acid isolation instrument and plasmid DNA was isolated using Cycle 3 (equipment software) according to manufacturer's instructions.

7.1.5. Preparation And Transformation Of *S. avermitilis* Protoplasts

Single colonies of *S. avermitilis* were isolated on 1/2 strength YPD-6. The mycelia were used to inoculate 10 ml of TSB medium in a 25 mm x 150 mm tube, which was then

incubated with shaking (300 rpm) at 28°C for 48 hrs. One ml of mycelia was used to inoculate 50 ml YEME medium. YEME medium contains per liter: Difco Yeast Extract - 3 g; Difco Bacto-peptone - 5 g; Difco Malt Extract - 3 g; Sucrose - 300 g. After autoclaving at 121°C for 25 min, the following were added: 2.5 M $MgCl_2 \cdot 6H_2O$ (separately autoclaved at 121°C for 25 min) - 2 ml; and glycine (20%) (filter-sterilized)- 25 ml.

The mycelia were grown at 30°C for 48-72 hrs and harvested by centrifugation in a 50 ml centrifuge tube (Falcon) at 3,000 rpm for 20 min. The supernatant was discarded and the mycelia were resuspended in P buffer, which contains: sucrose - 205 g; K_2SO_4 - 0.25 g; $MgCl_2 \cdot 6H_20$ - 2.02 g; H_2O - 600 ml; K_2PO_4 (0.5%) - 10 ml; trace element solution* - 20 ml; $CaCl_2 \cdot 2H_20$ (3.68%) - 100 ml; and MES buffer (1.0 M, pH 6.5) - 10 ml. (*Trace element solution contains per liter: $ZnCl_2$ - 40 mg; $FeCl_3 \cdot 6H_20$ - 200 mg; $CuCl_2 \cdot 2H_20$ - 10 mg; $MnCl_2 \cdot 4H_20$ - 10 mg; $Na_2B_4O_7 \cdot 10H_20$ - 10 mg; $(NH_4)_6 Mo_7O_{24} \cdot 4H_20$ - 10 mg). The pH was adjusted to 6.5, final volume was adjusted to 1 liter, and the medium was filtered hot through a 0.45 micron filter.

The mycelia were pelleted at 3,000 rpm for 20 min, the supernatant was discarded, and the mycelia were resuspended in 20 ml P buffer containing 2 mg/ml lysozyme. The mycelia were incubated at 35°C for 15 min with shaking, and checked microscopically to determine extent of protoplast formation. When protoplast formation was complete, the protoplasts were centrifuged at 8,000 rpm for 10 min. The supernatant was removed and the protoplasts were resuspended in 10 ml P buffer. The protoplasts were centrifuged at 8,000 rpm for 10 min, the supernatant was removed, the protoplasts were resuspended in 2 ml P buffer, and approximately 1 x 10^9 protoplasts were distributed to 2.0 ml cryogenic vials (Nalgene).

A vial containing 1 x 10^9 protoplasts was centrifuged at 8,000 rpm for 10 min, the supernatant was removed, and the protoplasts were resuspended in 0.1 ml P buffer. Two to 5 µg of transforming DNA were added to the protoplasts, immediately followed by the addition of 0.5 ml working T buffer. T buffer base contains: PEG-1000 (Sigma) - 25 g; sucrose - 2.5 g; H_2O - 83 ml. The pH was adjusted to 8.8 with 1 N NaOH (filter sterilized), and the T buffer base was filter-sterilized and stored at 4°C. Working T buffer, made the same day used, was T buffer base - 8.3 ml; K_2PO_4 (4 mM) - 1.0 ml; $CaCl_2 \cdot 2H_20$ (5 M) - 0.2 ml; and TES (1 M, pH 8) - 0.5 ml. Each component of the working T buffer was individually filter-sterilized.

Within 20 sec of adding T buffer to the protoplasts, 1.0 ml P buffer was also added and the protoplasts were centrifuged at 8,000 rpm for 10 min. The supernatant was discarded and the protoplasts were resuspended in 0.1 ml P buffer. The protoplasts were then plated on RM14 media, which contains: sucrose - 205 g; K_2SO_4 - 0.25 g; $MgCl_2 \cdot 6H_20$ - 10.12 g;

glucose - 10 g; Difco Casamino Acids - 0.1 g; Difco Yeast Extract - 5 g; Difco Oatmeal Agar - 3 g; Difco Bacto Agar - 22 g; dH_2O - 800 ml. The solution was autoclaved at 121°C for 25 min. After autoclaving, sterile stocks of the following were added: K_2PO_4 (0.5%) - 10 ml; $CaCl_2 \cdot 2H_20$ (5 M) - 5 ml; L-proline (20%) - 15 ml; MES buffer (1.0 M, pH 6.5) - 10 ml; trace element solution (same as above) - 2 ml; cycloheximide stock (25 mg/ml) - 40 ml; and 1N NaOH - 2 ml. Twenty-five ml of RM14 medium were aliquoted per plate, and plates dried for 24 hr before use.

The protoplasts were incubated in 95% humidity at 30°C for 20-24 hrs. To select thiostrepton resistant transformants, 1 ml of overlay buffer containing 125 µg per ml thiostrepton was spread evenly over the RM14 regeneration plates. Overlay buffer contains per 100 ml: sucrose - 10.3 g; trace element solution (same as above) - 0.2 ml; and MES (1 M, pH 6.5) - 1 ml. The protoplasts were incubated in 95% humidity at 30°C for 7-14 days until thiostrepton resistant ($Thio^r$) colonies were visible.

7.1.6. Transformation Of *Streptomyces lividans* Protoplasts

S. lividans TK64 (provided by the John Innes Institute, Norwich, U.K) was used for transformations in some cases. Methods and compositions for growing, protoplasting, and transforming *S. lividans* are described in Hopwood *et al.*, 1985, Genetic Manipulation of *Streptomyces*, A Laboratory Manual, John Innes Foundation, Norwich, U.K., and performed as described therein. Plasmid DNA was isolated from *S. lividans* transformants as described in Section 7.1.3, above.

7.1.7. Fermentation Analysis Of *S. avermitilis* Strains

S. avermitilis mycelia grown on 1/2 strength YPD-6 for 4-7 days were inoculated into 1 x 6 inch tubes containing 8 ml of preform medium and two 5 mm glass beads. Preform medium contains: soluble starch (either thin boiled starch or KOSO, Japan Corn Starch Co., Nagoya) - 20 g/L; Pharmamedia - 15 g/L; Ardamine pH - 5 g/L (Champlain Ind., Clifton, NJ); $CaCO_3$ - 2 g/L; 2x bcfa ("bcfa" refers to branched chain fatty acids) containing a final concentration in the medium of 50 ppm 2-(+/-)-methyl butyric acid, 60 ppm isobutyric acid, and 20 ppm isovaleric acid. The pH was adjusted to 7.2, and the medium was autoclaved at 121°C for 25 min.

The tube was shaken at a 17° angle at 215 rpm at 29°C for 3 days. A 2-ml aliquot of the seed culture was used to inoculate a 300 ml Erlenmeyer flask containing 25 ml of production medium which contains: starch (either thin boiled starch or KOSO) - 160 g/L; Nutrisoy (Archer Daniels Midland, Decatur, IL) - 10 g/L; Ardamine pH - 10 g/L; K_2HPO_4 - 2 g/L; $MgSO_4.4H_2O$ - 2 g/L; $FeSO_4.7H_2O$ - 0.02 g/L; $MnCl_2$ - 0.002 g/L; $ZnSO_4.7H_2O$ - 0.002 g/L; $CaCO_3$ - 14 g/L; 2x bcfa (as above); and cyclohexane carboxylic acid (CHC) (made up as a

20% solution at pH 7.0) - 800 ppm. The pH was adjusted to 6.9, and the medium was autoclaved at 121°C for 25 min. (As explained above, starter units other than CHC can be utilized instead (see, e.g., Table 1)).

After inoculation, the flask was incubated at 29°C for 12 days with shaking at 200 rpm. After incubation, a 2 ml sample was withdrawn from the flask, diluted with 8 ml of methanol, mixed, and the mixture centrifuged at 1,250 x g for 10 min to pellet debris. The supernatant was then assayed by HPLC using a Beckman Ultrasphere ODS column (25 cm x 4.6 mm ID) with a flow rate of 0.75 ml/min and detection by absorbance at 240 nm. The mobile phase was 86/8.9/5.1 methanol/water/ acetonitrile.

7.1.8. Isolation Of *S. avermitilis* PKS Genes

A cosmid library of *S. avermitilis* (ATCC 31272, SC-2) chromosomal DNA was prepared and hybridized with a ketosynthase (KS) probe made from a fragment of the *Saccharopolyspora erythraea* polyketide synthase (PKS) gene. A detailed description of the preparation of cosmid libraries can be found in Sambrook *et al.*, 1989, above. A detailed description of the preparation of *Streptomyces* chromosomal DNA libraries is presented in Hopwood *et al.*, 1985, above. Cosmid clones containing ketosynthase-hybridizing regions were identified by hybridization to a 2.7 Kb *Nde*I/*Eco*47III fragment from pEX26 (kindly supplied by Dr. P. Leadlay, Cambridge, UK). Approximately 5 ng of pEX26 were digested using *Nde*I and *Eco*47III. The reaction mixture was loaded on a 0.8% SeaPlaque GTG agarose gel (FMC BioProducts, Rockland, ME). The 2.7 Kb *Nde*I/*Eco*47III fragment was excised from the gel after electrophoresis and the DNA recovered from the gel using GELase™ from Epicentre Technologies using the Fast Protocol. The 2.7 Kb *Nde*I/*Eco*47III fragment was labeled with [α-^{32}P]dCTP (deoxycytidine 5'-triphosphate, tetra (triethylammonium) salt, [alpha-^{32}P]-) (NEN-Dupont, Boston, MA) using the BRL Nick Translation System (BRL Life Technologies, Inc., Gaithersburg, MD) following the supplier's instructions. A typical reaction was performed in 0.05 ml volume. After addition of 5 µl Stop buffer, the labeled DNA was separated from unincorporated nucleotides using a G-25 Sephadex Quick Spin™ Column (Boehringer Mannheim) following supplier's instructions.

Approximately 1,800 cosmid clones were screened by colony hybridization. Ten clones were identified that hybridized strongly to the *Sacc. erythraea* KS probe. *E. coli* colonies containing cosmid DNA were grown in LB liquid medium and cosmid DNA was isolated from each culture in the AutoGen 540™ automated nucleic acid isolation instrument using Cycle 3 (equipment software) according to manufacturer's instructions. Restriction endonuclease mapping and Southern blot hybridization analyses revealed that five of the clones contained overlapping chromosomal regions. An *S. avermitilis* genomic *Bam*HI

restriction map of the five cosmids (*i.e.*, pSE65, pSE66, pSE67, pSE68, pSE69) was constructed by analysis of overlapping cosmids and hybridizations (FIGURE 4).

7.1.9. Identification Of DNA That Modulates Avermectin B2:B1 Ratios And Identification Of An *aveC* ORF

The following methods were used to test subcloned fragments derived from the pSE66 cosmid clone for their ability to modulate avermectin B2:B1 ratios in AveC mutants. pSE66 (5 µg) was digested with *Sac*I and *Bam*HI. The reaction mixture was loaded on a 0.8% SeaPlaque™ GTG agarose gel (FMC BioProducts), a 2.9 Kb *Sac*I/*Bam*HI fragment was excised from the gel after electrophoresis, and the DNA was recovered from the gel using GELase™ (Epicentre Technologies) using the Fast Protocol. Approximately 5 µg of the shuttle vector pWHM3 (Vara *et al.*, 1989, J. Bacteriol. 171:5872-5881) was digested with *Sac*I and *Bam*HI. About 0.5 µg of the 2.9 Kb insert and 0.5 µg of digested pWHM3 were mixed together and incubated overnight with 1 unit of ligase (New England Biolabs, Inc., Beverly, MA) at 15°C, in a total volume of 20 µl, according to supplier's instructions. After incubation, 5 µl of the ligation mixture was incubated at 70°C for 10 min, cooled to rm temp, and used to transform competent *E. coli* DH5α cells (BRL) according to manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the 2.9 Kb *Sac*I/*Bam*HI insert was confirmed by restriction analysis. This plasmid was designated as pSE119.

Protoplasts of *S. avermitilis* strain 1100-SC38 (Pfizer in-house strain) were prepared and transformed with pSE119 as described in Section 7.1.5 above. Strain 1100-SC38 is a mutant that produces significantly more of the avermectin cyclohexyl-B2 form compared to avermectin cyclohexyl-B1 form when supplemented with cyclohexane carboxylic acid (B2:B1 of about 30:1). pSE119 used to transform *S. avermitilis* protoplasts was isolated from either *E. coli* strain GM2163 (obtained from Dr. B. J. Bachmann, Curator, *E. coli* Genetic Stock Center, Yale University), *E. coli* strain DM1 (BRL), or *S. lividans* strain TK64. Thiostrepton resistant transformants of strain 1100-SC38 were isolated and analyzed by HPLC analysis of fermentation products. Transformants of *S. avermitilis* strain 1100-SC38 containing pSE119 produced an altered ratio of avermectin cyclohexyl-B2:cyclohexyl-B1 of about 3.7:1 (TABLE 2).

Having established that pSE119 was able to modulate avermectin B2:B1 ratios in an AveC mutant, the insert DNA was sequenced. Approximately 10 µg of pSE119 were isolated using a plasmid DNA isolation kit (Qiagen, Valencia, CA) following manufacturer's instructions, and sequenced using an ABI 373A Automated DNA Sequencer (Perkin Elmer, Foster City, CA). Sequence data was assembled and edited using Genetic Computer Group

programs (GCG, Madison, WI). The DNA sequence and the *aveC* ORF are presented in FIGURE 1 (SEQ ID NO:1).

A new plasmid, designated as pSE118, was constructed as follows. Approximately 5 μg of pSE66 was digested with *Sph*I and *Bam*HI. The reaction mixture was loaded on a 0.8% SeaPlaque GTG agarose gel (FMC BioProducts), a 2.8 Kb SphI/*Bam*HI fragment was excised from the gel after electrophoresis, and the DNA was recovered from the gel using GELase™ (Epicentre Technologies) using the Fast Protocol. Approximately 5 μg of the shuttle vector pWHM3 was digested with *Sph*I and *Bam*HI. About 0.5 μg of the 2.8 Kb insert and 0.5 μg of digested pWHM3 were mixed together and incubated overnight with 1 unit of ligase (New England Biolabs) at 15°C in a total volume of 20 μl according to supplier's instructions. After incubation, 5 μl of the ligation mixture was incubated at 70°C for 10 min, cooled to rm temp, and used to transform competent *E. coli* DH5α cells according to manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the 2.8 Kb *Sph*I/*Bam*HI insert was confirmed by restriction analysis. This plasmid was designated as pSE118. The insert DNA in pSE118 and pSE119 overlap by approximately 838 nucleotides (FIGURE 4).

Protoplasts of *S. avermitilis* strain 1100-SC38 were transformed with pSE118 as above. Thiostrepton resistant transformants of strain 1100-SC38 were isolated and analyzed by HPLC analysis of fermentation products. Transformants of *S. avermitilis* strain 1100-SC38 containing pSE118 were not altered in the ratios of avermectin cyclohexyl-B2: avermectin cyclohexyl-B1 compared to strain 1100-SC38 (TABLE 2).

7.1.10. PCR Amplification Of The *aveC* Gene From *S. avermitilis* Chromosomal DNA

A ~1.2 Kb fragment containing the *aveC* ORF was isolated from *S. avermitilis* chromosomal DNA by PCR amplification using primers designed on the basis of the *aveC* nucleotide sequence obtained above. The PCR primers were supplied by Genosys Biotechnologies, Inc. (Texas). The rightward primer was: 5'-TCACGAAACCGGACACAC-3' (SEQ ID NO:6); and the leftward primer was: 5'- CATGATCGCTGAACCGAG-3' (SEQ ID NO:7). The PCR reaction was carried out with Deep Vent™ polymerase (New England Biolabs) in buffer provided by the manufacturer, and in the presence of 300 μM dNTP, 10% glycerol, 200 pmol of each primer, 0.1 μg template, and 2.5 units enzyme in a final volume of 100 μl, using a Perkin-Elmer Cetus thermal cycler. The thermal profile of the first cycle was 95°C for 5 min (denaturation step), 60°C for 2 min (annealing step), and 72°C for 2 min (extension step). The subsequent 24 cycles had a similar thermal profile except that the denaturation step was shortened to 45 sec and the annealing step was shortened to 1 min.

The PCR product was electrophoresed in a 1% agarose gel and a single DNA band of ~1.2 Kb was detected. This DNA was purified from the gel, and ligated with 25 ng of linearized, blunt pCR-Blunt vector (Invitrogen) in a 1:10 molar vector-to-insert ratio following manufacturer's instructions. The ligation mixture was used to transform One Shot™ Competent *E. coli* cells (Invitrogen) following manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the ~1.2 Kb insert was confirmed by restriction analysis. This plasmid was designated as pSE179.

The insert DNA from pSE179 was isolated by digestion with *Bam*HI/*Xba*I, separated by electrophoresis, purified from the gel, and ligated with shuttle vector pWHM3, which had also been digested with *Bam*HI/*Xba*I, in a total DNA concentration of 1 µg in a 1:5 molar vector-to-insert ratio. The ligation mixture was used to transform competent *E. coli* DH5α cells according to manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the ~1.2 Kb insert was confirmed by restriction analysis. This plasmid, which was designated as pSE186 (FIGURE 2, ATCC 209604), was transformed into *E. coli* DM1, and plasmid DNA was isolated from ampicillin resistant transformants.

7.2. Results

A 2.9 Kb *Sac*I/*Bam*HI fragment from pSE119 was identified that, when transformed into *S. avermitilis* strain 1100-SC38, significantly altered the ratio of B2:B1 avermectin production. *S. avermitilis* strain 1100-SC38 normally has a B2:B1 ratio of about 30:1, but when transformed with a vector comprising the 2.9 Kb *Sac*I/*Bam*HI fragment, the ratio of B2:B1 avermectin decreased to about 3.7:1. Post-fermentation analysis of transformant cultures verified the presence of the transforming DNA.

The 2.9 Kb pSE119 fragment was sequenced and a ~0.9 Kb ORF was identified (FIGURE 1) (SEQ ID NO:1), which encompasses a *Pst*I/*Sph*I fragment that had previously been mutated elsewhere to produce B2 products only (Ikeda *et al.*, 1995, above). A comparison of this ORF, or its corresponding deduced polypeptide, against known databases (GenEMBL, SWISS-PROT) did not show any strong homology with known DNA or protein sequences.

TABLE 2 presents the fermentation analysis of *S. avermitilis* strain 1100-SC38 transformed with various plasmids.

TABLE 2

S. avermitilis strain (transforming plasmid)	No. Transformants Tested	Avg. B2:B1 Ratio
1100-SC38 (none)	9	30.66
1100-SC38 (pWHM3)	21	31.3
1100-SC38 (pSE119)	12	3.7
1100-SC38 (pSE118)	12	30.4
1100-SC38 (pSE185)	14	27.9

8. EXAMPLE: CONSTRUCTION OF *S. AVERMITILIS* AveC MUTANTS

This example describes the construction of several different *S. avermitilis* AveC mutants using the compositions and methods described above. A general description of techniques for introducing mutations into a gene in *Streptomyces* is described by Kieser and Hopwood, 1991, Meth. Enzym. 204:430-458. A more detailed description is provided by Anzai *et al.*, 1988, J. Antibiot. XLI(2):226-233, and by Stutzman-Engwall *et al.*, 1992, J. Bacteriol. 174(1):144-154. These references are incorporated herein by reference in their entirety.

8.1. Inactivation Of The *S. avermitilis aveC* Gene

AveC mutants containing inactivated *aveC* genes were constructed using several methods, as detailed below.

In the first method, a 640 bp *Sph*I/*Pst*I fragment internal to the *aveC* gene in pSE119 (plasmid described in Section 7.1.9, above) was replaced with the *ermE* gene (for erythromycin resistance) from *Sacc. erythraea*. The *ermE* gene was isolated from pIJ4026 (provided by the John Innes Institute, Norwich, U.K.; see also Bibb *et al.*, 1985, *Gene* 41:357-368) by restriction enzyme digestion with *Bgl*II and *Eco*RI, followed by electrophoresis, and was purified from the gel. This ~1.7 Kb fragment was ligated into pGEM7Zf (Promega) which had been digested with *Bam*HI and *Eco*RI, and the ligation mixture transformed into competent *E. coli* DH5α cells following manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the ~1.7 Kb insert was confirmed by restriction analysis. This plasmid was designated as pSE27.

pSE118 (described in Section 7.1.9, above) was digested with *Sph*I and *Bam*HI, the digest electrophoresed, and the ~2.8 Kb *Sph*I/*Bam*HI insert purified from the gel. pSE119 was digested with *Pst*I and *Eco*RI, the digest electrophoresed, and the ~1.5 Kb *Pst*I/*Eco*RI insert

purified from the gel. Shuttle vector pWHM3 was digested with *Bam*HI and *Eco*RI. pSE27 was digested with *PstI* and *SphI*, the digest electrophoresed, and the ~1.7 Kb *PstI/SphI* insert purified from the gel. All four fragments (*i.e.*, ~2.8 Kb, ~1.5Kb, ~7.2Kb, ~1.7 Kb) were ligated together in a 4-way ligation. The ligation mixture was transformed into competent *E. coli* DH5α cells following manufacturer's instructions. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid was designated as pSE180 (FIGURE 3; ATCC 209605).

pSE180 was transformed into *S. lividans* TK64 and transformed colonies identified by resistance to thiostrepton and erythromycin. pSE180 was isolated from *S. lividans* and used to transform *S. avermitilis* protoplasts. Four thiostrepton resistant *S. avermitilis* transformants were identified, and protoplasts were prepared and plated under non-selective conditions on RM14 media. After the protoplasts had regenerated, single colonies were screened for the presence of erythromycin resistance and the absence of thiostrepton resistance, indicating chromosomal integration of the inactivated *aveC* gene and loss of the free replicon. One Erm^r $Thio^s$ transformant was identified and designated as strain SE180-11. Total chromosomal DNA was isolated from strain SE180-11, digested with restriction enzymes *Bam*HI, *Hind*III, *PstI*, or *SphI*, resolved by electrophoresis on a 0.8% agarose gel, transferred to nylon membranes, and hybridized to the *ermE* probe. These analyses showed that chromosomal integration of the *ermE* resistance gene, and concomitant deletion of the 640 bp *PstI/SphI* fragment had occurred by a double crossover event. HPLC analysis of fermentation products of strain SE180-11 showed that normal avermectins were no longer produced (FIGURE 5A).

In a second method for inactivating the *aveC* gene, the 1.7 Kb *ermE* gene was removed from the chromosome of *S. avermitilis* strain SE180-11, leaving a 640 bp *PstI/SphI* deletion in the *aveC* gene. A gene replacement plasmid was constructed as follows: pSE180 was partially digested with *XbaI* and an ~11.4 Kb fragment purified from the gel. The ~11.4 Kb band lacks the 1.7 Kb *ermE* resistance gene. The DNA was then ligated and transformed into *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the correct insert was confirmed by restriction analysis. This plasmid, which was designated as pSE184, was transformed into *E. coli* DM1, and plasmid DNA isolated from ampicillin resistant transformants. This plasmid was used to transform protoplasts of *S. avermitilis* strain SE180-11. Protoplasts were prepared from thiostrepton resistant transformants of strain SE180-11 and were plated as single colonies on RM14. After the protoplasts had regenerated, single colonies were screened for the absence of both erythromycin resistance and thiostrepton resistance, indicating chromosomal integration of the inactivated *aveC* gene and loss of the free replicon containing the *ermE* gene. One Erm^s $Thio^s$ transformant was identified and designated as SE184-1-13. Fermentation analysis of

SE184-1-13 showed that normal avermectins were not produced and that SE184-1-13 had the same fermentation profile as SE180-11.

In a third method for inactivating the *aveC* gene, a frameshift was introduced into the chromosomal *aveC* gene by adding two G's after the C at nt position 471 using PCR, thereby creating a *Bsp*E1 site. The presence of the engineered *Bsp*E1 site was useful in detecting the gene replacement event. The PCR primers were designed to introduce a frameshift mutation into the *aveC* gene, and were supplied by Genosys Biotechnologies, Inc. The rightward primer was: 5'-GGTTCCGGATGCCGTTCTCG-3' (SEQ ID NO:8) and the leftward primer was: 5'-AACTCCGGTCGACTCCCCTTC-3' (SEQ ID NO:9). The PCR conditions were as described in Section 7.1.10 above. The 666 bp PCR product was digested with *Sph*I to give two fragments of 278 bp and 388 bp, respectively. The 388 bp fragment was purified from the gel.

The gene replacement plasmid was constructed as follows: shuttle vector pWHM3 was digested with *Eco*RI and *Bam*HI. pSE119 was digested with *Bam*HI and *Sph*I, the digest electrophoresed, and a ~840 bp fragment was purified from the gel. pSE119 was digested with *Eco*RI and *Xmn*I, the digest was resolved by electrophoresis, and a ~1.7 Kb fragment was purified from the gel. All four fragments (*i.e.*, ~7.2 Kb, ~840 bp, ~1.7 Kb, and 388 bp) were ligated together in a 4-way ligation. The ligation mixture was transformed into competent *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the correct insert was confirmed by restriction analysis and DNA sequence analysis. This plasmid, which was designated as pSE185, was transformed into *E. coli* DM1 and plasmid DNA isolated from ampicillin resistant transformants. This plasmid was used to transform protoplasts of *S. avermitilis* strain 1100-SC38. Thiostrepton resistant transformants of strain 1100-SC38 were isolated and analyzed by HPLC analysis of fermentation products. pSE185 did not significantly alter the B2:B1 avermectin ratios when transformed into *S. avermitilis* strain 1100-SC38 (TABLE 2).

pSE185 was used to transform protoplasts of *S. avermitilis* to generate a frameshift mutation in the chromosomal *aveC* gene. Protoplasts were prepared from thiostrepton resistant transformants and plated as single colonies on RM14. After the protoplasts had regenerated, single colonies were screened for the absence of thiostrepton resistance. Chromosomal DNA from thiostrepton sensitive colonies was isolated and screened by PCR for the presence of the frameshift mutation integrated into the chromosome. The PCR primers were designed based on the *aveC* nucleotide sequence, and were supplied by Genosys Biotechnologies, Inc. (Texas). The rightward PCR primer was: 5'-GCAAGGATACGGGGACTAC-3' (SEQ ID NO:10) and the leftward PCR primer was: 5'-GAACCGACCGCCTGATAC-3' (SEQ ID NO:11), and the PCR conditions were as described

in Section 7.1.10 above. The PCR product obtained was 543 bp and, when digested with *Bsp*E1, three fragments of 368 bp, 96 bp, and 79 bp were observed, indicating chromosomal integration of the inactivated *aveC* gene and loss of the free replicon.

Fermentation analysis of *S. avermitilis* mutants containing the frameshift mutation in the *aveC* gene showed that normal avermectins were no longer produced, and that these mutants had the same fermentation HPLC profile as strains SE180-11 and SE184-1-13. One Thio^s transformant was identified and designated as strain SE185-5a.

Additionally, a mutation in the *aveC* gene that changes nt position 520 from G to A, which results in changing the codon encoding a tryptophan (W) at position 116 to a termination codon, was produced. An *S. avermitilis* strain with this mutation did not produce normal avermectins and had the same fermentation profile as strains SE180-11, SE184-1-13, and SE185-5a.

Additionally, mutations in the *aveC* gene that change both: (i) nt position 970 from G to A, which changes the amino acid at position 266 from a glycine (G) to an aspartate (D), and (ii) nt position 996 from T to C, which changes the amino acid at position 275 from tyrosine (Y) to histidine (H), were produced. An *S. avermitilis* strain with these mutations (G266D/Y275H) did not produce normal avermectins and had the same fermentation profile as strains SE180-11, SE184-1-13, and SE185-5a.

The *S. avermitilis aveC* inactivation mutant strains SE180-11, SE184-1-13, SE185-5a, and others provided herewith, provide screening tools to assess the impact of other mutations in the *aveC* gene. pSE186, which contains a wild-type copy of the *aveC* gene, was transformed into *E. coli* DM1, and plasmid DNA was isolated from ampicillin resistant transformants. This pSE186 DNA was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by HPLC analysis of fermentation products. The presence of the functional *aveC* gene *in trans* was able to restore normal avermectin production to strain SE180-11 (FIGURE 5B).

8.2. Analysis Of Mutations In The *aveC* Gene That Alter Class B2:B1 Ratios

As described above, *S. avermitilis* strain SE180-11 containing an inactive *aveC* gene was complemented by transformation with a plasmid containing a functional *aveC* gene (pSE186). Strain SE180-11 was also utilized as a host strain to characterize other mutations in the *aveC* gene, as described below.

Chromosomal DNA was isolated from strain 1100-SC38, and used as a template for PCR amplification of the *aveC* gene. The 1.2 Kb ORF was isolated by PCR amplification

using primers designed on the basis of the *aveC* nucleotide sequence. The rightward primer was SEQ ID NO:6 and the leftward primer was SEQ ID NO:7 (see Section 7.1.10, above). The PCR and subcloning conditions were as described in Section 7.1.10. DNA sequence analysis of the 1.2 Kb ORF shows a mutation in the *aveC* gene that changes nt position 337 from C to T, which changes the amino acid at position 55 from serine (S) to phenylalanine (F). The *aveC* gene containing the S55F mutation was subcloned into pWHM3 to produce a plasmid which was designated as pSE187, and which was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by HPLC analysis of fermentation products. The presence of the *aveC* gene encoding a change at amino acid residue 55 (S55F) was able to restore normal avermectin production to strain SE180-11 (FIGURE 5C); however, the cyclohexyl B2:cyclohexyl B1 ratio was about 26:1, as compared to strain SE180-11 transformed with pSE186, which had a ratio of B2:B1 of about 1.6:1 (TABLE 3), indicating that the single mutation (S55F) modulates the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1.

Another mutation in the *aveC* gene was identified that changes nt position 862 from G to A, which changes the amino acid at position 230 from glycine (G) to aspartate (D). An *S. avermitilis* strain having this mutation (G230D) produces avermectins at a B2:B1 ratio of about 30:1.

8.3. Mutations That Reduce The B2:B1 Ratio

Several mutations were constructed that reduce the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1, as follows.

A mutation in the *aveC* gene was identified that changes nt position 588 from G to A, which changes the amino acid at position 139 from alanine (A) to threonine (T). The *aveC* gene containing the A139T mutation was subcloned into pWHM3 to produce a plasmid which was designated pSE188, and which was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by HPLC analysis of fermentation products. The presence of the mutated *aveC* gene encoding a change at amino acid residue 139 (A139T) was able to restore avermectin production to strain SE180-11 (FIGURE 5D); however, the B2:B1 ratio was about 0.94:1, indicating that this mutation reduces the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1. This result was unexpected because published results, as well as the results of mutations described above, have only demonstrated either inactivation of the *aveC* gene or increased production of the B2 form of avermectin relative to the B1 form (TABLE 3).

Because the A139T mutation altered the B2:B1 ratios in the more favorable B1 direction, a mutation was constructed that encoded a threonine instead of a serine at amino acid position 138. Thus, pSE186 was digested with *Eco*RI and cloned into pGEM3Zf (Promega) which had been digested with *Eco*RI. This plasmid, which was designated as pSE186a, was digested with *Apa*I and *Kpn*I, the DNA fragments separated on an agarose gel, and two fragments of ~3.8 Kb and ~0.4 Kb were purified from the gel. The ~1.2 Kb insert DNA from pSE186 was used as a PCR template to introduce a single base change at nt position 585. The PCR primers were designed to introduce a mutation at nt position 585, and were supplied by Genosys Biotechnologies, Inc. (Texas). The rightward PCR primer was: 5'-GGGGGCGGGCCCGGGTGCGGAGGCGGAAATGCCCCTGGCGACG-3' (SEQ ID NO:12); and the leftward PCR primer was: 5'-GGAACCGACCGCCTGATACA-3' (SEQ ID NO:13). The PCR reaction was carried out using an Advantage GC genomic PCR kit (Clonetech Laboratories, Palo Alto, CA) in buffer provided by the manufacturer in the presence of 200 μM dNTPs, 200 pmol of each primer, 50 ng template DNA, 1.0 M GC-Melt and 1 unit KlenTaq Polymerase Mix in a final volume of 50 μl. The thermal profile of the first cycle was 94°C for 1 min; followed by 25 cycles of 94°C for 30 sec and 68°C for 2 min; and 1 cycle at 68°C for 3 min. A PCR product of 295 bp was digested with *Apa*I and *Kpn*I to release a 254 bp fragment, which was resolved by electrophoresis and purified from the gel. All three fragments (~3.8 Kb, ~0.4 Kb and 254 bp) were ligated together in a 3-way ligation. The ligation mixture was transformed into competent *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid was designated as pSE198.

pSE198 was digested with *Eco*RI, cloned into pWHM3, which had been digested with *Eco*RI, and transformed into *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the correct insert was confirmed by restriction analysis and DNA sequence analysis. This plasmid DNA was transformed into *E. coli* DM1, plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid, which was designated as pSE199, was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by HPLC analysis of fermentation products. The presence of the mutated *aveC* gene encoding a change at amino acid residue 138 (S138T) was able to restore normal avermectin production to strain SE180-11; however, the B2:B1 ratio was 0.88:1 indicating that this mutation reduces the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1 (TABLE 3). This B2:B1 ratio is even lower

than the 0.94:1 ratio observed with the A139T mutation produced by transformation of strain SE180-11 with pSE188, as described above.

Another mutation was constructed to introduce a threonine at both amino acid positions 138 and 139. The ~1.2 Kb insert DNA from pSE186 was used as a PCR template. The PCR primers were designed to introduce mutations at nt positions 585 and 588, and were supplied by Genosys Biotechnologies, Inc. (Texas). The rightward PCR primer was: 5'-GGGGGCGGGCCCGGGTGCGGAGGCGGAAATGCCGCTGGCGACGACC-3' (SEQ ID NO:14); and the leftward PCR primer was: 5'-GGAACATCACGGCATTCACC-3' (SEQ ID NO:15). The PCR reaction was performed using the conditions described immediately above in this Section. A PCR product of 449 bp was digested with *Apa*I and *Kpn*I to release a 254 bp fragment, which was resolved by electrophoresis and purified from the gel. pSE186a was digested with *Apa*I and *Kpn*I, the DNA fragments separated on an agarose gel, and two fragments of ~3.8 Kb and ~0.4 Kb were purified from the gel. All three fragments (~3.8 Kb, ~0.4 Kb and 254 bp) were ligated together in a 3-way ligation, and the ligation mixture was transformed into competent *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid was designated as pSE230.

pSE230 was digested with *Eco*RI, cloned into pWHM3, which had been digested with *Eco*RI, and transformed into *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the correct insert was confirmed by restriction analysis and DNA sequence analysis. This plasmid DNA was transformed into *E. coli* DM1, plasmid DNA isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid, which was designated as pSE231, was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by fermentation. The presence of the double mutated *aveC* gene, encoding S138T/A139T, was able to restore normal avermectin production to strain SE180-11; however, the B2:B1 ratio was 0.84:1 showing that this mutation further reduces the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1 (TABLE 3), over the reductions provided by transformation of strain SE180-11 with pSE188 or pSE199, as described above.

Another mutation was constructed to further reduce the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1. Because the S138T/A139T mutations altered the B2:B1 ratios in the more favorable B1 direction, a mutation was constructed to introduce a threonine at amino acid position 138 and a phenylalanine at amino acid position 139. The ~1.2 Kb insert DNA from pSE186 was used as a PCR template. The PCR primers were designed to

introduce mutations at nt positions 585 (changing a T to A), 588 (changing a G to T), and 589 (changing a C to T), and were supplied by Genosys Biotechnologies, Inc. (Texas). The rightward PCR primer was: 5'-GGGGGCGGGCCCGGGTGCGGAGGCGGAAATGCCGC TGGCGACGTTC-3' (SEQ ID NO:16); and the leftward PCR primer was: 5'-GGAACATCACGGCATTCACC-3' (SEQ ID NO:15). The PCR reaction was carried out using an Advantage GC genomic PCR kit (Clonetech Laboratories, Palo Alto, CA) in buffer provided by the manufacturer in the presence of 200 μM dNTPs, 200 pmol of each primer, 50 ng template DNA, 1.1 mM Mg acetate, 1.0 M GC-Melt and 1 unit Tth DNA Polymerase in a final volume of 50 μl. The thermal profile of the first cycle was 94°C for 1 min; followed by 25 cycles of 94°C for 30 sec and 68°C for 2 min; and 1 cycle at 68°C for 3 min. A PCR product of 449 bp was digested with *Apa*I and *Kpn*I to release a 254 bp fragment, which was resolved by electrophoresis and purified from the gel. All three fragments (~3.8 Kb, ~0.4 Kb and 254 bp) were ligated together in a 3-way ligation. The ligation mixture was transformed into competent *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid was designated as pSE238.

pSE238 was digested with *Eco*RI, cloned into pWHM3, which had been digested with *Eco*RI, and transformed into *E. coli* DH5α cells. Plasmid DNA was isolated from ampicillin resistant transformants and the presence of the correct insert was confirmed by restriction analysis and DNA sequence analysis. This plasmid DNA was transformed into *E. coli* DM1, plasmid DNA was isolated from ampicillin resistant transformants, and the presence of the correct insert was confirmed by restriction analysis. This plasmid, which was designated as pSE239, was used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated, the presence of erythromycin resistance was determined, and Thio^r Erm^r transformants were analyzed by HPLC analysis of fermentation products. The presence of the double mutated *aveC* gene encoding S138T/A139F was able to restore normal avermectin production to strain SE180-11; however, the B2:B1 ratio was 0.75:1 showing that this mutation further reduced the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1 (TABLE 3) over the reductions provided by transformation of strain SE180-11 with pSE188, pSE199, or pSE231 as described above.

TABLE 3

S. avermitilis strain (transforming plasmid)	No. Transformants Tested	Relative B2 Conc.	Relative B1 Conc.	Avg. B2:B1 Ratio
SE180-11 (none)	30	0	0	0
SE180-11 (pWHM3)	30	0	0	0
SE180-11 (pSE186)	26	222	140	1.59
SE180-11 (pSE187)	12	283	11	26.3
SE180-11 (pSE188)	24	193	206	0.94
SE180-11 (pSE199)	18	155	171	0.88
SE180-11 (pSE231)	6	259	309	0.84
SE180-11 (pSE239)	20	184	242	0.75

Additional mutations were constructed to further reduce the amount of cyclohexyl-B2 produced relative to cyclohexyl-B1 using the technique of DNA shuffling as described in Stemmer, 1994, Nature 370:389-391; and Stemmer, 1994, Proc. Natl. Acad. Sci. USA 91:10747-10751; and further described in United States Patent Nos. 5,605,793; 5,811,238; 5,830,721; and 5,837,458.

DNA shuffled plasmids containing mutated *aveC* genes were transformed into competent *dam dcm E. coli* cells. Plasmid DNA was isolated from ampicillin resistant transformants, and used to transform protoplasts of *S. avermitilis* strain SE180-11. Thiostrepton resistant transformants of strain SE180-11 were isolated and screened for the production of avermectins with a cyclohexyl-B2:cyclohexyl-B1 ratio of 1:1 or less. The DNA sequence of plasmid DNA from SE180-11 transformants producing avermectins with a B2:B1 ratio of 1:1 or less was determined.

Eight transformants were identified that produced reduced amounts of cyclohexyl-B2 relative to cyclohexyl-B1. The lowest B2:B1 ratio achieved among these transformants was 040:1 (TABLE 4). Plasmid DNA was isolated from each of the eight transformants and the DNA sequence determined to identify the mutations in the *aveC* gene. The mutations are as follows.

pSE290 contains 4 nucleotide mutations at nt position 317 from T to A, at nt position 353 from C to A, at nt position 438 from G to A, and at nt position 1155 from T to A. The nucleotide change at nt position 317 changes the amino acid at AA position 48 from D to E and the nucleotide change at nt position 438 changes the amino acid at AA position 89 from A to T. The B2:B1 ratio produced by cells carrying this plasmid was 0.42:1 (TABLE 4).

pSE291 contains 4 nucleotide mutations at nt position 272 from G to A, at nt position 585 from T to A, at nt position 588 from G to A, and at nt position 708 from G to A. The nucleotide change at nt position 585 changes the amino acid at AA position 138 from S to T, the nucleotide change at nt position 588 changes the amino acid at AA position 139 from A to T, and the nucleotide change at nt position 708 changes the amino acid at AA position 179 from G to S. The B2:B1 ratio produced by cells carrying this plasmid was 0.57:1 (TABLE 4).

pSE292 contains the same four nucleotide mutations as pSE290. The B2:B1 ratio produced by cells carrying this plasmid was 0.40:1 (TABLE 4).

pSE293 contains 6 nucleotide mutations at nt 24 from A to G, at nt position 286 from A to C, at nt position 497 from T to C, at nt position 554 from C to T, at nt position 580 from T to C, and at nt position 886 from A to T. The nucleotide change at nt position 286 changes the amino acid at AA position 38 from Q to P, the nucleotide change at nt position 580 changes the amino acid at AA position 136 from L to P, and the nucleotide change at nt position 886 changes the amino acid at AA position 238 from E to D. The B2:B1 ratio produced by cells carrying this plasmid was 0.68:1 (TABLE 4).

pSE294 contains 6 nucleotide mutations at nt 469 from T to C, at nt position 585 from T to A, at nt position 588 from G to A, at nt position 708 from G to A, at nt position 833 from C to T, and at nt position 1184 from G to A. In addition, nts at positions 173, 174, and 175 are deleted. The nucleotide change at nt position 469 changes the amino acid at AA position 99 from F to S, the nucleotide change at nt position 585 changes the amino acid at AA position 138 from S to T, the nucleotide change at nt position 588 changes the amino acid at AA position 139 from A to T, and the nucleotide change at nt position 708 changes the amino acid from AA position 179 from G to S. The B2:B1 ratio produced by cells carrying this plasmid was 0.53:1 (TABLE 4).

pSE295 contains 2 nucleotide mutations at nt 588 from G to A and at nt 856 from T to C. The nucleotide change at nt position 588 changes the amino acid at AA position 139 from A to T and the nucleotide change at nt position 856 changes the amino acid at AA position 228 from M to T. The B2:B1 ratio produced by cells carrying this plasmid was 0.80:1 (TABLE 4).

pSE296 contains 5 nucleotide mutations at nt position 155 from T to C, at nt position 505 from G to T, at nt position 1039 from C to T, at nt position 1202 from C to T, and at nt position 1210 from T to C. The nucleotide change at nt position 505 changes the amino acid at AA position 111 from G to V and the nucleotide change at nt position 1039 changes the amino acid at AA position 289 from P to L. The B2:B1 ratio produced by cells carrying this plasmid was 0.73:1 (TABLE 4).

pSE297 contains 4 nucleotide mutations at nt position 377 from G to T, at nt position 588 from G to A, at nt position 633 from A to G, and at nt position 1067 from A to T. The

nucleotide change at nt position 588 changes the amino acid at AA position 139 from A to T, the nucleotide change at nt position 633 changes the amino acid at AA position 154 from K to E, and the nucleotide change at nt position 1067 changes the amino acid at AA position 298 from Q to H. The B2:B1 ratio produced by cells carrying this plasmid was 0.67:1 (TABLE 4).

TABLE 4

S. avermitilis strain (transforming plasmid)	No. Transformants Tested	Relative B2 Conc.	Relative B1 Conc.	Avg. B2:B1 Ratio
SE180-11 (none)	4	0	0	0
SE180-11 (pWHM3)	4	0	0	0
SE180-11 (pSE290)	4	87	208	0.42
SE180-11 (pSE291)	4	106	185	0.57
SE180-11 (pSE292)	4	91	231	0.40
SE180-11 (pSE293)	4	123	180	0.68
SE180-11 (pSE294)	4	68	129	0.53
SE180-11 (pSE295)	4	217	271	0.80
SE180-11 (pSE296)	1	135	186	0.73
SE180-11 (pSE297)	1	148	221	0.67

An additional round of DNA shuffling was conducted to further reduce the amount of cyclohexyl-B2 avermectin produced relative to cyclohexyl-B1 avermectin as follows.

Semi-synthetic shuffling

The best clone was shuffled using the method described in PCT International Publication WO 97/20078 by Maxygen Inc. Individual oligonucleotides encoding beneficial substitutions best corresponding to decreased B2:B1 ratio were added to the shuffling reaction at 5 molar excess over the *aveC* template strands. Each nucleotide mismatch of the oligonucleotide was flanked by 20 nucleotides of perfect identity to ensure proper incorporation during the shuffling reaction. Oligonucleotides were purchased from Operon Technologies (Alameda, CA).

HTP growth of S. avermitilis

Independent clones were picked from the transformation plates and inoculated into 200 µl R5 medium (Kieser, T., *et al.*, "Practical Streptomyces Genetics", 2000, Norwich, U.K.,

John Innes Foundation) in deep 96-well seed plates and grown at 30°C with shaking. In each well, a glass-bead was dispensed for dispersion of mycelia and agitation of the culture. During this time, the cultures attained even and dense growth. After 4-5 days, 20 µl of the seed medium culture was dispensed to production plates and the remaining volume was frozen as master plates at –80°C after the addition of glycerol to the final concentration of 20%. The production plates were incubated at 30° under humidity for 12-14 days. Sporulation of the strains occurred after 5-8 days of incubation. The production plates were made essentially as described in PCT International Publication WO 99/41389 by Pfizer Inc., with the exception of adding 1% agarose to ensure a solid surface.

Extraction and ESI-MS/MS screening

A total of 1 ml ethyl acetate was added to each well and incubated shaking at room temperature for 20 minutes. Approximately 750 µl of the ethyl acetate-phase was recovered, transferred to a 96-well plate and set to evaporate over night. The precipitate was resuspended in 100 µl methanol 1mM NaCl of which 5 µl solution was injected into mass spectrometer by an autosampler in a 96-well format and analyzed directly in the flow injection phase without liquid chromatography or other separation. The compounds were ionized by electrospray ionization and two separate channels were monitored on two MS/MS transitions. The MS/MS transition for B1 sodiated ion is from m/z 921 to m/z 777 and for B2 sodiated ion is from m/z 939 to m/z 795 in positive mode. A Finnigan TSQ-7000, Micromass Quattro-LC mass spectrometer and a Leap Technology Twin-Pal autosampler were used for this high throughput screening. Integration of the separate B1 and B2 chromatograms for each well location identified the hits.

Fifty-seven (57) new combinations of amino acid substitutions were identified that can produce ratios of cyclohexyl B2:cyclohexyl B1 avermectins of 0.35:1 or less (FIGURE 6). Several of these new mutations can produce ratios of cyclohexyl B2:cyclohexyl B1 avermectins of about 0.30:1 or less; several can produce ratios of cyclohexyl B2:cyclohexyl B1 avermectins of about 0.25:1 or less, and several can produce ratios of cyclohexyl B2:cyclohexyl B1 avermectins of about 0.20:1 or less. Two (2) new mutations were identified that can produce ratios of cyclohexyl B2:cyclohexyl B1 avermectins of 0.37 or 0.38.

DEPOSIT OF BIOLOGICAL MATERIALS

The following plasmids were deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, MD, 20852, USA, on January 29, 1998, and were assigned the following accession numbers:

Plasmid	Accession No.
plasmid pSE180	209605
plasmid pSE186	209604

The current address of the American Type Culture Collection is 10801 University Blvd, Manassas, VA, 20110, USA.

All patents, patent applications, and publications cited above are incorporated herein by reference in their entirety.

The present invention is not to be limited in scope by the specific embodiment described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

18 JUL 2005

WHAT IS CLAIMED IS :

1. A polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the Streptomyces avermitilis aveC allele, the S. avermitilis AveC gene product- encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the aveC ORF of S. avermitilis as presented in FIGURE 1 (SEQ ID NO : 1), or a degenerate variant thereof, which includes one or more silent changes to the nucleotide sequence according to the degeneracy of the genetic code; but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO : 2, such that cells of S. avermitilis strain ATCC 53692 in which the wild-type aveC allele has been inactivated and that express the polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2: 1 ratio of avermectins that is reduced compared to the ratio produced by cells of S. avermitilis strain ATCC 53692 that instead express only the wild-type aveC allele, wherein when the class 2: 1 avermectins are cyclohexyl B2: cyclohexyl B1 avermectins the ratio of class 2: 1 avermectins is 0.35 : 1 or less.

2. The polynucleotide molecule of claim 1, wherein the ratio of cyclohexyl B2: cyclohexyl B1 avermectins is about 0.20 : 1 or less.

3. The polynucleotide molecule of claim 1, wherein the combination of amino acid substitutions comprises a combination selected from the group consisting of:

(a) D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L;
(b) G40S, D48E, L136P, G179S, E238D;
(c) D48E, L136P, R163Q, G179S;
(d) D48E, L136P, R163Q, G179S, E238D;
(e) D48E, L136P, R163Q, G179S, A200G, E238D;
(f) D48E, L136P, G179S, E238D;
(g) D48E, A61T, L136P, G179S, E238D;
(h) D48E, A61T, L136P, G179S;
(i) D48E, A89T, S138T, A139T, G179S ;
(j) D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L;
(k) D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L;
(l) D48E, L136P, G179S, A198G, E238D, P289L;
(m) D48E, A61T, S138T, A139F, G179S, A198G, P289L;
(n) D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L;
(o) Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D;
(p) D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L;
(q) D48E, L136P, G179S, R250W;

(r) D48E, A89T, S138T, A139T, R163Q, G179S;
(s) D48E, L136P, G179S, A198G, P289L;
(t) D48E, F78L, A89T, L136P, G179S;
(u) D48E, A89T, S138T, A139T, G179S, E238D, F278L;
(v) D48E, A89T, L136P, R163Q, G179S;
(w) D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L;
(x) D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S;
(y) D48E, A89T, S90G, L136P, R163Q, G179S, E238D;
(z) D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L;
(aa) D48E, A89T, S138T, A139T, G179S;
(ab) D48E, L136P, R163Q, G179S, S231L;
(ac) D48E, L136P, S138T, A139F, G179S, V196A, E238D;
(ad) D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D;
(ae) G35S, D48E, A89T, S138T, A139T, G179S, P289L;
(af) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D;
(ag) D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D;
(ah) S41G, D48E, A89T, L136P, G179S;
(ai) D48E, A89T, L136P, R163Q, G179S, P252S;
(aj) D48E, A89T, L136P, G179S, F234S;
(ak) D48E, A89T, L136P, R163Q, G179S, E238D;
(al) Q36R, D48E, A89T, L136P, G179S, E238D;
(am) D48E, A89T, L136P, R163Q, G179S;
(an) D48E, A89T, S138T, G179S;
(ao) D48E, A89T, L136P, G179S, E238D;
(ap) D48E, A89T, L136P, K154E, G179S, E238D;
(aq) D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L;
(ar) D48E, A89T, S138T, A139F, G179S, V196A, E238D;
(as) D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L;
(at) D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L;
(au) D48E, A89T, L136P, G179S;
(av) D48E, A89T, V120A, L136P, G179S;
(aw) D48E, A61T, A89T, S138T, A139F,G179S, V196A, A198G, E238D;
(ax) D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D;
(ay) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L;
(az) D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D;
(ba) D48E, A89T, S138T, A139F, G179S, A198G, V220A;

(bb) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L;
(bc) D48E, A61T, A89T, L136P, G179S, P289L;
(bd) D48E, A89T, S138T, A139T, G179S, V196A, E238D, P289L; and
(be) D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D.

4. A polynucleotide molecule comprising a nucleotide sequence that is otherwise the same as the Streptomyces avermitilis aveC allele, the S. avermitilis AveC gene product- encoding sequence of plasmid pSE186 (ATCC 209604) or the nucleotide sequence of the aveC ORF of S. avermitilis as presented in FIGURE 1 (SEQ ID NO : 1), or a degenerate variant thereof, which includes one or more silent changes to the nucleotide sequence according to the degeneracy of the genetic code, but which nucleotide sequence further comprises mutations encoding a combination of amino acid substitutions at amino acid residues corresponding to the amino acid positions of SEQ ID NO : 2, such that cells of S. avermitilis strain ATCC 53692 in which the wild-type aveC allele has been inactivated and that express a polynucleotide molecule comprising the mutated nucleotide sequence are capable of producing a class 2: 1 ratio of avermectins that is reduced compared to the ratio produced by cells of S. avermitilis strain ATCC 53692 that instead express only the wild-type aveC allele, wherein when the class 2: 1 avermectins are cyclohexyl B2: cyclohexyl B1 avermectins, the ratio of class 2: 1 avermectins is reduced to about 0.40 : 1 or less, and wherein the combination of amino acid substitutions comprises a combination selected from the group consisting of: (bf) D48E, S138T, A139T, G179S, E238D; and (bg) Y28C, Q38R, D48E, L136P, G179S, E238D.

5. A host cell comprising the polynucleotide of claim 1 or claim 4.

6. A method for making a strain of Streptomyces avermitilis, comprising (i) mutating the aveC allele in a cell of a strain of S. avermitilis, which mutation results in a combination of amino acid substitutions in the AveC gene product or (ii) introducing into a cell of a strain of S. avermitilis a mutated aveC allele or variant thereof which includes one or more silent changes to the nucleotide sequence according to the degeneracy of the genetic code encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions in the AveC gene product comprises a combination selected from the group consisting of:

(a) D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L;
(b) G40S, D48E, L136P, G179S, E238D;
(c) D48E, L136P, R163Q, G179S;
(d) D48E, L136P, R163Q, G179S, E238D;
(e) D48E, L136P, R163Q, G179S, A200G, E238D;
(f) D48E, L136P, G179S, E238D;

(g) D48E, A61T, L136P, G179S, E238D;
(h) D48E, A61T, L136P, G179S;
(i) D48E, A89T, S138T, A139T, G179S;
(j) D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L;
(k) D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L;
(l) D48E, L136P, G179S, A198G, E238D, P289L;
(m) D48E, A61T, S138T, A139F, G179S, A198G, P289L;
(n) D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L;
(o) Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D;
(p) D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L;
(q) D48E, L136P, G179S, R250W;
(r) D48E, A89T, S138T, A139T, R163Q, G179S;
(s) D48E, L136P, G179S, A198G, P289L;
(t) D48E, F78L, A89T, L136P, G179S;
(u) D48E, A89T, S138T, A139T, G179S, E238D, F278L;
(v) D48E, A89T, L136P, R163Q, G179S;
(w) D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L;
(x) D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S;
(y) D48E, A89T, S90G, L136P, R163Q, G179S, E238D;
(z) D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L;
(aa) D48E, A89T, S138T, A139T, G179S;
(ab) D48E, L136P, R163Q, G179S, S231L;
(ac) D48E, L136P, S138T, A139F, G179S, V196A, E238D;
(ad) D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D;
(ae) G35S, D48E, A89T, S138T, A139T, G179S, P289L;
(af) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D;
(ag) D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D;
(ah) S41G, D48E, A89T, L136P, G179S;
(ai) D48E, A89T, L136P, R163Q, G179S, P252S;
(aj) D48E, A89T, L136P, G179S, F234S;
(ak) D48E, A89T, L136P, R163Q, G179S, E238D;
(al) Q36R, D48E, A89T, L136P, G179S, E238D;
(am) D48E, A89T, L136P, R163Q, G179S;
(an) D48E, A89T, S138T, G179S;
(ao) D48E, A89T, L136P, G179S, E238D;
(ap) D48E, A89T, L136P, K154E, G179S, E238D;

(aq) D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L;
(ar) D48E, A89T, S138T, A139F, G179S, V196A, E238D,
(as) D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L;
(at) D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L;
(au) D48E, A89T, L136P, G179S ;
(av) D48E, A89T, V120A, L136P, G179S;
(aw) D48E, A61T, A89T, S138T, A139F, G179S, V196A, A198G, E238D;
(ax) D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D;
(ay) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L;
(az) D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D;
(ba) D48E, A89T, S138T, A139F, G179S, A198G, V220A;
(bb) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L;
(bc) D48E, A61T, A89T, L136P, G179S, P289L;
(bd) D48E, A89T, S1. 38T, A139T, G179S, V196A, E238D, P289L; and
(be) D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D.

7. A method for making a strain of Streptomyces avermitilis, comprising (i) mutating the aveC allele in a cell of a strain of S. avermitilis, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of S. avermitilis a mutated aveC allele or variant thereof which includes one or more silent changes to the nucleotide sequence according to the degeneracy of the genetic code encoding an AveC gene product comprising a combination of amino acid substitutions, wherein cells comprising the mutated aveC allele or degenerate variant are capable of producing cyclohexyl B2: cyclohexyl B1 avermectins in a ratio of 0.35 : 1 or less.

8. The method of claim 7, wherein the combination of amino acid substitutions in the AveC gene product comprises a combination selected from the group consisting of:

(a) D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L;
(b) G40S, D48E, L136P, G179S, E238D;
(c) D48E, L136P, R163Q, G179S ;
(d) D48E, L136P, R163Q, G179S, E238D;
(e) D48E, L136P, R163Q, G179S, A200G, E238D;
(f) D48E, L136P, G179S, E238D;
(g) D48E, A61T, L136P, G179S, E238D;
(h) D48E, A61T, L136P, G179S ;
(i) D48E, A89T, S138T, A139T, G179S ;
(j) D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L;

(k) D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L;
(l) D48E, L136P, G179S, A198G, E238D, P289L;
(m) D48E, A61T, S138T, A139F, G179S, A198G, P289L;
(n) D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L;
(o) Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D;
(p) D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L;
(q) D48E, L136P, G179S, R250W;
(r) D48E, A89T, S138T, A139T, R163Q, G179S;
(s) D48E, L136P, G179S, A198G, P289L;
(t) D48E, F78L, A89T, L136P, G179S;
(u) D48E, A89T, S138T, A139T, G179S, E238D, F278L;
(v) D48E, A89T, L136P, R163Q, G179S;
(w) D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L;
(x) D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S;
(y) D48E, A89T, S90G, L136P, R163Q, G179S, E238D;
(z) D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L;
(aa) D48E, A89T, S138T, A139T, G179S;
(ab) D48E, L136P, R163Q, G179S, S231L;
(ac) D48E, L136P, S138T, A139F, G179S, V196A, E238D;
(ad) D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D;
(ae) G35S, D48E, A89T, S138T, A139T, G179S, P289L;
(af) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D;
(ag) D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D;
(ah) S41G, D48E, A89T, L136P, G179S;
(ai) D48E, A89T, L136P, R163Q, G179S, P252S;
(aj) D48E, A89T, L136P, G179S, F234S;
(ak) D48E, A89T, L136P, R163Q, G179S, E238D;
(al) Q36R, D48E, A89T, L136P, G179S, E238D;
(am) D48E, A89T, L136P, R163Q, G179S;
(an) D48E, A89T, S138T, G179S;
(ao) D48E, A89T, L136P, G179S, E238D;
(ap) D48E, A89T, L136P, K154E, G179S, E238D;
(aq) D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L;
(ar) D48E, A89T, S138T, A139F, G179S, V196A, E238D;
(as) D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L;
(at) D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L;

(au) D48E, A89T, L136P, G179S;
(av) D48E, A89T, V120A, L136P, G179S;
(aw) D48E, A61T, A89T, S138T, A139F,G179S, V196A, A198G, E238D;
(ax) D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D;
(ay) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L;
(az) D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D;
(ba) D48E, A89T, S138T, A139F, G179S, A198G, V220A;
(bb) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L;
(bc) D48E, A61T, A89T, L136P, G179S, P289L;
(bd) D48E, A89T, S138T, A139T, G179S, V196A, E238D, P289L; and
(be) D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D.

9. The method of claim 7, wherein the ratio of cyclohexyl B2:cyclohexyl B1 avermectins is about 0.20:1 or less.

10. A method for making a novel strain of *Streptomyces avermitilis*, comprising (i) mutating the *aveC* allele in a cell of a strain of *S. avermitilis*, which mutation results in a combination of amino acid substitutions in the AveC gene product, or (ii) introducing into a cell of a strain of *S. avermitilis* a mutated *aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions, wherein the combination of amino acid substitutions is selected from the group consisting of:

(bf) D48E, S138T, A139T, G179S, E238D; and
(bg) Y28C, Q38R, D48E, L136P, G179S, E238D.

11. A cell of a *Streptomyces* species that comprises a mutated *S. avermitilis aveC* allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of:

(a) D48E, A61T, A89T, S138T, A139T, G179S, A198G, P289L;
(b) G40S, D48E, L136P, G179S, E238D;
(c) D48E, L136P, R163Q, G179S;
(d) D48E, L136P, R163Q, G179S, E238D;
(e) D48E, L136P, R163Q, G179S, A200G, E238D;
(f) D48E, L136P, G179S, E238D;
(g) D48E, A61T, L136P, G179S, E238D;
(h) D48E, A61T, L136P, G179S;
(i) D48E, A89T, S138T, A139T, G179S;

(j) D48E, A61T, L136P, G179S, A198G, P202S, E238D, P289L;
(k) D48E, A61T, L136P, S138T, A139F, G179S, E238D, P289L;
(l) D48E, L136P, G179S, A198G, E238D, P289L;
(m) D48E, A61T, S138T, A139F, G179S, A198G, P289L;
(n) D48E, L84P, G111V, S138T, A139T, G179S, A198G, P289L;
(o) Y28C, D48E, A61T, A89T, S138T, A139T, G179S, E238D;
(p) D48E, A61T, A107T, S108G, L136P, G179S, S192A, E238D, P289L;
(q) D48E, L136P, G179S, R250W;
(r) D48E, A89T, S138T, A139T, R163Q, G179S;
(s) D48E, L136P, G179S, A198G, P289L;
(t) D48E, F78L, A89T, L136P, G179S;
(u) D48E, A89T, S138T, A139T, G179S, E238D, F278L;
(v) D48E, A89T, L136P, R163Q, G179S;
(w) D48E, A61T, A89T, G111V, S138T, A139F, G179S, E238D, P289L;
(x) D25G, D48E, A89T, L136P, S138T, A139T, V141A, I159T, R163Q, G179S;
(y) D48E, A89T, S90G, L136P, R163Q, G179S, E238D;
(z) D48E, A61T, A89T, G111V, S138T, A139T, G179S, E238D, P289L;
(aa) D48E, A89T, S138T, A139T, G179S;
(ab) D48E, L136P, R163Q, G179S, S231L;
(ac) D48E, L136P, S138T, A139F, G179S, V196A, E238D;
(ad) D48E, A61T, A89T, F99S, S138T, A139T, G179S, E238D;
(ae) G35S, D48E, A89T, S138T, A139T, G179S, P289L;
(af) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D;
(ag) D48E, A89T, G111V, S138T, A139T, G179S, A198G, E238D;
(ah) S41G, D48E, A89T, L136P, G179S;
(ai) D48E, A89T, L136P, R163Q, G179S, P252S;
(aj) D48E, A89T, L136P, G179S, F234S;
(ak) D48E, A89T, L136P, R163Q, G179S, E238D;
(al) Q36R, D48E, A89T, L136P, G179S, E238D;
(am) D48E, A89T, L136P, R163Q, G179S;
(an) D48E, A89T, S138T, G179S;
(ao) D48E, A89T, L136P, G179S, E238D;
(ap) D48E, A89T, L136P, K154E, G179S, E238D;
(aq) D48E, A89T, S138T, A139T, K154R, G179S, V196A, P289L;
(ar) D48E, A89T, S138T, A139F, G179S, V196A, E238D;
(as) D48E, A61T, A89T, L136P, G179S, V196A, A198G, P289L;

(at) D48E, A61T, S138T, A139F, G179S, G196A, E238D, P289L;
(au) D48E, A89T, L136P, G179S; (av) D48E, A89T, V120A, L136P, G179S;
(aw) D48E, A61T, A89T, S138T, A139F, G179S, V196A, A198G, E238D;
(ax) D48E, A61T, A89T, G111V, S138T, A139F, G179S, V196A, E238D;
(ay) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, P289L;
(az) D48E, A61T, A89T, L136P, S138T, A139F, G179S, A198G, E238D;
(ba) D48E, A89T, S138T, A139F, G179S, A198G, V220A;
(bb) D48E, A61T, A89T, S138T, A139T, G179S, V196A, E238D, R239H, P289L;
(bc) D48E, A61T, A89T, L136P, G179S, P289L;
(bd) D48E, A89T, S138T, A139T, G179S, V196A, E238D, P289L; and
(be) D48E, A61T, A89T, S138T, A139F, G179S, V196A, E238D.

12. The cell of claim 11, which is a cell of Streptomyces avermitilis.

13. A cell of a Streptomyces species, comprising a mutated S. avermitilis aveC allele or degenerate variant thereof encoding an AveC gene product comprising a combination of amino acid substitutions selected from the group consisting of

(bf) D48E, S138T, A139T, G179S, E238D; and
(bg) Y28C, Q38R, D48E, L136P, G179S, E238D.