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ORIGINAL PAPER

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Identification of a gene cluster encoding meilingmycin biosynthesis among multiple polyketide synthase contigs isolated from *Streptomyces nanchangensis* NS3226

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Abstract A cluster encoding genes for the biosynthesis of meilingmycin, a macrolide antibiotic structurally similar to avermectin and milbemycin α11, was identified among seven uncharacterized polyketide synthase gene clusters isolated from *Streptomyces nanchangensis* NS3226 by hybridization with PCR products using primers derived from the sequences of *aveE*, *aveF* and a thioesterase domain of the avermectin biosynthetic gene cluster. Introduction of a 24.1-kb deletion by targeted gene replacement resulted in a loss of meilingmycin production, confirming that the gene cluster encodes biosynthesis of this important anthelminthic antibiotic compound. A sequenced 8.6-kb fragment had *aveC* and *aveE* homologues (*meiC* and *meiE*) linked together, as in the avermectin gene cluster, but the arrangement of *aveF* (*meiF*) and the thioesterase homologues differed. The results should pave the way to producing novel insecticidal compounds by generating hybrids between the two pathways.

Keywords Antibiotic biosynthetic genes · Macrolide antibiotic · Polyketide synthase · Avermectin · Gene replacement in *Streptomyces*

Introduction

Streptomyces nanchangensis NS3226 is a new species (Ouyang et al. 1984) that was isolated from the rhizosphere of a tea plant, *Thea oleosa*, in Nanchang, China. Early studies indicated that *S. nanchangensis* NS3226 produces compounds that kill several harmful agricultural and forest insects and mites, including species of *Aphis, Tetranychus, Dendrolimus*, and *Thosea*. At least two of the compounds have been characterized as insecticidal antibiotics (Ouyang et al. 1993). One is the polyether nanchangmycin (Sun et al. 2002), which structurally and biologically resembles dianemycin (Czerwinski and Steinrauf 1971). Nanchangmycin shows activity against some gram-positive bacteria, fungi, and chicken coccidial parasites and therefore used in intensive poultry rearing. The other compound is a 16-membered macrolide, meilingmycin (Sun et al. 2002), which is slightly different from milbemycin α11 (Takahashi et al. 1993) and has an aglycone and antiparasitic activity similar to that of avermectin (Fig. 1) produced by *Streptomyces avermitilis* (Burg et al. 1979). Both meilingmycin and nanchangmycin are very active against a broad spectrum of harmful nematodes and insects, and are non-toxic for mammals and plants (Ouyang et al. 1993). In addition, both compounds are synthesized by modular type I polyketide synthases.

We previously reported the isolation of eight independent polyketide synthase gene clusters (A–H) in *S. nanchangensis* NS3226. Cluster A was confirmed to be responsible for biosynthesis of the polyether nanchangmycin (Sun et al. 2002). In a continued attempt to identify the meilingmycin biosynthesis pathway genes among the other seven polyketide synthase gene clusters, an effort was made to replace fragments of chromosomal DNA corresponding to the polyketide synthase clusters B and C, which are about 132 and 104 kb long, respectively, using gene disruption and replacement techniques; however, none of the mutants lacked meilingmycin production, suggesting that clusters B and C are not involved in meilingmycin biosynthesis.

As shown in Fig. 1, meilingmycin and avermectin have very similar aglycones, which would possibly involve similar genes for identical biosynthetic steps. We therefore decided to take advantage of the reported sequence for avermectin biosynthesis to detect the meilingmycin gene cluster. Three candidate homologous genes that could be shared by both pathways were chosen: (1) *aveE* (1,371 bp, GenBank accession no. AB032367), whose deduced

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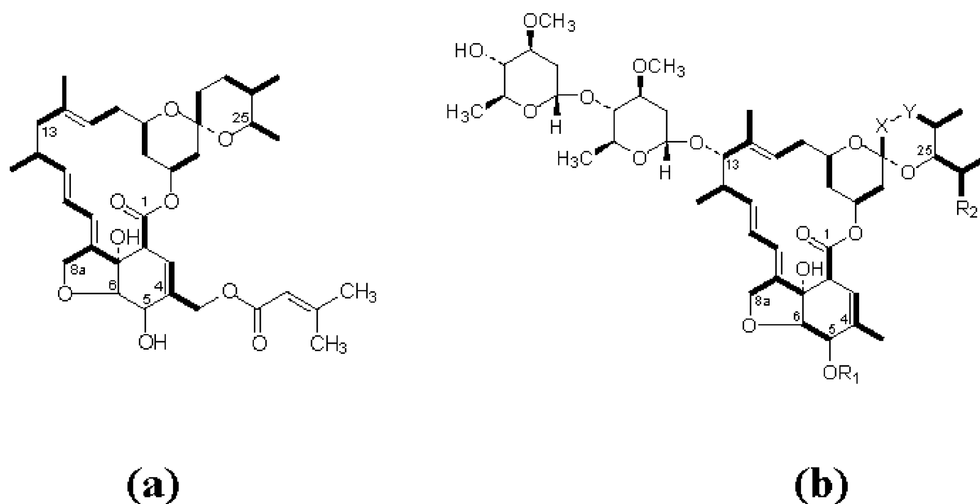


Fig. 1 Meilingmycin (**a**) (Ouyang et al. 1984), a macrolide antibiotic structurally and biologically similar to avermectin (**b**). Meilingmycin differs from avermectin in having no α-L-oleandrose attached at position 13 of the macrolide ring but with addition of an isopantenoic acid moiety at position 4. The difference in the side group at position 25 probably reflects flexibility in the choice of starter unit. R1 (-H or $-CH_3$) and R2 ($-CH_3$ or C_2H_5) represent variable moieties, and X-Y could be CH=CH or CH2-CH(OH) for different avermectin components (see (Ikeda and Omura 1997) for details)

amino acid sequence resembles the consensus sequence of cytochrome P450 hydroxylases and is believed to be involved in furan ring formation at C6–C8a of avermectin (Ikeda et al. 1999); (2) *aveF* (909 bp, GenBank accession no. AB032524), a gene responsible for post-polyketide modification in avermectin biosynthesis, which encodes a C5-ketoreductase whose deduced amino acid sequence shows good similarity to members of the NADPH-binding consensus motif GxxxGxGxxxAxxxA (Persson et al. 1991) in its N terminus, and which was demonstrated to catalyze the NADPH-specific reduction of 5-oxoavermectins to avermectin "B" components in cell-free extracts of *S. avermitilis* (Ikeda and Omura 1997); (3) a terminal thioesterase domain, at the terminus of AVES4, adjacent to the acyl carrier protein domain of module 12 in the *ave* gene cluster (Ikeda et al. 1999). This thioesterase plays a key role in the release and cyclization of enzyme-bound intermediates during the biosynthesis of essentially all of the identified macrolide antibiotics (Hopwood 1997). Although there is no extensive homology among thioesterases from different *Streptomyces* polyketide synthase genes, it was hoped that sequences might be conserved for such similar compounds in two different producers. The successful amplifications by PCR of the above three potentially similar genes formed the initial base for the successful identification of a gene cluster determining the biosynthesis of meilingmycin. The gene cluster identified was confirmed to be directly relevant to meilingmycin biosynthesis by targeted gene replacement followed by HPLC analysis of the compounds produced by the engineered mutants, and the sequenced genes encoding the AveE, AveF and thioesterase homologues in *S. nanchangensis* NS3226 were found to be very similar to their *S. avermitilis* counterparts.

Materials and methods

Bacterial strains, plasmid vectors, and growth media

S. nanchangensis NS3226 is a wild-type strain producing nanchangmycin and meilingmycin (Ouyang et al. 1984). Strains SYH22c and SYH22e are two meilingmycin blocked mutants of NS3226 created by gene replacement. *S. avermitilis* NRRL8165 is the wild-type producer of avermectin. *Escherichia coli* DH5α (Hanahan 1983) was used for construction of gene replacement vectors, and *E. coli* ET12567 carrying pUZ8002 (a RK2 derivative defective in its own *ori*T while supplying transfer functions to *ori*T-carrying plasmids) (MacNeil et al. 1992; Flett et al. 1997) was used as donor for conjugation. A genomic library of *S. nanchangensis* NS3226, constructed using pHZ1358 as a cosmid vector, was the same as described by Sun et al. (2002).

GS solid medium (2% soluble starch, 0.1% KNO_3, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% NaCl, 0.001% $FeSO_4$, 2% agar, pH 7.5) was used for *S. nanchangensis* sporulation and TSBY liquid medium (3% Difco tryptic soy broth powder, 10.3% sucrose, 1% Difco yeast extract, pH 7.2) for growing mycelium and isolation of total DNA of *S. nanchangensis*; YEME liquid medium (Kieser et al. 2000) was used for *S. avermitilis*; YD solid medium (0.4% Difco yeast extract, 1% maltose extract, 0.4% glucose, 0.2% $MgCl_2$, 0.15% $CaCl_2$, 2% agar, pH 7.5) was used for conjugation between *E. coli* and *Streptomyces*; LA or LB medium (Sambrook et al. 1989) was used for *E. coli*.

For *Streptomyces*, thiostrepton and apramycin were used at concentrations of $5\,\mu g\,ml^{-1}$ and $10\,\mu g\,ml^{-1}$, respectively, in solid medium and half the concentration was used in liquid medium when needed. For *E. coli*, apramycin, kanamycin, chloramphenicol, and ampicillin were added at concentrations of 30, 30, 25, and $100\,\mu g\,ml^{-1}$, respectively.

DNA manipulation

DNA manipulation, Southern hybridization, and transformation of *E. coli* were carried out by standard procedures (Sambrook et al. 1989). Conjugation between *E. coli* and *Streptomyces* was done as previously described (Sun et al. 2002). A 3.2-kb *Sac*I fragment corresponding to the region of the DEBS3 gene encoding the KR4 (ketoreductase), ACP6 (acyl carrier protein) domains of module 5 and KS6 (ketosynthase) and AT7 (acyltransferase) domains of module 6 from the *Saccharopolyspora erythraea* erythromycin polyketide synthase gene cluster (Donadio et al. 1991; Bevitt et al. 1992; Donadio and Katz 1992) was excised from an *E. coli* plasmid kindly supplied by P.F. Leadlay for use as a probe for detecting the polyketide synthase region of the *mei* cluster.

DNA amplification, sequencing, and computer analysis

Oligonucleotide primers 5′-GAGTGCTCTCGCCCCCACCC-3′ (ave-E1) and 5′-CGACGACCGCGACCGTAACG-3′ (ave-E2) were used for PCR-amplification of partial *aveE*. 5′-ATTCCCGTTGCCGTCACTCG-3′ (aveF-1) and 5′-GCGGTTCATGTCGGTGGCGG-3′ (aveF-2) were used for PCR amplification of partial *aveF*. 5′-CCTCATCTGCCTGCCCACCG-3′ (TE-1) and 5′-

TGATGCGATTTCTGTCAGCC-3′ (TE-2) were used for PCR-amplification of partial aveTE. PCR amplification was done using a HYBAID PCR machine. Each reaction mixture contained 50 ng of genomic or cosmid DNA, 0.5 µM of required primers, 0.2 mM of dNTPs, 1.2 mM of $MgCl_2$ and 2.5% DMSO in a final volume of 40 µl. After addition of 2.5 U DNA polymerase (Roche), the DNA template was denatured at 94 °C for 8 min. Amplification was carried out by 35 cycles of annealing (40 s at 60 °C), extension (1 min at 72 °C) and denaturation (30 s at 94 °C. DNA fragments of 680, 705, and 625 bp were expected when using *aveE, aveF* and thioesterase primers, respectively. PCR products were then purified from 1% agarose gels using the Gene Clean kit (Bio 101).

DNA was sequenced using the Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The nucleotide sequences were read from an Applied Biosystems PRISM 377 sequencer. The sequence data were analyzed with the Frame-Plot 2.3 online program (Ishikawa and Hotta 1999). DNA and deduced protein sequence similarity searches were carried out using BLAST (Altschul et al. 1997; Altschul and Koonin 1998; Schaffer et al. 2001) and FASTA (Pearson 1990). Multiple alignments of sequences were done using BioEdit Sequence Alignment Editor (Hall 1999).

Fermentation

S. nanchangensis NS3226 and its derivatives from 7-day-old plates were used to inoculate 250-ml flasks containing 30 ml of B2-1 liquid medium (3% corn powder, 1% corn starch, 1% soy bean powder, 0.2% KH_2PO_4, 0.05% $MgSO_4$, 0.05% NaCl, 0.2% $(NH_4)_2SO_4$, 1% $CaCO_3$, pH 7.5). The cultures were incubated on a rotary shaker (220 rpm) at 28 °C for 7 days.

HPLC analysis

After the mycelium was harvested by centrifugation at 3,000 rpm for 10 min, the pellets were extracted with an equal volume of methanol for 12 h. The extract was directly applied to an HPLC column run under the following conditions: Waters Xterra RP18 (5 µm, 3.9×150 mm) at 25 °C, mobile phase of acetonitrile-water (57:43, v/v) during 0–30 min, 70:30 (v/v) during 30–32 min, 80:20 (v/v) during 32–34 min, 90:10 (v/v) during 34–50 min with a flow rate of 0.8 ml/min. The effluent was monitored at 234 nm with a Waters 996 photodiode array detector. The data were processed with a Waters Millennium Chromatography Manager.

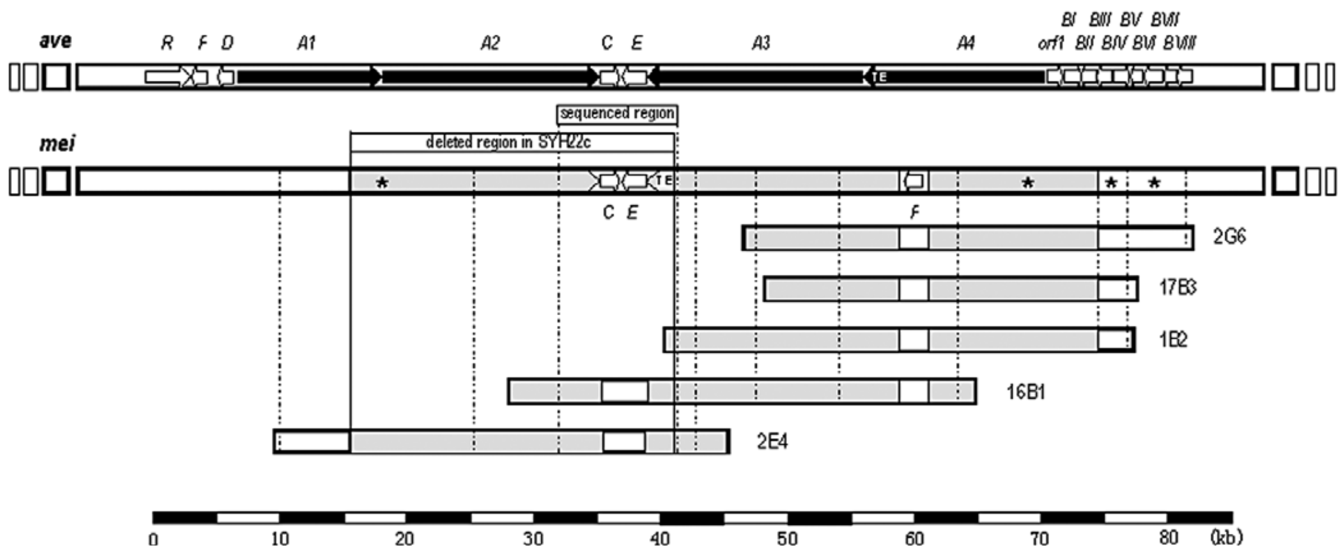
Results

S. nanchangensis NS3226 contains homologues of avermectin biosynthetic genes

The PCR experiment was carried out using non-degenerate oligonucleotides as primers (aveE-1 and aveE-2). This PCR amplification yielded a fragment running at 680 bp on an agarose gel, as expected. The sizes of the PCR products from *S. avermitilis* NRRL8165 and *S. nanchangensis* NS3226 were indistinguishable (not shown). Similarly, a PCR experiment using a pair of non-degenerate primers (aveF-1 and aveF-2) spanning the conserved NADPH-binding region of the *aveF* sequence of *S. avermitilis* NRRL8165 resulted in amplification of the desired fragment (705 bp) when chromosomal DNA of *S. avermitilis* NRRL8165 or of *S. nanchangensis* NS3226 was used as template (not shown). Finally, when non-degenerate PCR thioesterase primers (TE-1 and TE-2) were used under the same conditions, a 625-bp fragment encoding almost the entire thioesterase domain was generated by PCR amplification of *S. avermitilis* NRRL8165 and *S. nanchangensis* NS3226 genomic DNA (not shown).

The three PCR products were sequenced. The sequences of the *aveE-*, *aveF-* and thioesterase-specified PCR products in NS3226 were very similar to those of *aveE*, *aveF* and the region in the thioesterase domain of NRRL8165 (70.6% identities to *aveE*, 65.9% identities to *aveF*, and 75.7% identities to thioesterase at the nucleotide level, respectively).

Fig. 2 Relative locations of the homologous genes between the *mei* and *ave* clusters. Overlapping cosmids covering most (if not all) genes for meilingmycin biosynthesis are shown under the *mei* cluster. *Hatching* indicates polyketide synthase regions, as detected by Southern hybridization with the *Streptomyces erythraea* erythromycin polyketide synthase probes, or DNA sequences from the 'sequenced region'. The position of the sequenced *meiF* gene in the specific 8.6-kb *Pvu*II fragment is arbitrarily placed and thus inaccurate. *Vertical continuous and dotted lines* indicate the positions of *Bam*HI and *Pvu*II sites, respectively. Replacement of sequences labeled 'deleted region in SYH22c' abolished meilingmycin production. *Top* Organization of the sequenced *ave* gene cluster for avermectin biosynthesis. Polyketide synthase genes are shown as *solid black arrows* and others as *open arrows* (see Ikeda et al. 1999 for details of the gene functions). *Asterisks* indicate a region with more than one unmapped *Pvu*II site



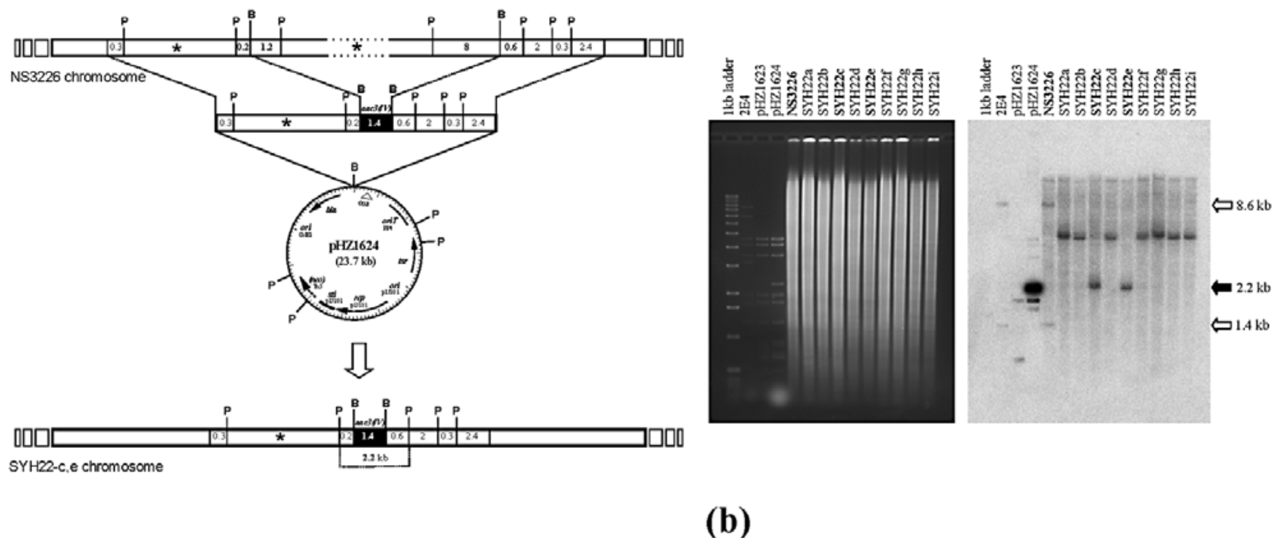


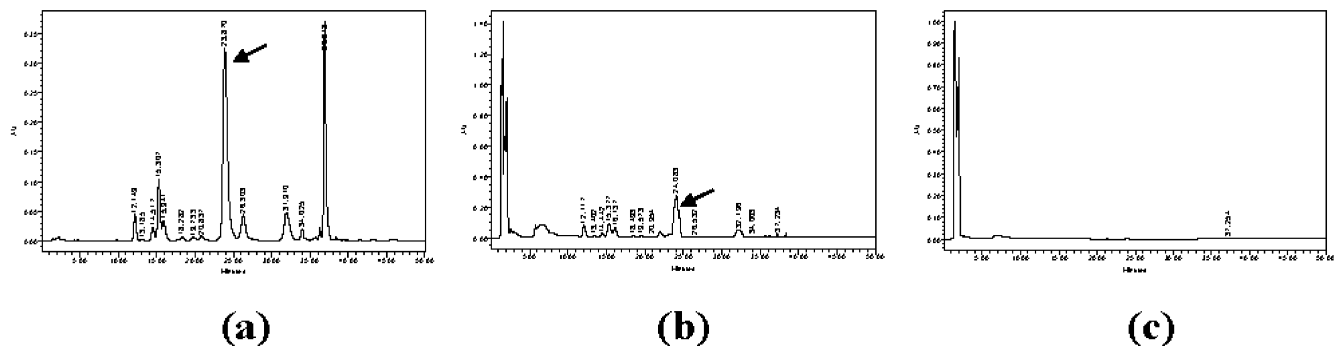
Fig. 3a, b Gene replacement. **a** Replacement of part of polyketide synthase gene cluster H in strain NS3226 using pHZ1624, constructed by substitution of the internal *Bam*HI fragment of cosmid 2E4 by *aac3(IV)*. A total of ca. 24.1 kb of contiguous DNA between the two *Bam*HI sites should be deleted and replaced by the 1.4-kb *aac3(IV)* fragment. The genome of a mutant strain with the expected deletion should have a new, 2.2-kb *Pvu*II fragment. B, *Bam*HI; P, *Pvu*II. The *asterisk* represents multiple *Pvu*II fragments whose exact order has not been determined. **b** Ethidium-bromide-stained agarose gel and Southern transfer probed with labeled 2.2-kb *Pvu*II fragment of pHZ1624 containing *aac3(IV)*. All the DNA samples were digested with *Pvu*II. Only SYH22c and SYH22e have the expected gene replacement among independent Apr^R Thio^S derivatives (SYH22a–i). The new 2.2-kb fragment (*solid arrow*) formed after gene replacement is the sum of 0.2 kb and 0.6 kb (deletion of the 24.1-kb *Bam*HI fragment out of the original *Pvu*II fragment, as indicated by *open arrows*) plus 1.4 kb containing *aac3(IV)*. The size markers (1-kb ladder) and *Pvu*II digests of the control plasmids (pHZ1623 and pHZ1624) are indicated on the *left* of the gel. Some weak signals might be the result of non-specific hybridization with the probe carrying polyketide synthase

Fig. 4a–c HPLC analysis of meilingmycin production by the strains after gene replacement. Meilingmycin was extracted from the mycelium of NS3226 (**b**) and SYH22c (**c**) by methanol extraction. Pure meilingmycin mixture (**a**) was used as standard. *Arrows* point to peaks corresponding to meilingmycin structurally illustrated in Fig. 1, which can be seen in **a** and **b** but not in **c**

Identification of cluster H as the meilingmycin biosynthetic gene cluster using homologous PCR probes

To identify a specific cluster involved in meilingmycin production, 30 out of 75 cosmids representing cluster A, which was known to encode nanchangmycin biosynthesis (Sun et al. 2002) (as an additional control), and seven uncharacterized polyketide synthase gene clusters B–H were digested with *Pvu*II before fractionation on agarose gels and transfer to nylon membranes for Southern blotting. The probes were generated by PCR using NS3226 as template and *aveE*, *aveF*, or thioesterase primers. The *aveE* and thioesterase probes generated from NS3226 hybridized to an 8.6-kb *Pvu*II fragment of two overlapping cosmids (2E4 and 16B1), while the *aveF*-specific probe hybridized only to an 8.6-kb *Pvu*II fragment of cosmid 16B1 in cluster H (Sun et al. 2002) (Fig. 2).

Three additional cosmids (1B2, 17B3, and 2G6, Fig. 2) to the right of cosmid 16B1 were obtained by Southern hybridization against a cosmid library constructed in pHZ1358 (Sun et al. 2002) using the *aveF*-specific PCR fragment of 16B1 (Fig. 2) as a probe, in order to obtain genes likely to encode most (if not all) of the *mei* biosynthetic pathway. The three cosmids were used as templates for more specific PCR amplifications using *aveE*-, *aveF*- and thioesterase-specific primers. Bands corresponding in size to the original PCR fragments were obtained in all cases (not shown).



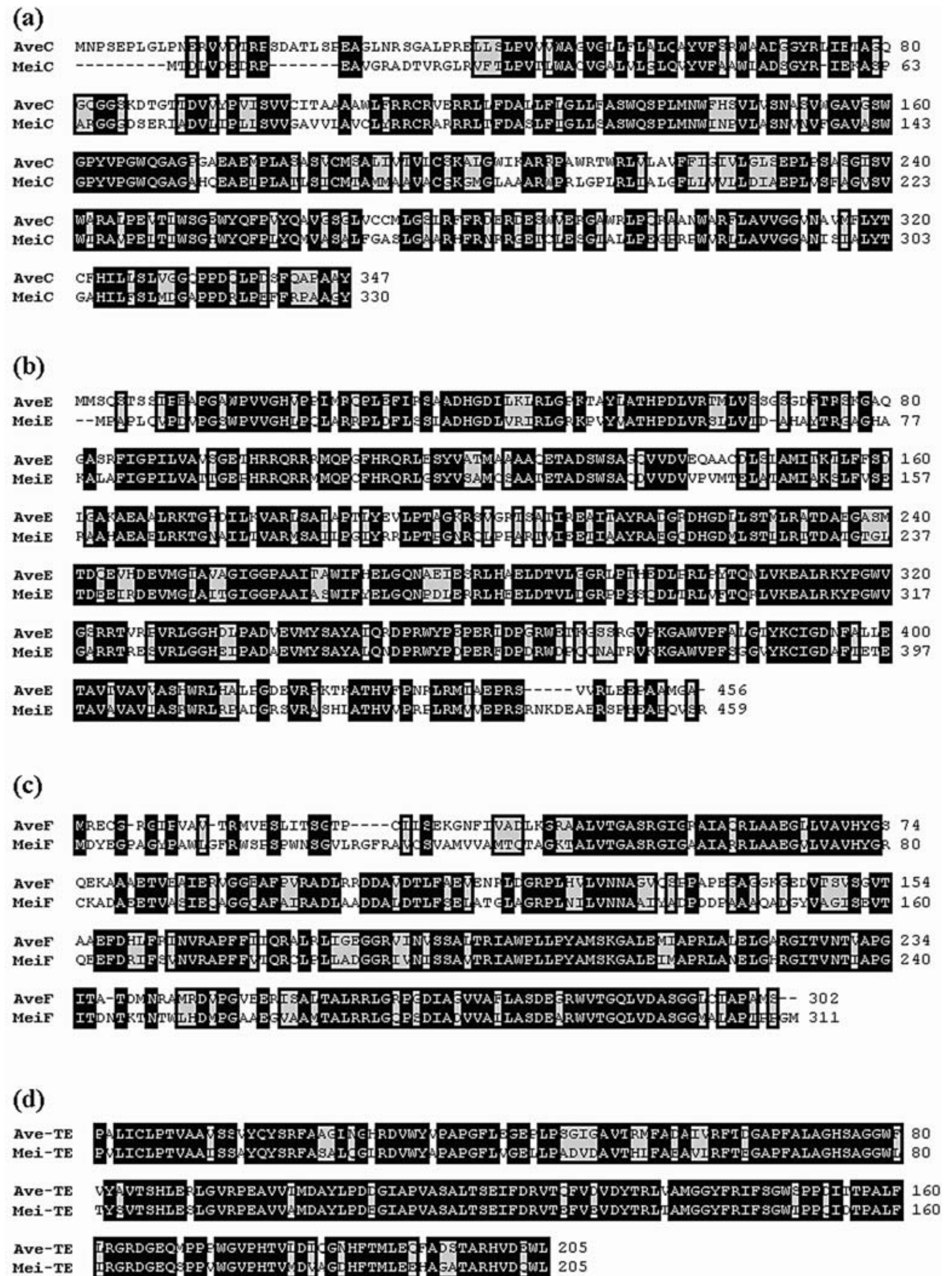


Fig. 5 Alignments of amino acid sequences between AveC and MeiC (**a**), AveE and MeiE (**b**), AveF and MeiF (**c**) and Ave-thioesterase and Mei-thioesterase (**d**). Identical amino acids are shaded in *black* and similar amino acids are shaded in *gray*

Confirmation of the meilingmycin biosynthetic gene cluster by generating a large deletion in cluster H by gene replacement

The above experiments suggested that cluster H may encode meilingmycin biosynthesis. A gene replacement experiment used a vector derived from cosmid 2E4. A 1.4-kb *Bam*HI fragment carrying the apramycin resistance gene (*aac3(IV)*) was cloned into a unique *Bam*HI site of an intermediate construct (pHZ1623) created by self-ligation of cosmid 2E4 after complete digestion with *Bam*HI to remove an internal ca. 24.1-kb fragment. The resulting plasmid (pHZ1624, Fig. 3a) thus carried *aac3(IV)* between two arm fragments in their natural orientation. pHZ1624 was then introduced into NS3226 by conjugation from *E. coli* ET12567/pUZ8002 (MacNeil et al. 1992; Flett et al. 1997) into strain NS3226. Nine candidate recombinants (SYH22a–i, Fig. 3b) were obtained by selecting for Apr^R and screening for $Thio^S$ after only one cycle of non-selective growth, because of the exceptional instability of the pHZ1358-derived vectors in many of the hosts tested (Sun et al. 2002). Southern blotting indicated that the expected gene replacement (Fig. 3a) had occurred in SYH22c and SYH22e but not in the other seven Apr^R $Thio^S$ mu-

tants, SYH22a, b, d, f–i (Fig. 3b), which may have an unknown but specific region replaced by *aac3(IV)*.

The disruptants were examined by HPLC analysis for production of meilingmycin (Fig. 4). No peaks of meilingmycin components were observed from the SYH22c (Fig. 4c) and SYH22e extracts as compared with the meilingmycin standard sample (Fig. 4a) and a sample isolated from the wild-type strain NS3226 (Fig. 4b), indicating that the meilingmycin biosynthetic gene cluster had been disrupted. As controls, the "false" gene disruptants (SHY22a, b, d, f–i) still produced meilingmycin.

Relative locations of the identified homologous genes in the meilingmycin (*mei*) and avermectin (*ave*) gene clusters

The region covered by the five cosmids was compared for its distribution of polyketide synthase and non-polyketide synthase regions as well as for the relative positions of the known *aveE*, *aveF*, and thioesterase homologues with the well-characterized avermectin biosynthetic gene cluster (Fig. 2). Firstly, an 8.6-kb *Pvu*II fragment that was shown to contain *meiC* and *meiE* in the non-polyketide synthase region of the *mei* cluster was sequenced. This region separates the polyketide synthase region into two parts because both genes are flanked by two incomplete polyketide synthase genes. The nucleotide (993 bp) and deduced amino acid (330) sequences of *meiC* were 62.4% and 50.9% identical (73.0% similar) to the nucleotide (1,044 bp) and amino acid (347) sequences of *aveC*, while the nucleotide (1,380 bp) and amino acid (459) sequences of *meiE* were 67.2% and 59.7% identical (78.4% similar) to the nucleotide (1,371 bp) and amino acid (456) sequences of *aveE* (Fig. 5). The divergent transcription of *meiC* and *meiE*, the same as for *aveC* and *aveE* (Fig. 2), provided an orientation for the alignment of the *mei* and *ave* clusters for further detailed comparisons. Although it would not have been meaningful to compare the sizes of the *mei* polyketide synthase region, which was only roughly determined by Southern hybridization with the totally sequenced *ave* polyketide synthase, there are organizational differences between the two gene clusters. The different polyketide synthase distribution could also be determined from the position of the unique thioesterase domain, immediately next to *meiE* in the *mei* cluster, but 17 kb distant and separated by AVES3 in the *ave* cluster (Fig. 2). *aveF,* which is located second from the leftmost ORF in the the *ave* cluster, outside the polyketide synthase region, lies between the two polyketide synthase regions in the *mei* cluster (Fig. 2), as detected by Southern hybridization using PCR-generated *aveF*-specific probe. The nucleotide and deduced amino acid sequences of *mei*-thioesterase were 76.1% and 78.5% identical (92.7% similar) to those of *ave*-thioesterase, whereas the nucleotide and deduced amino acid sequences of *aveF* and *meiF* were 65.4% and 56.6% (74.6% similar), respectively (Fig. 5).

The 8.6-kb DNA and deduced protein sequences carrying *aveC* and *aveE* in the central part of the *mei* gene cluster, and sequences of the *aveF* gene and its deduced protein sequence, reported here have been deposited in GenBank under the accession numbers AY129009 and AY262284, respectively.

Discussion

Experiments in which stepwise targeted gene disruption and replacement techniques were used to search among the multiple polyketide synthase clusters isolated from *S. nanchangensis* NS3226 for one involved in meilingmycin biosynthesis met with no success. The major reason for the failure was our focus on the larger clusters among the eight contigs isolated, based on the assumption that the *mei* cluster was likely to be large. It turned out that the meilingmycin pathway genes are located on one of the smallest contigs (cluster H), initially isolated with only two cosmids. PCR amplification of the likely shared genes between the *ave* and *mei* clusters implicated cluster H as relevant to meilingmycin biosynthesis; this cluster was subsequently extended by three additional cosmids. The PCR approach based on prediction of the unique gene(s) allowed us to focus on a limited numbers of cosmids (five out of 78 cosmids covering eight polyketide synthase clusters) for further detailed investigation by more laborious gene disruption and replacement experiments.

The data presented here serve to identify the *mei* gene cluster, although we still cannot be sure whether the isolated DNA covers the entire region necessary for meilingmycin production. The results suggest that the *mei* and *ave* gene clusters are similar in many ways albeit differences clearly exist. Genes similar to *aveBI* to *aveBVIII*, located to the right of the *ave* cluster, which are involved in α-L-oleandrose biosynthesis would not be expected in the *mei* cluster as meilingmycin has no α-L-oleandrose attached at position 13 of the macrolide ring; however, one would expect gene(s) unique to meilingmycin biosynthesis, e.g., coding for the addition of an isopantenoic acid moiety, probably derived from valine, at position 4. Comparisons with the published *ave* sequence (Ikeda et al. 1999) should identify the potential differences in the *mei* cluster corresponding to the two nonfunctional domains (DH in module 7 and KR in module 10) of the *ave* cluster, the possible presence of two additional active domains (DH and ER) catalyzing dehydration and enoyl reduction to remove a hydroxyl group at C13 in the *mei* cluster (in contrast to only one, inactive DH domain present in the corresponding part of the *ave* cluster), and whether a flexible or a different starter contributed to the variation in the side group at position 25. An extensive characterization of the differences between the two pathways should help in the generation of novel antibiotic compounds by deletion, addition, or exchange of polyketide synthase domains and non-polyketide synthase genes between the two clusters so as to modify or exchange side groups and create altered meilingmycin (or avermectin) derivatives. These compounds might have enhanced potency or an altered spectrum of activity. Such work is now in progress.

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