Genetic Localization and Molecular Characterization of the *nonS* Gene Required for Macrotetrolide Biosynthesis in *Streptomyces griseus* DSM40695

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The macrotetrolides are a family of cyclic polyethers derived from tetrramerization, in a stereospecific fashion, of the enantiomeric nonactic acid (NA) and its homologs. Isotope labeling experiments established that NA is of polyketide origin, and biochemical investigations demonstrated that 2-methyl-6,8-dihydroxynon-2E-enoic acid can be converted into NA by a cell-free preparation from *Streptomyces lividans* that expresses *nonS*. These results lead to the hypothesis that macrotetrolide biosynthesis involves a pair of enantiospecific polyketide pathways. In this work, a 55-kb contiguous DNA region was cloned from *Streptomyces griseus* DSM40695, a 6.3-kb fragment of which was sequenced to reveal five open reading frames, including the previously reported *nonR* and *nonS* genes. Inactivation of *nonS* in vivo completely abolished macrotetrolide production. Complementation of the *nonS* mutant by the expression of *nonS* in trans fully restored its macrotetrolide production ability, with a distribution of individual macrotetrolides similar to that for the wild-type producer. In contrast, fermentation of the *nonS* mutant in the presence of exogenous (+)-NA resulted in the production of nonactin, monactin, and dinactin but not in the production of trinactin and tetractin. These results prove the direct involvement of *nonS* in macrotetrolide biosynthesis. The difference in macrotetrolide production between in vivo complementation of the *nonS* mutant by the plasmid-borne *nonS* gene and fermentation of the *nonS* mutant in the presence of exogenously added (+)-NA suggests that *NonS* catalyzes the formation of (−)-NA and its homologs, supporting the existence of a pair of enantiospecific polyketide pathways for macrotetrolide biosynthesis in *S. griseus*. The latter should provide a model that can be used to study the mechanism by which polyketide synthase controls stereochemistry during polyketide biosynthesis.

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The macrotetrolides are a family of cyclic polyethers produced by a number of *Streptomyces* species (9, 16, 18). Nonactin (NON), the smallest homolog and a symmetric member of the family, was first isolated in 1955 (14). Its structure was initially deduced from spectroscopic analysis and was later confirmed by X-ray crystallography (15, 27), revealing that the intriguing molecular topology of NON consists of the (+)(−)(+)(−)-ester linkage of the enantiomeric nonactic acid (NA) building blocks. The other members of this family are homologs of NON. They contain homononactic acid (HNA) and/or bishomononacetic acid (BNA) as building blocks, and they all have the same relative configuration (Fig. 1). NA-type building blocks have also been identified in several macrotetrolides (24), including the pamamycins (34, 35).

The macrotetrolides exert a broad spectrum of biological activities (56), ranging from antibacterial, antifungal, antitumor (11), antiprotozoan, antiparasitic, and insecticidal activities to immunosuppressive activities (12, 47–49, 56). In fact, comparative studies on the immunosuppressive activities of tetractin and cyclosporin, the latter being the most widely used immunosuppressant agent, showed that these two compounds were approximately equally effective and that tetractin has the advantage of low toxicity (49). The biological activities of the macrotetrolides are generally traced to their ionophoric properties (30, 38, 46), and the potencies of these activities appear to parallel the size of the alkyl substituents of the macrotetrolides: tetractin is often the most potent member of the family, while NON is generally inactive.

The biosynthesis of NON has been extensively studied by in vivo feeding experiments with 13C-, 2H-, and 18O-labeled precursors and biosynthetic intermediates (2–5, 13, 45) and by isolation of both enantiomers of NA and the dimeric NA (17). These results established unambiguously the polyketide origin of NON, the assembly of which from one molecule each of propionate and succinate and two molecules of acetate must have invoked (i) the rare use of succinate as an intact four-carbon fragment (C-3 to C-6) and (ii) the derivation of a three-carbon unit (C-7 to C-9) from two molecules of acetate (one of which is activated in the form of malonate) (Fig. 2). Feeding experiments with 13C- and 18O-doubly-labeled precursors indicated that the C-3-O bond is formed during closure of the tetrahydrofuran ring, presumably by an intramolecular Michael addition of the 6-hydroxy group onto the enone moiety of 2-methyl-6,8-dihydroxynon-2E-enoic acid (NEA) (3, 4). The involvement of the latter step in NON biosynthesis was further substantiated by the efficient and enantiospecific incorporation of both (6R,8R)-NEA into NON (45) and by the drastic reduction of NON production upon the addition of an NEA analog into the fermentation medium, which presumably acts as a suicide inhibitor for this enzymatic step (39). Sequential and stereospecific oligomerization of (+)- and (−)-NA into NON was supported by the isolation of both enantiomers of NA and the dimeric NA (17), which could be viewed as derailed biosynthetic intermediates from the enzyme complex. In vitro synthesis of macrotetrolides from NA and its homologs was also demonstrated in the presence of an enzyme preparation that appeared to be a heteromeric complex with an apparent molecular weight of 350,000 (2, 36).
Genetic analysis of NON biosynthesis was initiated by cloning the nonR resistance gene from *Streptomyces griseus*. By screening for tetractin resistance in *Streptomyces lividans* TK64, Plater and Robinson (37) isolated several overlapping clones that harbored a total of 9 kb of *TK64*. Plater and Robinson (37) isolated several overlapping clones, including clones that harbored a total of 9 kb of *TK64*, and subsequently completed the cloning and DNA sequence determination of *orfX*-named it NONS—and characterized NONS as the nonactate synthase that catalyzes the formation of NA from NEA via an intramolecular Michael addition. However, the latter study fell short of shedding light on the stereospecificity of the monomeric building blocks (B).

**FIG. 1.** Structures of macrotetrolides (A) and their monomeric building blocks (B).

Analysis of NON biosynthesis was as follows: initial denaturation at 94°C for 5 min, 24 to 36 cycles of 45 s at 94°C, 1 min at 60°C, and 2 min at 72°C. To prepare the nonR probe for PCR, the following pair of primers was used: 5'-ACACTCGCCACGGCCTTGG-3' (forward primer) and 5'-CGTCGCTGAACCTGCATGTC-3' (reverse primer). A distinctive product with the predicted size of 1.3 kb was amplified from *S. griseus* DSM40695 and was cloned into pGEM-T to yield pBS2001. Automated DNA sequencing was carried out on an ABI Prism 377 DNA Sequencing System (Perkin-Elmer/ABI) and the sequences were deposited in GenBank (accession number AJ011072). The nucleotide sequence of the insert was determined by the dideoxynucleotide chain-termination method.

**SMITH ET AL. ANTIMICROB. AGENTS CHEMOTHER.**

**MATeRIALS AND METHODS**

**Bacterial strains, plasmids, and biochemicals.** *Escherichia coli* DH5α (42), *E. coli* XL1-Blue MR (Strategene, La Jolla, Calif.), *E. coli* ET1012567 (29), *S. griseus* DSM40695 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) (also known as *Streptomyces* subsp. *griseus* ETHA7796), and *Micrococcus luteus* ATCC 9343 (American Type Culture Collection, Rockville, Md.) were used in this work. pDHS (20), pOJ446 (8), pWHM3 (50), and pWHM79 (43) were described previously. pUO9090 was a gift from M. C. Martin (Universidad de Oviedo, Oviedo, Spain), and other plasmids were from standard commercial sources. Ampicillin, apramycin, and NON were from Sigma (St. Louis, Mo.), and tetrastrepton was from S. J. Lucania (Bristol-Myers Squibb, Princeton, N.J.). Racemate methyl nonactate was a gift from P. Metz (52).

**Media and culture conditions.** *E. coli* strains carrying plasmids were grown in Luria-Bertani (LB) medium and were selected with appropriate antibiotics (42). *S. griseus* strains were grown on GMP (55) at 28°C for sporulation and in Trypticase soy broth (23) supplemented with 5 mM MgCl₂ and 0.5% glycine at 28°C and 300 rpm (Incubator Shaker series 25, New Brunswick Scientific Co., Inc., Edison, N.J.) for isolation of genomic DNA. For protoplast preparation, *S. griseus* strains were grown in SGGP (54) supplemented with 0.5% glycine at 28°C and 300 rpm for 25 h; the resulting mycelia were washed with water, resuspended in P buffer (23), and digested with lysozyme (1 mg/ml) at 37°C for 45 min. For regeneration, protoplasts were plated on R1M (55) and incubated at 28°C. For macrotetrolide production, *S. griseus* strains were cultured by a three-stage fermentation procedure described by Ashworth and Robinson (4).

**DNA isolation and manipulation.** Plasmid preparation and DNA extraction were carried out by commercial kits (Qiagen, Santa Clarita, Calif.). Genomic *S. griseus* DNA was isolated by standard protocols (23, 40). Restriction enzymes and other molecular biology reagents were from commercial sources, and digestion and ligations were done by standard methods (42). For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed by the protocols provided by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

PCR primers were synthesized at the Protein Structure Laboratory, University of California, Davis. PCR was carried out on a Gene Amp PCR System 2400 (Perkin-Elmer/ABI, Foster City, Calif.) with Taq polymerase and buffer from Promega (Madison, Wis.), which consisted of 5 ng of *S. griseus* genomic or plasmid DNA as template, 25 pmol of each primer, 25 μM deoxyribonucleoside triphosphates, 5% dimethyl sulfoxide, 2 U of Taq polymerase, and 1X buffer in a final volume of 50 μL. The PCR temperature program was as follows: initial denaturation at 94°C for 5 min, 24 to 36 cycles of 45 s at 94°C, 1 min at 60°C, and 2 min at 72°C. To prepare the nonR probe for PCR, the following pair of primers was used: 5'-ACACTCGCCACGGCCTTGG-3' (forward primer) and 5'-CGTCGCTGAACCTGCATGTC-3' (reverse primer). A distinctive product with the predicted size of 1.3 kb was amplified from *S. griseus* DSM40695 and was cloned into pGEM-T to yield pBS2001. Automated DNA sequencing was carried out on an ABI Prism 377 DNA Sequencing System (Perkin-Elmer/ABI) and the sequences were deposited in GenBank (accession number AJ011072). The nucleotide sequence of the insert was determined by the dideoxynucleotide chain-termination method.

To screen the genomic library, colonies from four LB plates that contained 1 mg of DNA. Restriction enzyme mapping of and chromosomal walking from these overlapping cosmids confirmed to be positive by Southern hybridization (42). Further restriction enzyme mapping of and chromosomal walking from these overlapping cosmids led to the genetic localization of a 55-kb contiguous DNA region, as represented in FIG. 1B. The resulting cosmid clones were confirmed to be positive by Southern hybridization (42). Further restriction enzyme mapping of and chromosomal walking from these overlapping cosmids led to the genetic localization of a 55-kb contiguous DNA region, as represented in FIG. 1B. The resulting cosmid clones were confirmed to be positive by Southern hybridization (42).

**FIG. 2A.** Structures of macrotetrolides (A) and their monomeric building blocks (B).

Analysis of NON biosynthesis was initiated by cloning the nonR resistance gene from *Streptomyces griseus*. By screening for tetractin resistance in *Streptomyces lividans* TK64, Plater and Robinson (37) isolated several overlapping clones that harbored a total of 9 kb of *S. griseus* genomic DNA and determined the DNA sequence of a 3.3-kb fragment to reveal three complete open reading frames (ORFs), including *orfX*-named it NONS—and characterized NONS as the nonactate synthase that catalyzes the formation of NA from NEA via an intramolecular Michael addition. However, the latter study fell short of shedding light on the stereospecificity of the monomeric building blocks (B).

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of pGEM-3zf to yield pBS2005, the DNA sequence of which was subsequently determined (see Fig. 3B).

Construction of nonS mutants by gene disruption and replacement. For gene disruption, a 0.7-kb internal fragment of nonS was amplified by PCR from pBS2005 with primers 5'-TCTAGACCTGACGCTCTCCCGC-3' (forward primer) and 5'-GAATTCTCCGTGAACGCGGCC-3' (reverse primer) and was cloned into pGEM-T to yield pBS2006. The latter plasmid was digested with EcoRI and XbaI, and the resulting 0.7-kb EcoRI-XbaI fragment was cloned into the same sites of pDH5 (20) to yield pBS2007 (see Fig. 4A).

For gene replacement, a 2.2-kb HindIII fragment that contained nonS was subeloned from pBS2005 into the same site of pGEM-3zf to yield pBS2008. The latter plasmid was digested with EcoRI and HindIII, and the resulting 2.2-kb EcoRI-HindIII fragment with the desired orientation was cloned into the same sites of pDH5 to yield pSB2009. The aac(3)IV apramycin resistance gene was rescued from pUO9090 as a 1.5-kb BglII-BamHI fragment and was inserted into the unique BglII site of pBS2009 to yield pBS2010, in which the nonS gene was insertionally inactivated by aac(3)IV transcribed in the opposite direction in reference to the direction of transcription for nonS (see Fig. 5A).

FIG. 2. (A) A pair of enantiospecific polyketide pathways proposed for macrotetrolide biosynthesis in S. griseus. Arrows with broken lines indicate intermediates that have been either isolated or confirmed by feeding experiments. (B) Monomeric building block compositions of individual macrotetrolides.

<table>
<thead>
<tr>
<th>Macrotetrolide</th>
<th>Number of monomeric building blocks</th>
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<tbody>
<tr>
<td></td>
<td>(-)-NA</td>
</tr>
<tr>
<td>NON</td>
<td>2</td>
</tr>
<tr>
<td>Monactin</td>
<td>2</td>
</tr>
<tr>
<td>Dinactin</td>
<td>2</td>
</tr>
<tr>
<td>Trinactin</td>
<td>1</td>
</tr>
<tr>
<td>Tetranactin</td>
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To introduce pBS2007 and pBS2010 into <i>S. griseus</i> by polyethylene glycol (PEG)-mediated protoplast transformation (23), these plasmids were propagated in <i>E. coli</i> ET12556 (29), and the resulting double-stranded DNA was denatured by alkaline treatment (21). The double-stranded DNA (10 μg) and 25% PEG 1000 in P buffer (200 μl) (23) were sequentially added to 100 μl of <i>S. griseus</i> protoplasts (10<sup>9</sup>) in P buffer. The resulting solution was mixed immediately and was spread onto R1M plates. After incubation at 28°C for 16 to 20 h, the plates were selected with thiostrepton (final concentration, 25 μg/ml) for pBS2007 and apramycin (final concentration, 50 μg/ml) for pBS2010; incubation continued until colonies appeared (3 to 5 days). These transformants that were derived from pBS2007 and that were resistant to both apramycin and thiostrepton were identified as <i>S. griseus</i> SB2002 mutants.

<i>S. griseus</i> SB2002 was further grown in 20 ml of liquid SGGP medium for two rounds without any antibiotic and for one round with apramycin (final concentration, 50 μg/ml) each of which was at 28°C and 300 rpm for 20 h, and was then plated onto solid SGGP medium with apramycin (final concentration, 50 μg/ml) and incubated at 28°C for 2 days. The resulting colonies were selected on SGGP plates with apramycin (final concentration, 50 μg/ml) and thiostrepton (final concentration, 25 μg/ml), respectively. Those colonies that were thiostrepton sensitive and apramycin resistant were identified as <i>S. griseus</i> SB2003 mutants.

**Construction of nonS expression plasmids**. pBS2011 was constructed by cloning a 2.2-kb EcoRI-HindIII fragment that contained the intact nonS gene from pBS2008 into the same sites of pWHM5. To clone the erm<sup>E</sup> promoter, pWHM5 (43) was digested with EcoRI and HindIII, and the resulting 0.45-kb EcoRI-BamHI fragment was inserted into the same sites of pBS2001 to yield pBS2012, in which the expression of nonS is under the control of the erm<sup>E</sup> promoter (7).

**Analysis of macrotetrolide production**. Macrotetrolides were isolated by the procedure described by Ashworth and Robinson (4) and were analyzed by either thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) procedure described by Ashworth and Robinson (4) and were analyzed by either TLC, or bioassay. For TLC, Keisiglet 60 F<sub>254</sub> glass plates (gel thickness, 0.25 mm; Merck, Rahway, N.J.) were used and developed with CHCl<sub>3</sub>-ethyl acetate (1:2; vol/vol). Individual macrotetrolides were purified by preparative TLC and were subjected to electron-spray mass spectral analysis. The latter was performed on a VG BioQ spectrometer at the Facility for Advanced Instrumentation of the University of California, Davis. For HPLC analysis, the method of Herlt (19) was used, by which a Microsc-MV C<sub>18</sub> column (4.0 by 250 mm; 5 μm; pore size 100 A; Rainin, Walnut Creek, Calif.), isocratic elution with tetrahydrofuran-H<sub>2</sub>O (60:40; vol/vol) at flow rate of 0.8 ml/min on a Dynamax gradient HPLC system (Rainin), and an evaporative light-scattering detector (Alltech, Deerfield, Ill.) were used. Macrotetrolide distribution was determined from the average of several fermentations. For the bioassay, either the fermentation supernatant (200 μl) or the acetone extract (10 μl) was added to stainless steel cylinders or filter paper disks, respectively, both of which were placed on LB plates presoaked with an overnight <i>M. luteus</i> culture (0.01%; vol/vol). The plates were incubated at 37°C for 24 h, and macrotetrolide production was estimated by measuring the sizes of the inhibition zones.

**Feeding of (±)-nonactic acid to nonS mutants**. To prepare (±)-NA from racemic methyl nonanolate, 10 mg of the methyl nonanolate was dissolved in methanol (200 μl), and this solution was mixed with 2 N NaOH (200 μl). The resulting mixture was shaken at room temperature for 3 h, and hydrolysis was quantitatively terminated by TCA (15% w/v) and toluene in presence of HCl (2 N; vol/vol). The mixture was then neutralized by addition of concentrated HCl (33 μl) and was evaporated to dryness. The residue was dissolved in H<sub>2</sub>O (6.0 ml; pH 8.5), sterilized by filtration (pore size, 0.22 μm; Millipore, Bedford, Mass.), and stored at 4°C for feeding experiments. To 25 ml of a production culture of <i>S. griseus</i> DSM40695, SB2001, or SB2003, a total of 8.2 mg of (±)-NA was administered in three equal portions on days 2, 3, and 4, respectively. Fermentation continued for 2 additional days, and macrotetrolides were isolated and analyzed by the procedures described above.

**Nucleotide sequence accession number**. The nucleotide sequence reported here has been deposited in the GenBank database under accession numbers AF263011 and AF263012.

**RESULTS**

**Cloning of the non gene cluster from <i>S. griseus</i> DSM40695 and sequence analysis of the nonR locus**. Plater and Robinson (37) cloned the non<sup>R</sup> gene from <i>S. griseus</i> and demonstrated that Non<sup>R</sup> confers macrolide resistance on <i>S. lividans</i> in the presence of K<sup>+</sup> ion. Given that the fact that antibiotic production genes have been found in nearly all cases to be clustered in one region of the bacterial chromosome that consists of structural, resistance, and regulatory genes (22), we proceeded to isolate and sequence the DNA flanking the non<sup>R</sup> gene in order to identify the non gene cluster. We amplified a 1.3-kb non<sup>R</sup> fragment (pBS2001) by PCR and used it as a probe to screen an <i>S. griseus</i> genomic library that was constructed in the <i>E. coli</i>-<i>Streptomyces</i> shuttle vector pOJ446 (8). Of the 2,400 colonies screened by colony hybridization, 54 positive clones were identified, and 12 of these were selected for further analysis. Restriction enzyme mapping and Southern analysis showed that 11 of the 12 clones contained a single 6.3-kb BamHI fragment that hybridizes to the non<sup>R</sup> probe. Additional chromosomal walking from this locus eventually led to the localization of a 55-kb contiguous DNA region covered by 30 overlapping cosmids, as exemplified by pBS2002, pBS2003, and pBS2004 (Fig. 3A).

The 6.3-kb BamHI fragment was subcloned (pBS2005), and its nucleotide sequence was determined. (During our work, this region was also sequenced [GenBank accession number AF074603] and, very recently, was reported by Priestly and coworkers [51].) Five complete ORFs, transcribed in the same direction, were identified, including non<sup>R</sup> and orf<sup>B</sup>, (also called nonB) described previously by Plater and Robinson (37) and nonS reported very recently by Priestley and coworkers (53) (Fig. 3B). Immediately upstream of nonS are two additional ORFs, non<sup>L</sup> and nonD, which encode a 555-amino-acid protein with a molecular weight of 59,595 and an isoelectric point of 5.83 and a 569-amino-acid protein with a molecular weight of 61,566 and an isoelectric point of 4.98, respectively. NonL is homologous to a homologue of a coenzyme A (CoA) ligases or ligase-like domains, including the loading domains of the Rif PKS from <i>Amicyclolatopsis mediterranei</i> S699 (33% identity and 43% similarity) (6), the FKS506 PKS from <i>Streptomyces</i> sp. strain MA6548 (32% identity and 42% similarity) (33), and the second type I PKS from the raphamycin-producing strain <i>Streptomyces hygroscopicus</i> ATCC 29253 (28% identity and 40% similarity) (41). These CoA ligase-like domains of type I PKS catalyze the ATP-dependent activation of free carboxylic acids into the corresponding acyl CoAs before they can be loaded onto the PKS complex for polyketide biosynthesis. An equivalent role could be proposed for NonL, although the substrate for NonL is yet to be determined. The fact that the addition of ATP has no effect on the in vitro synthesis of macrolitols from NA and its homologs argues against the notion that the latter acids are activated into acyl CoAs that involve a CoA ligase like NonL before incorporation into macrolitols (2, 36). NonD shows low but significant end-to-end similarity to several glutaryl 7-aminoccephalosporanic acid (7-ACA) acylases, including the ones from <i>Bacillus subtilis</i> (28.4% identity and 40.8% similarity) (1) and from <i>Zymomonas mobilis</i> (19% identity and 27.3% similarity) (GenBank accession number AF124775), as well as to several proteins of unknown function, such as the hypothetical protein Rv2800 from <i>Mycobacterium tuberculosis</i> (31% identity and 40% similarity; GenBank accession number Z81331) and the hypothetical protein from a <i>Synechocystis</i> sp. (26% identity and 39% similarity; GenBank accession number D90903). Since glutaryl 7-ACA acylase catalyzes the amide hydrolysis of glutaryl 7-ACA to yield 7-ACA, it is not clear how NonD could play a role in macrolide biosynthesis.

**Inactivation of the nonS gene in <i>S. griseus</i> DSM40695 by gene disruption and replacement**. To examine if the cloned gene cluster encodes macrolide biosynthesis, nonS was inactivated by both insertional disruption and replacement with a mutant copy to generate macrolide-nonproducing mutant strains. To effect gene disruption, pBS2007—a pH5 derivative that contains a 0.7-kb internal fragment of nonS—was introduced into <i>S. griseus</i> via PEG-mediated protoplast transformation. One of the thiostrepton-resistant transformants was selected and was named <i>S. griseus</i> SB2001. Since
pBS2007 is derived from the Streptomyces-nonreplicating plasmid pDH5. S. griseus SB2001 must have resulted from integration of pBS2007 into the S. griseus chromosome by insert-directed homologous recombination (Fig. 4A).

To confirm that targeted nonS disruption has occurred by a single-crossover homologous recombination event, Southern analysis of genomic DNA from S. griseus SB2001 was performed with either pDH5 or the 0.7-kb fragment of nonS as a probe. As shown in Fig. 4B, a distinctive band of the predicted size of 11 kb was detected with pDH5 as a probe in S. griseus SB2001 (lane 2); this band was absent from the wild-type strain S. griseus DSM40695 (Fig. 4B, lane 1). Complementarily, when the 0.7-kb fragment of nonS was used as a probe, the 6.3-kb band in the wild-type strain (Fig. 4C, lane 1) was shifted to a 11-kb fragment in the SB2001 mutant strain (Fig. 4C, lane 2), as would be expected for disruption of nonS by a single-crossover homologous recombination event (Fig. 4A).

To effect gene replacement, pBS2010—a pDH5 derivative that contains a mutant copy of nonS generated by inserting the aac(3)IV apramycin resistance gene from pUO9090 into the unique BglII site of nonS—was similarly introduced into S. griseus via PEG-mediated protoplast transformation. Transformants resistant to both thiostrepton and apramycin (five colonies) were isolated and identified as S. griseus SB2002, which had resulted from a single-crossover homologous recombination event as confirmed by Southern analysis (data not shown). Direct screening for colonies resistant to apramycin only was unsuccessful, suggesting that the double-crossover homologous recombination event occurs at a very low frequency. To encourage the double-crossover event, the SB2002 strain was cultured sequentially in a liquid medium for several rounds without any added antibiotic and for one round in the presence of apramycin. The resulting culture was then selected on solid medium containing thiostrepton or apramycin. Colonies that are thiostrepton sensitive and apramycin resistant were selected as representatives of nonS replacement mutants.

To confirm that targeted nonS replacement has occurred by a double-crossover homologous recombination event, Southern analysis of genomic DNA from S. griseus SB2003 was performed with either acc(3)IV or nonS as a probe. As shown in Fig. 5B, a distinctive band of the predicted size of 7.8 kb was detected with acc(3)IV as a probe in S. griseus SB2003 (Fig. 5B, lane 3); this band was absent from the wild-type strain S. griseus DSM40695 (Fig. 5B, lane 2). Complementarily, when nonS was used as a probe, the 6.3-kb band in the wild-type strain (Fig. 5C, lane 2) was shifted to a 7.8-kb fragment in the SB2003 mutant strain (Fig. 5C, lane 3), as would be expected for replacement of nonS with the mutant copy by a double-crossover homologous recombination event (Fig. 5A).

Characterization of the nonS mutants as macrotetralide nonproducers. S. griseus DSM40695 produces a mixture of

FIG. 3. (A) Restriction map of a 55-kb contiguous DNA region from S. griseus as represented by three overlapping cosmid clones. (B) Genetic organization of the nonDLSRB genes within the non cluster. nonR probe, the 1.3-kb fragment from pBS2001; B, BglII.

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macrotetrolides (NON, monactin, dinactin, trinactin, and tetranactin at 9.3:23:100:81:18), as judged by HPLC analysis of acetone extracts of the freeze-dried mycelia with an evaporative light-scattering detector (Fig. 6A) (19). While no apparent difference in growth characteristics and morphologies between the wild-type and the mutant strains was observed, macrotetrolide production is completely abolished in either the SB2001 nonS disruption mutant strain or the SB2003 nonS replacement mutant strain (Fig. 6B and D, respectively). Macrotetrolide production was also determined by assaying the antibacterial activity of the fermentation broth or the acetone extracts against M. luteus ATCC 9431. Consistent with the HPLC analysis, acetone extracts or aliquots of fermentation broth from either the SB2001 or SB2003 strains showed a complete lack of activity, while a clear inhibition zone was observed from that of the wild-type organism as well as from that of the positive control with an authentic sample of NON (data not shown).

In vivo complementation of the nonS mutants by expression of nonS in trans. The ability of the wild-type nonS gene to complement the inactivated nonS gene in vivo was tested in the S. griseus SB2003 mutant strain. Two nonS expression constructs were made in pWHM3 (50), pBS2011 and pBS2012, the latter of which uses the constitutive ermE* promoter (7) to ensure efficient expression of nonS. Both the pBS2011 and pBS2012 constructs, as well as the pWHM3 vector as a control, were introduced via PEG-mediated protoplast transformation into S. griseus. Acetone extracts from fermentations of three transformants of each variant were analyzed for macrotetrolide production by HPLC. While no adverse effect on the growth characteristics of S. griseus SB2003 was observed upon introduction of pWHM3, pBS2011 clearly restored macrotetrolide production to the SB2003 strain, albeit the yields of these metabolites were low in comparison with the yields from the wild-type strain (Fig. 6A versus F). The latter phenotype could be caused by the poor expression of nonS in pBS2011. This belief was indeed supported by the observation that introduction of pBS2012 into the SB2003 mutant resulted in production of the macrotetrolides to a comparable level.
with the distribution of individual macrotetrolides (NON, monactin, dinactin, triactin, and tetranactin at 15:37:100:54:11) being very similar to that for the wild-type strain (Fig. 6A versus G). These data confirm that the abolishment of macrotetrolide production in the SB2003 strain is due to the specific inactivation of nonS.

Fermentation of the nonS mutants in the presence of exogenously added (±)-NA. To investigate if nonS governs one of the enantiospecific pathways that leads to the biosynthesis of (+)- or (−)-NA and its homologs, S. griseus SB2001 and SB2003, as well as the wild-type strain as a control, were cultured in medium supplemented with exogenous (±)-NA. Acetone extracts from fermentations of all three strains were analyzed for macrotetrolide production by HPLC. No significant differences in either the total production yield or the distribution of individual macrotetrolides were observed when the wild-type strain was cultured in the presence of exogenous (±)-NA. In contrast, additions of (±)-NA to the fermentation medium only partially restored macrotetrolide production to SB2001 (Fig. 6C) and SB2003 (Fig. 6E); only NON, monactin, and dinactin were produced, but trinactin and tetranactin were not found. In contrast to the wild-type strain that produces dianactin as the major macrotetrolide, SB2001 and SB2003, in the presence of exogenous (±)-NA, synthesis of NON as the predominant product (NON, monactin, and dinactin at 100:20:1.6). The latter was, in fact, expected, since, unlike the formation of NON, which can be synthesized from the exogenously added (±)-NA, the formation of either monactin or dinactin requires one or two molar equivalents of endogenous (+)-HNA (Fig. 2B), respectively, the concentration of which is apparently very low in comparison with the large excess of (±)-NA added exogenously. Both NON and monactin were isolated and subjected to electron-spray mass spectral analysis with authentic samples of NON and monactin as references. The isolated NON and monactin yielded the characteristic ions as the authentic standards with molecular weights of 737.4 (M + H\(^+\)), 754.6 (M + NH\(_4\)\(^+\)), 759.7 (M + Na\(^+\)), and 775.6 (M + K\(^+\)) for NON and of 751.4 (M + H\(^+\)), 768.5 (M + NH\(_4\)\(^+\)), 773.6 (M + Na\(^+\)), and 789.6 (M + K\(^+\)) for monactin. Close examination of the macrotetrolide structures reveals that, in addition to the (±)-NA monomer, the production of monactin and dinactin requires only (+)-HNA, and the production of trinactin and tetranactin requires both (+)- and (−)-HNA. The synthesis of either (+)- or (−)-HNA, if abolished as the result of nonS inactivation, cannot be circumvented by exogenous supplementation of (±)-NA (Fig. 2B).

**DISCUSSION**

Polyketides are structurally classified into four major groups: aromatic polyketides, macrolides, polyenes, and polyethers. Recent advances in genetic and biochemical studies of polyketide biosynthesis have provided a mechanistic explanation of how PKSs achieve the vast structural diversity by varying the similar sets of biosynthetic reactions, leading to the successful cloning and characterization of multiple sets of gene clusters that encode aromatic polyketide, macrolide, or polyene biosynthesis (22). However, genetic analysis of polyether biosynthesis has heretofore met with little success, despite considerable effort. The only published reports were the cloning of *Streptomyces longisporoflavus* in *nrB1* and *nrB2*, which confer tetracosin resistance, by Leadlay and coworkers (28); the cloning from *S. griseus* of nonR, which confers tetracosin resistance, by Plater and Robinson (37); and the cloning from *S. griseus* of nonS, which exhibits nonactate synthase activity upon heterologous expression, by Priestley and Earle (39). Very recently, Priestley and coworkers (51) also reported on the DNA sequence analysis of 15.5-kb flanking the *nonRS* genes. While those studies certainly have set an excellent stage for further investigation, they fell short of proving the direct involvement of the cloned genes in either tetracosin or macrotetrolide biosynthesis. Thus, our report here on the genetic localization of a 55-kb contiguous DNA region, inactivation of *nonS* in *S. griseus* for the generation of macrotetrolide-nonproducing mutants, the in vivo complementation of *nonS* mutants by expression of the *nonS* gene in *trans*, and the fermentation of the *nonS* mutants in the presence of exogenous (±)-NA for the restoration of macrotetrolide production established unambiguously the successful cloning of the *non* biosynthesis gene cluster. As it has been demonstrated for genetic characterizations of *nonS*, we envisage that the genetic system developed here for *S. griseus* should enable us to analyze in detail the entire non gene cluster involved in macrotetrolide biosynthesis.

The intriguing molecular topology of the macrotetrolides, which consists of the (+)(−)(+)−ester linkage of the enantiomeric NA and its homologs, has inspired us and others (45, 53) to propose that the biosynthesis of these enantiomeric polyketide intermediates results from a pair of enantiospecific pathways (Fig. 2A). This hypothesis is consistent with the efficient and enantiospecific incorporations of the N-capryllysine thioester of both (6′R,8R)-NEA and (6′S,8S)-NEA into NON (45) and with the in vivo transformation of the racemic N-octyllysine thioester of NEA into NA by a cell-free preparation made from either an *S. griseus* or an *S.
lividans strain that heterologously expresses the nonS gene (53). It is imagined that a pair of enantiospecific PKS enzymes catalyzes the assembly of the two enantiomers of NEA from one molecule each of acetate, propionate, and succinate and that, on the basis of the paradigm of PKSs, the occurrence of NEA homologs—consequently, HNA and BNA—resulted from a less-than-strict starter unit specificity exhibited by the PKS enzymes. To test this hypothesis, we set out to inactivate nonS by targeted gene disruption (SB2001 mutant strain) or gene replacement (SB2003 mutant strain). We reasoned that inactivation of nonS should abolish the biosynthesis of at least one set of the enantiomeric monomers, consequently rendering the nonS mutants macrotetrolide nonproducers, as depicted in Fig. 2B. Examination of SB2001 and SB2003 confirmed that macrotetrolide production has been completely abolished in both strains (Fig. 6B and D). Complementarily, the nonS mutation can be fully complemented in trans by a plasmid-born copy of nonS. Thus, introduction of pBS1012—in which the expression of nonS is under the control of the ermE* promoter—into SB2003 not only restored macrotetrolide production to the mutant strain to the wild-type level but also restored the distribution of individual macrotetrolides so that it was similar to that in the wild type (Fig. 6A versus G). These results not only confirm that the abolishment of macrotetrolide production in SB2003 results from the specific inactivation of nonS but also indicate that NonS alone is responsible for the synthesis of at least one enantiomeric set of NA and its homologs.

To probe if nonS governs only one or both enantiospecific pathways that lead to the synthesis of (−)- or (+)-NA and its homologs, we cultured SB2001 and SB2003 in the presence of exogenous (±)-NA. We reasoned, as summarized in Fig. 2B, that if nonS governs both enantiospecific pathways, addition of (±)-NA to the nonS mutants should result in the production of NON only because the synthesis of the other macrotetrolides requires endogenous (−)- or (+)-HNA, or both. The biosynthesis of the latter has been abolished as the result of nonS inactivation. The same phenotype could also occur, however, if nonS is responsible only for the synthesis of (±)-NA and its homologs, since NON is the only macrotetrolide whose biosynthesis does not require (±)-HNA. In contrast, if nonS is responsible only for the synthesis of (−)-NA and its homologs, addition of (±)-NA to the nonS mutants should result in the production of NON, monactin, and dinactin; trinactin and tetranactin should not be found due to the lack of endogenous (−)-HNA. While no significant difference in either the total production yield or the distribution of individual macrotetrolides was observed when the wild-type strain was cultured in the presence of exogenous (±)-NA, only NON, monactin, and dinactin were detected from SB2001 (Fig. 6C) and SB2003 (Fig. 6E) upon fermentation in the presence of exogenous (±)-NA; the identities of these macrotetrolides were confirmed by both HPLC and electron-spray mass spectral analysis in comparison with authentic standards (dinactin was not analyzed by mass spectral analysis due to its trace quantity). These data agree well with the hypothesis that nonS is responsible only for the synthesis of (−)-NA and its homologs, providing strong support that macrotetrolide biosynthesis in S. griseus involves a pair of enantiospecific pathways. Indeed, sequence analysis of the non cluster has revealed an ORF 18.2 kb upstream of nonS. The deduced amino acid sequence of this ORF shows significant end-to-end similarity to NonS (21% identity and 29% similarity) as well as to the well-characterized mitochondrial 2-enoyl-CoA hydratase-1 (23% identity and 38% similarity) (31). NonS shows almost an identical level of homology to mitochondrial 2-enoyl-CoA hydratase-1 (25% identity and 37% similarity). Experiments are in progress to investigate if the latter enzyme, complementary to NonS, is responsible for the synthesis of (±)-NA and its homologs.

It remains obscure how modular PKS enzymes control the stereochemistry during the polyketide chain assembly at either the alkyl-branched center, such as C-2 of NA, or the oxygenated center (derived from the β-keto group of the polyketide intermediates), such as C-8 and C-6 of NA (Fig. 1B). Progress has recently been made in this direction by studying the 6-deoxyerythronolide B synthase in vivo or in vitro, leading to the finding that the stereochemistry of the alkyl-branched center or the oxygenated center is controlled by the ketoacyl synthase (KS) (10, 31, 52) or the ketoreductase (KR) (10, 25), respectively, in the specific cases examined. However, these studies fall short of providing a satisfactory explanation at the molecular level of how a given KS or KR exhibits its stereospecificity; sequence comparison among various KS or KR domains has also failed to reveal any conserved motifs that could potentially be ascribed to their opposite stereospecificities. We envisaged that PKS enzymes from a pair of enantiospecific polyketide pathways should provide an ideal model that could be used to study the stereochemistry of polyketide biosynthesis. Since these enzymes must recognize and process a set of enantio- meric substrates and intermediates, any difference in their primary amino acid sequences and secondary protein structures will provide an understanding of the factors by which PKS enzyme controls stereochemistry during polyketide biosynthesis. The current work indicates the existence of such a pair of enantiospecific polyketide pathways for macrotetrolide biosynthesis in S. griseus, setting the stage for in-depth investigations. Furthermore, enantiospecific pairs of enzymes are relatively rare in nature, and only in a few cases have such enzymes been isolated and characterized comparatively (44). Studies with individual enzymes for macrotetrolide biosynthesis, therefore, shall shed light on the formation of enantiomeric natural products in general. The cloning of the non cluster and the establishment of a genetic system in S. griseus have made such studies possible.

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