‘Streptomyces nanchangensis’, a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters

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Several independent gene clusters containing varying lengths of type I polyketide synthase genes were isolated from ‘Streptomyces nanchangensis’ NS3226, a producer of nanchangmycin and meilingmycin. The former is a polyether compound similar to dianemycin and the latter is a macrolide compound similar to milbemycin, which shares the same macrolide ring as avermectin but has different side groups. Clusters A–H spanned about 133, 132, 104, 174, 122, 54, 37 and 59 kb, respectively. Two systems were developed for functional analysis of the gene clusters by gene disruption or replacement. (1) Streptomyces phage φC31 and its derived vectors can infect and lysogenize this strain. (2) pSET152, an Escherichia coli plasmid with φC31 attP site, and pHZ1358, a Streptomyces–Escherichia coli shuttle cosmid vector, both carrying oriT from RP4, can be mobilized from E. coli into NS3226 by conjugation.

pHZ1358 was shown to be generally useful for generating mutant strains by gene disruption and replacement in NS3226 as well as in several other Streptomyces strains. A region in cluster A (~ 133 kb) seemed to be involved in nanchangmycin production because replacement of several DNA fragments in this region by an apramycin resistance gene [aac3(IV)] gave rise to nanchangmycin non-producing mutants.

Keywords: antibiotic biosynthetic genes, dianemycin, avermectin, polyketide synthase, gene replacement in Streptomyces

INTRODUCTION

Streptomycetes produce a wide variety of commercially important polyketide compounds, including the well-known macrolide, polyene and polyether antibiotics which exhibit antibacterial, antifungal, anthelmintic, antitumour and immunosuppressive activities. Biosynthesis of these antibiotics is catalysed by a large family of polyketide synthases (PKSs) using malonyl-CoA, methylmalonyl-CoA and ethylmalony-CoA as extender units for building the polyketide backbone (Hopwood, 1997; Katz, 1997; Leadlay, 1997).

Many gene clusters encoding the enzymes of polyketide biosynthesis have been cloned and characterized (see Schweke et al., 1995; Xue et al., 1998; Ikeda et al., 1999, for examples). Modular type I PKSs consisting of several large multifunctional proteins catalyse the biosynthesis of ‘complex’ or ‘reduced’ polyketides, the founding example being the erythromycin PKS (Cortes et al., 1990; Donadio et al., 1991). Combinatorial biosynthesis has been especially successful with many actinomycete PKSs following extensive structural and functional studies, and has led to the production of many ‘unnatural natural products’ (Cane et al., 1998; Hopwood, 1997; Hutchinson, 1998; Katz & McDaniel, 1999).

‘Streptomyces nanchangensis’ was isolated from the soil in Nanchang, China (Ouyang et al., 1984). ‘Str.
The structure of nanchangmycin, a polyether antibiotic with similar structure to diaminemycin. The 14 bonds presumably formed by the PKSs are numbered; bold lines indicate the building units. Acetate is used as the starter unit. Condensation steps 3, 6, 8, and 9 would involve malonyl extender units and the others, methylmalonate units. Condensation steps 10 and 11 would be followed by ketoreduction, and steps 3, 7, and 12 by ketoreduction and dehydration. Step 2, 4, 8, and 14 are followed by ketoreduction, dehydration, and enol reduction, and steps 1, 5, 9, and 13 by no keto group modification. Oxidation occurred at the methyl group of step 1 and double bonds are formed at steps 3 and 7. Six-membered ring formation would involve hemiketal formation between a keto double bond and a hydroxyl at step 11. Cascade cyclizations form between keto groups at step 1, 5, and 9, and oxidized double bonds at steps 3 and 7, respectively.

'N. nanchangensis' produces at least two kinds of insecticidal antibiotics (Ouyang et al., 1993). The polyether nanchangmycin (Fig. 1) structurally and biologically resembles diaminemycin (Czerwinski & Steinauf, 1971), which is used in poultry farming. The 16-membered macrolide meilingmycin (Fig. 2a) resembles milbemycin, which is used in poultry farming. The 16-membered nanchangmycin. Inhibition zones were visible after 12 h incubation at 37°C for growth of mycelium and isolation of total DNA, R2YE medium (2% soluble starch, 0.7% NaCl, 0.01% FeSO₄, 2% agar, pH 7.5) or YEME liquid medium (Kieser et al., 2000) at 28°C for growth of mycelium and isolation of total DNA, R2YE agar medium for transformation and protoplast regeneration (Kieser et al., 2000), YD medium (0.4% Difco yeast extract, 1% maltose extract, 0.4% glucose, 0.2% MgCl₂, 0.15% CaCl₂, 2% agar, pH 7.5) for conjugation and B2-1 liquid medium (3% corn powder, 1% corn starch, 1% soy bean powder, 0.2% KH₂PO₄, 0.05% MgSO₄, 0.05% NaCl, 0.2% (NH₄)₂SO₄, 1% CaCO₃, pH 7.5) for fermentation. Protoplast preparation was according to Hopwood et al. (1985).

E. coli strains were cultured according to Sambrook et al. (1989). Cosmid clones were selected after infection of E. coli LE392 on L agar containing 100 µg ampicillin ml⁻¹ or 10 µg apramycin ml⁻¹. For Streptomyces, apramycin and thiostrepton were both used at 10 µg ml⁻¹ in GS agar medium and at 5 µg ml⁻¹ in liquid media.

**METHODS**

**Bacterial strains, phages and plasmids.** These are described in Table 1. pHZ1351 was constructed by the incorporation of pIJ2925 [a pUC18 derivative with modified polylinker (Janssen & Bibb, 1993)] into the unique EcoRI site of pHZ209, a pIJ101-derived sti' vector (Z. Deng, unpublished). A spontaneous deletion that removes a 693 bp pIJ101 sequence (nt 7740–8432) and creates a NacI site at the junction (CGACGG–CGCCCTG) is localized in a region that is thought to be important for plasmid maintenance (Kieser et al., 1982). pHZ1358 is a derivative of pHZ1351 for cosmid cloning and conjugation from E. coli. pHZ1358 was constructed by first removing the two PsI fragments (0.35 and 0.95 kb) of pHZ1351 and then replacing the BglII fragment containing pIJ2925 with SuperCos 1 (Evans et al., 1989) linearized with BglII. Secondly, the 2.2 kb HindIII–XbaI fragment of SuperCos 1 was replaced with a 0.8 kb HindIII–XbaI fragment from pHZ132 (Hu et al., 1994) containing oriT.

**DNA probes.** Five probes from the Saccharopolyspora erythraea erythromycin PKS gene cluster (Bievitt et al., 1992; Donadio & Katz, 1992; Donadio et al., 1991) were excised from Escherichia coli plasmids kindly supplied by P. F. Leadlay (Cambridge University, UK) and purified from agarose gels using the Gene Clean kit (Bio101). The DEBS (6-deoxyerythronolide B synthase) probe is a 3.2 kb SacI fragment containing genes for KR4 (ketoreductase 4), ACP6 (acyl carrier protein 6), KS6 (ketosynthase 6) and AT7 (acyltransferase 7) domains; the KR4 probe is a 3.75 bp Smal fragment containing the gene for KR in the DEBS3 PKS; the AT7 probe is an 854 bp Smal fragment containing most of the gene for AT in the DEBS3 PKS; the KS5 probe is a 519 bp SacI–SatI fragment containing most of the fifth KS function of DEBS3; the TE (thioesterase) probe is a 621 bp Smal fragment containing the thioesterase-cyclase function of DEBS3.

**Culture techniques, transformation and conjugation.** 'Str. nanchangensis' NS3226 and its derivatives were grown on GS medium (2% soluble starch, 0.1% KNO₃, 0.05% K₂HPO₄, 0.05% MgSO₄, 7H₂O, 0.05% NaCl, 0.01% FeSO₄, 2% agar, pH 7.5) or YEME liquid medium (Kieser et al., 2000) at 28°C for growth of mycelium and isolation of total DNA, R2YE agar medium for transformation and protoplast regeneration (Kieser et al., 2000), YD medium (0.4% Difco yeast extract, 1% maltose extract, 0.4% glucose, 0.2% MgCl₂, 0.15% CaCl₂, 2% agar, pH 7.5) for conjugation and B2-1 liquid medium (3% corn powder, 1% corn starch, 1% soy bean powder, 0.2% KH₂PO₄, 0.05% MgSO₄, 0.05% NaCl, 0.2% (NH₄)₂SO₄, 1% CaCO₃, pH 7.5) for fermentation. Protoplast preparation was according to Hopwood et al. (1985).

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**Cloning techniques.** Plasmid and total DNA was isolated from Streptomyces strains using 'procedure 3' of Hopwood et al. (1985). For the generation of cosmid libraries, total DNA samples were partially digested with MboI, dephosphorylated with calf intestinal alkaline phosphatase and size fractionated by sucrose gradient centrifugation (Hopwood et al., 1985). The DNA fragments were mixed at a 1:1 molar ratio with BamHI-digested cosmid vectors and ligated at ~200 µg DNA ml⁻¹. Packaging was done with λ packaging mixes prepared according to Sambrook et al. (1989).

**Antibiotic assay.** Production of nanchangmycin was detected using a bioassay and HPLC. The strains were grown at 28°C for 5–7 d on GS agar medium. Agar plugs were transferred to L agar containing Bacillus cereus 1126, which is sensitive to nanchangmycin. Inhibition zones were visible after 12 h incubation at 37°C. For HPLC analyses, the strains were cultured in 40 ml B2-1 fermentation medium in 250 ml baffled flasks at 28°C and shaken at 220 r.p.m. for 7 d. After the mycelia were harvested by centrifugation at 600 g for 10 min,
Multiple PKS gene clusters of ‘Str. nanchangensis’

**RESULTS**

Hybridization with an erythromycin PKS gene probe from Sac. erythraea identified eight independent PKS gene clusters from ‘Str. nanchangensis’ NS3226

Probing Southern transfers of total DNA of NS3226 with a labelled 3–2 kb fragment from the DEBS3 erythromycin (ery) PKS gene (containing KR4, ACP6, KS6 and AT7 as a contiguous fragment) of Sac. erythraea involved in the biosynthesis of the 14-membered macrolide erythromycin gave many strong signals at low stringency (6x SSC, 65 °C). To isolate the hybridizing sequences a genomic library of NS3226 DNA was constructed using the bifunctional cosmid vector pHZ1351 (see Fig. 6b). Ninety cosmids with a mean insert size of 35–40 kb hybridized to the 3-2 kb ery probe at low stringency and were picked from a library with a total of 1920 clones. Comparison of the PvuII digestion patterns of the 90 cosmids revealed multiple overlapping bands. Moreover, at least one and up to 14 PvuII fragments from each of the 90 cosmids hybridized to the 3-2 kb ery probe, indicating a large number of partially homologous PKS-encoding sequences.

Ordering of the cosmids was achieved by comparing PvuII (and BamHI) digests of the cosmids. Cosmids producing similar bands were grouped and fractionated again on agarose gels until the most likely order of the cosmids was found (Figs 3 and 4). The order of the cosmids and restriction maps were further confirmed by Southern hybridization at high stringency (0.2x SSC, 65 °C) to prove the same origins of similar bands, using stepwise probes along the putative contigs (Figs 3 and 4). Seventy-five out of the 90 cosmids were thus grouped into eight independent clusters. Cluster A (Fig. 3) consists of 17 cosmid clones that hybridized to the ery PKS probe and spans ~ 133 kb of continuous DNA. An ~ 89 kb PKS-homologous region was located in the central part of the cluster; 28 kb flanking the left side, and at least 14 kb to the right of the PKS-homologous regions did not hybridize to the ery probe even at low stringency (6x SSC, 65 °C). Cluster B (Fig. 4) has an ~ 87 kb PKS-homologous region in a ~ 132 kb contig (17 cosmids) and cluster C (Fig. 4) has an ~ 86 kb PKS-homologous region in a ~ 104 kb contig (7 cosmids). PKS-homologous regions in other clusters (D, ~ 174 kb; E, ~ 122 kb; F, ~ 54 kb; G, ~ 37 kb; H, ~ 59 kb; Fig. 4), as detected using the 3–2 kb ery probe only containing KR4, ACP6, KS6 and AT7, are either not concentrated in one or two contiguous regions (e.g. clusters D and E in Fig. 4) or not as large as in clusters A–C (~ 10 kb in cluster F, ~ 11 kb in cluster G and ~ 33 kb in cluster H) along each contig.

Use of small probes encoding the KR4, AT7 and KS5 domains of the ery PKS refined the hybridization studies. The KR4, AT7 and KS5 probes give multiple signals in different regions of all the eight clusters (not shown), indicating that every gene cluster covered by the overlapping cosmids contains typical repetitive PKS modules. None of the cosmids clones, however, hybridized to the TE probe of the erythromycin pathway.

Localization of contigs to five different ‘Str. nanchangensis’ Asel fragments separated by PFGE

Asel digestion of ‘Str. nanchangensis’ NS3226 DNA generates ~ 19 macro-restriction fragments (Fig. 5) that can be separated by PFGE. The precise number of bands is not certain because several bands are clearly multiple, but ~ 17 bands can be detected in a single gel. These were transferred to nylon membrane and hybridized at high stringency (0.2x SSC, 65 °C) against representative cosmids from each cluster as probes. Clearly, 32P-

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The products were extracted with an equal volume of methanol. The extract was directly applied to HPLC on a Waters Xterra RP18 5 μm (3.9 x 150 mm) column. The mobile phase was acetonitrile/water (85:15) and the flow rate was 1 ml min⁻¹ at room temperature. The effluent was monitored at 243 nm with a Waters 996 photodiode array detector. The data were processed with a Waters Millennium Chromatography Manager.

**Sequence analysis.** Regions with spontaneous mutations in the pIJ101-derived vector pHZ1351 were sequenced using pBluescript II SK(+) or SK(−) as vectors. Sequencing reactions were done using the Amersham Thermosequenase sequencing kit containing fluorescent dye terminators. M13 reactions were done using the Amersham Thermosequenase and 4 min 60 °C. The bands. Moreover, at least one and up to 14 patterns of the 90 cosmids revealed multiple overlapping cosmids and restriction maps were further confirmed by Southern hybridization at high stringency (0.2x SSC, 65 °C) to prove the same origins of similar bands, using stepwise probes along the putative contigs (Figs 3 and 4). Seventy-five out of the 90 cosmids were thus grouped into eight independent clusters. Cluster A (Fig. 3) consists of 17 cosmid clones that hybridized to the ery PKS probe and spans ~ 133 kb of continuous DNA. An ~ 89 kb PKS-homologous region was located in the central part of the cluster; 28 kb flanking the left side, and at least 14 kb to the right of the PKS-homologous regions did not hybridize to the ery probe even at low stringency (6x SSC, 65 °C). Cluster B (Fig. 4) has an ~ 87 kb PKS-homologous region in a ~ 132 kb contig (17 cosmids) and cluster C (Fig. 4) has an ~ 86 kb PKS-homologous region in a ~ 104 kb contig (7 cosmids). PKS-homologous regions in other clusters (D, ~ 174 kb; E, ~ 122 kb; F, ~ 54 kb; G, ~ 37 kb; H, ~ 59 kb; Fig. 4), as detected using the 3–2 kb ery probe only containing KR4, ACP6, KS6 and AT7, are either not concentrated in one or two contiguous regions (e.g. clusters D and E in Fig. 4) or not as large as in clusters A–C (~ 10 kb in cluster F, ~ 11 kb in cluster G and ~ 33 kb in cluster H) along each contig.

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**Fig. 2.** The structure of meilingmycin (a), a macrolide structurally and biologically similar to milbemycin z11, and avermectin (b). Meilingmycin differs from avermectin in having no x-l-oleandrose attached at position 13 of the macrolide ring (implication of an extra or functional dehydratase and enoyl reductase domains for step 13) but has an isopantenoic acid moiety at position 4, which is probably derived from valine. The difference in the side group at position 25 probably reflects the flexibility of the starter unit in avermectin biosynthesis.
Table 1. Strains, phages and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Characteristics*</th>
<th>Source or reference</th>
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<tr>
<td>'Str. nanchangensis' strains</td>
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<td></td>
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<tr>
<td>NS3226</td>
<td>Wild-type producer for nanchangmycin and melilingmycin</td>
<td>Ouyang et al. (1984)</td>
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<td>SYH1</td>
<td>Non-producer for nanchangmycin generated by gene replacement of region N1 in cluster A (see Fig. 3)</td>
<td>This work (see Fig. 7a)</td>
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<tr>
<td>SYH2</td>
<td>As above, but replacing region N2 of cluster A (see Fig. 3)</td>
<td>This work</td>
</tr>
<tr>
<td>SYH8</td>
<td>As above, but replacing region N3 of cluster A (see Fig. 3)</td>
<td>This work</td>
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<td>Str. lividans 66 strains</td>
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<td>JT46</td>
<td>str-6 pro-2</td>
<td>Tsai &amp; Chen (1987)</td>
</tr>
<tr>
<td>E. coli K-12 strains</td>
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<td>DH15α</td>
<td>F– recA lacZΔM15</td>
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<td>ET12567</td>
<td>dam dcm hsdS</td>
<td>MacNeil et al. (1992)</td>
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<td>LE392</td>
<td>supE44 supF58 hsdR514; used for infection with in vitro-packaged cosmids</td>
<td>Borck et al. (1976)</td>
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<td>Bacillus subtilis strain</td>
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<tr>
<td>1126</td>
<td>Indicator strain sensitive to nanchangmycin</td>
<td>Ouyang et al. (1984)</td>
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<td>φC31 derivative; c+ att' tsr</td>
<td>Hopwood et al. (1985)</td>
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<td>φC31 derivative; c+ att' tsr vph</td>
<td>Hopwood et al. (1985)</td>
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<td>φC31 derivative; c+ att' tsr vph</td>
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<td>φC31 derivative; c+ att' tsr vph</td>
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<td>φC31 derivative; c+ att' tsr vph</td>
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<td>pHZ1358</td>
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<td>pIJ922</td>
<td>SCP2* derivative; tsr LonE</td>
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<td>pIJ61</td>
<td>SLP1.2 derivative; tsr LonE</td>
<td>Thompson et al. (1982)</td>
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<td>pJV1 derivative; tsr mel</td>
<td>Bailey et al. (1986)</td>
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<td>pSET152</td>
<td>aac3(IV) lacZ repα catα att' conf oriT</td>
<td>Bierman et al. (1992)</td>
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<td>pH2358 derivative of cosmid 11A8 used for replacement of region N1 in cluster A; tsr aac3(IV) Ltz LonE sti Ltz+ oriT</td>
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<td>This work (see Fig. 3)</td>
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*Ltz (lethal zygosis), pock formation caused by plasmid transfer; mel, tyrosinase gene for melanin production; oriT, origin of transfer of plasmid RK2; tsr, thiostrepton resistance gene; aac3(IV), apramycin resistance gene; sti, origin for second-strand synthesis of the multicopy plasmid pJJ101 (Deng et al., 1988).
fragments, together with cluster C, which were not differentiated further by digestions with DraI and SspI because both enzymes resulted in smearing and poor resolution of the chromosomal fragments on PFGE.

**A gene cloning system for 'Str. nanchangensis' NS3226**

The regeneration frequency of NS3226 protoplasts was only 0.02% (measured by comparing microscopic protoplast counts with the numbers of colonies on regeneration medium). Typically, about 6% of the colonies were derived from nonprotoplasted units that survived dilution in water. Several plasmids failed to give Thio<sup>R</sup> (thiostrepton resistant) NS3226 protoplast transformants, even with several micrograms of pure plasmid DNA. These included the sti<sup>−</sup> pIJ101 derivatives pIJ702 and pIJ486 isolated from Streptomyces lividans JT46; sti<sup>−</sup> pIJ101 derivatives pHZ1351 and pHZ1358 (Fig. 6) isolated from *E. coli* DH5α; SCP2<sup>+</sup>-derived low-copy number plasmid vector pIJ922 (Lydiate et al., 1985); SLP1.2-derived vector pIJ61 (Hopwood et al., 1985); pJV1-derived multi-copy number plasmid vector pWOR120 (Bailey et al., 1986), the last three isolated from *S. lividans* JT46. Transformation attempts using the denatured DNA of pIJ702 and pHZ1351 (Fig. 6a) (Oh & Chater, 1997) also failed. *Streptomyces* phage φC31 and its derived att<sup>+</sup> vector KC301, and att<sup>−</sup> vectors KC505, KC515, KC516, KC518, however, could infect 'Str. nanchangensis' NS3226 and form lysogens. Another *Streptomyces* phage, φHAU3, and its derivatives, pIJ8300 and pIJ8301 (Zhou et al., 1994) could also infect, but not lysogenize, NS3226.

The infection and lysogenization by φC31 and its derivatives of NS3226 prompted us to test the possibility of integrating φC31 attP-containing plasmid in NS3226. A pUC18-derived *E. coli* plasmid, pSET152 (Bierman et al., 1992), was chosen for this experiment as it carries the φC31 attP site as well as oriT from the *E. coli* conjugative plasmid RP4. Transfer of this plasmid by conjugation from *E. coli* to NS3226 could be tested to avoid difficult

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**Fig. 3.** Overlapping cosmids covering genes for nanchangmycin biosynthesis. Part of the 'Str. nanchangensis' genome containing genes for the biosynthesis of nanchangmycin. Vertical continuous and dotted lines indicate the positions of *BamH* and *Pvu*II sites, respectively. The 17 boxes below indicate individual cosmid clones. Hatching indicates regions hybridizing with the *Sac. erythraea* erythromycin PKS gene probes. Probes 1–8 (black stippled boxes) were used to confirm ordering of the positive cosmid clones. Replacement of sequences labelled N1–N3 abolished nanchangmycin production. The asterisks represent multiple *Pvu*II fragments whose exact orders have not been determined. A scale is presented below the clones.
protoplast transformation of the strain. pSET152 was transferred into NS3226 at a high frequency (≈ 10^-6 of the initial viable recipient population), and pSET152 was found to be integrated into the chromosome of strain NS3226 via attP in all of the exconjugants tested. pHZ1358 (Fig. 6b) can also be transferred by conjugation from E. coli ET12567 carrying an integrated copy of RP4 into NS3226, but it seemed to be very unstable (≈ 90% loss after one round of non-selective growth). This feature allowed pHZ1358 (Fig. 6b) to be used efficiently for generating NS3226 mutants by gene replacement (see below).
Cluster A contains genes for nanchangmycin biosynthesis

Cosmid 11A8, with a 37 kb insert in the middle of cluster A (Fig. 3), contains the tsr gene suitable for selection in Streptomyces. Its CoIE1 origin of replication is non-functional in strain NS3226. The pIJ101 origin of replication and sti (second-strand origin of replication) (Deng et al., 1988) are functional but result in extremely unstable replication in strain NS3226. For targeted gene replacement, 26 kb of the strain NS3226 DNA insert in cosmid 11A8 was replaced by a 1–3 kb apramycin resistance (AprR) determinant [aac3(IV)]. This was achieved first by complete digestion of 11A8 DNA by BamHI and religation to obtain pHZ1552 in E. coli. The internal 26 kb (marked ‘N1’ in Fig. 3) of the 37 kb insert in 11A8 was found to be deleted and was further replaced by a 1–4 kb BamHI fragment carrying aac3(IV) inserted between 4–8 kb (proximal to bla) and 5–8 kb (proximal to oriT) fragments flanking both sides of the deleted region, giving pHZ1553 (Fig. 7a).

pHZ1553 was transferred by conjugation from E. coli ET12567 carrying RP4 into strain NS3226. About 10^7 exconjugants per donor were obtained which were initially selected to be ThioR. These colonies were also AprR and thus presumably contained pHZ1553 either replicating autonomously or integrated into the host genome by homologous recombination. Such colonies were inoculated onto GS medium containing apramycin and screened by replica plating for thiostrepton sensitivity. About 90% of the colonies was ThioS and thus had probably lost the pIJ101-derived vector part of pHZ1553 (Fig. 7a, step 2). This was confirmed for two AprR ThioR isolates by probing a Southern transfer of total DNA (Fig. 7b) with a labelled 17-4 kb PstI fragment from pHZ1553 (Fig. 7a). Two of the presumed double crossover recombinants were tested for their ability to inhibit Bacillus cereus 1126. The NS3226 control gave inhibition zones of about 16 mm diameter while the two candidate strains grew normally but produced no inhibition zone. The non-production of nanchangmycin was further confirmed by HPLC analy-
**Fig. 7.** Schematic representation (a) of the replacement of part of the PKS gene cluster A in strain NS3226 using pHZ1553, constructed by substitution of the internal BamHI fragment of cosmid 11A8 (Fig. 3) by aac3(IV). Refer to (b) for details of other parts of the vector. A total of ~26 kb contiguous fragments between the two BamHI sites in NS3226 should be deleted and replaced by the 1-4 kb aac3(IV) fragment. The genome of SYH1 should have a 16–5 kb new PvuII fragment. B, BamHI; P, PvuII. (b) Ethidium bromide-stained agarose gel and Southern transfer probed with the labelled 17–4 kb PvuII fragment of pHZ1553 containing aac3(IV), and 4–8 and 5–8 kb flanking PKS-homologous regions. All the DNA samples were digested with PvuII. SYH1a and SYH1b are two independent Apr^R Thio^2^ derivatives of NS3226. The black arrow points to the 16–5 kb new fragment formed as a sum of 1–4 kb aac3(IV) plus 5–1 kb and part of the 18 kb PKS-homologous regions in NS3226 after gene replacement, which is clearly smaller than the original fragment in NS3226 as indicated by a white arrow. The positions of the size markers (1 kb ladder and λ DNA digested with HindIII) are indicated to the left and right of the gel, respectively. A 1–6 kb hybridizing signal detected in the 1 kb ladder (size markers) is the result of hybridization between this fragment (originating from pBR322) and part of the probe fragment carrying bla.

**Fig. 8.** HPLC analysis of nanchangmycin production by the strains after gene replacement. Nanchangmycin was extracted from the mycelium of NS3226 (a) and SYH1a (b) by methanol extraction. Pure nanchangmycin (c) was used as standard. Peaks corresponding to nanchangmycin can be seen in (a) and (c) but not in (b).
Another gene replacement experiment resulted in removal of ~120 kb DNA sequence, perhaps containing an almost complete gene cluster for nanchangmycin biosynthesis, from the chromosome of NS3226. Again, the vector (pHZ1586) was derived from pHZ1358 (Fig. 6b). The 8.4 kb DNA sequence flanking the leftmost BamHI sites (shown in Fig. 3) of the cosmid 2G2 is designated ‘left arm’ and 4.3 kb DNA sequence flanking the rightmost BamHI sites (Fig. 3) of the cosmid 2F7 is designated ‘right arm’ for the desired double crossovers, and aac3(IV) is inserted between the two BamHI sites. The NS3226 derivative SYH8 obtained after double crossing over was confirmed by Southern hybridization (not shown) to have the N3 region (Fig. 3) removed from the chromosome and no nanchangmycin production could be detected either by bioassay using B. cereus 1126 as the indicator strain or by HPLC analysis.

DISCUSSION

It is striking that a 3.2 kb heterologous type I PKS gene fragment from DEBS3 of the erythromycin producer Sac. erythraea strongly hybridized to 90 of the 1920 cosmids in the NS3226 cosmid library, corresponding to ~5% of the genomic sequence. Ordering of the hybridizing clones produced eight separate contigs each consisting of 2–17 cosmids clones and covering 37–174 kb. Respective single cosmid probes, from each of the eight PKS clusters hybridized to five different ‘Str. nanchangensis’ Asel fragments fractionated by PFGE, suggesting that multiple PKS gene clusters had been isolated. However, the presence of eight separate contigs here should not be simply taken as conclusive evidence that ‘Str. nanchangensis’ has eight independent PKS gene clusters encoding eight non-aromatic polyketide biosynthetic pathways, because none of them had been confirmed by DNA sequencing and only one of the gene clusters was verified to encode nanchangmycin production. The other known secondary metabolite produced by ‘Str. nanchangensis’, meilingmycin, is assumed to be encoded by another cluster, but we know nothing about the other six clusters whose encoded products could be relevant to polyketide synthesis.

Our data presented here imply that micro-organisms may encode many more secondary metabolites than are readily detectable in standard fermentation. While the overwhelming study of polyketide biosynthetic gene clusters in micro-organisms, especially Streptomyces, is experiencing many exciting breakthroughs, the genetic, and thus their encoded metabolite, diversities might still be far from thorough understanding. The identification of six to eight PKS gene clusters in a single Sorangium cellulosum strain, which is known to produce only epothilone, had also been reported by Santi et al. (2000). We suspect that type I PKS-like pathways could be a common theme among many different bacteria, apart from most commonly studied actinomycetes. Using conserved or even heterologous PKS probes for identifying unknown PKS gene clusters (or potential secondary metabolite pathways), especially in micro-organisms other than, for example, Streptomyces or Myxobacterium, for discovering the new polyketide compounds may have significant implications.

The identification and isolation of multiple PKS genes in a single strain could provide an opportunity for isolating ‘Str. nanchangensis’ derivatives that produce only one of the antibiotics but in increased quantities, as the biosynthesis of compounds derived from similar precursors in the same cell might be competitive. Thus an increase in production, or selective antibiotic production, could be achieved by selective cluster-specific mutagenesis by gene replacement, as we have demonstrated. Indeed, the deletion of a large DNA fragment by gene replacement (not shown) in cluster C (Fig. 4) resulted in at least a threefold increase of the nanchangmycin production as detected by bioassay, but no obvious increase for meilingmycin production was detected in the disrupted strains for clusters A–C (data not shown). An example of successful selective production of avermectin compounds, obviating the need for separation of avermectin and oligomycin by knocking out oligomycin production in an avermectin producer by Tn4560 mutagenesis had been reported by Ikeda & Omura (1995). The unknown or unwanted clusters could be deleted in a stepwise manner. Such targeted deletions should have no danger of introducing deleterious mutations elsewhere in the chromosome, which would otherwise affect, for example, cell growth.

A search of the isolated multiple PKS clusters for the ones that could be involved in nanchangmycin or meilingmycin biosynthesis led to the discovery of the cluster that is essential for the former. Several DNA fragments in clusters A–C respectively were disrupted or replaced for targeted mutations to achieve this result, although none of the three clusters were found to encode meilingmycin production. To our knowledge, the identification of a large region involved in nanchangmycin production would constitute a first documented example of what seems to be a complete polymer antibiotic biosynthesis gene cluster. The coverage of 89 kb PKS-homologous DNA (Fig. 3) involved in the biosynthesis of nanchangmycin (Fig. 1) strongly supports the hypothesis of a modular organization for polymer biosynthesis, as has been reported for macrolide and other antibiotics. We tend to think that the interruption of the 89 kb PKS-homologous region by a 12 kb PKS-nonhomologous region is not a division of two independent PKS clusters, but all necessary for nanchangmycin production. Based on erythromycin and avermectin examples, a ‘standard’ PKS module appears to be encoded by ~5 kb of DNA (Bevitt et al., 1992; Donadio & Katz, 1992; MacNeil et al., 1992). The DNA encoding the PKS for the nanchangmycin polymer production would require at least 70 kb of genetic information; enough for 14 modules. The 89 kb PKS-homologous region might be a maximal estimate because four PvuII end fragments flanking the two separate contigs (Fig. 3) may only have a partial PKS-homologous region. The most interesting and possibly unique enzymes for polymer biosynthesis would be the cyclase(s) that
catalyses the cascade of cyclizations to form the poly-
ether structure, but such new information will have to
wait until the gene cluster is sequenced: this work is now
in progress. The genetic information required for such
enzymes [or/and additional genes necessary for the side-
group (Fig. 1) biosynthesis] could either be located in the
12 kb DNA sandwiched between two PKS-homologous
regions or flanking both sides of the PKS-homologous
regions. The interrupted PKS regions in a single PKS
pathway had also been reported in other clusters,
including avermectin (MacNeil et al., 1992; Ikeda et al.,
1999).

‘Str. nanchangensis’ NS3226 is refractory to trans-
formation by plasmid DNA from E. coli or Str. lividans,
possibly because of the poor frequency of protoplast
regeneration, and/or strong restriction system(s). Such
problems were circumvented effectively by the develop-
ment of mobilized conjugation of Streptomyces—E.
coli shuttle cosmid vectors, also carrying oriT from RP4,
from E. coli to ‘Str. nanchangensis’. Most significant is
the construction and efficient utilization of replicative
Streptomyces plasmids derived from pIJ101 for gen-
erating mutations by targeted gene disruption and
replacement experiments, which were used in this study
for the demonstration that a specific gene cluster was
involved in antibiotic production. Different from the
suicide plasmids or temperature-sensitive Streptomyces
plasmid vectors frequently used so far for gene dis-
ruption and replacement, pHZ1351 and pHZ1358 (Fig.
6) are replicative but genetically very unstable in ‘Str.
nanchangensis’. Fewer than 1 in 1000000 colonies lost
thiostrepton resistance and thus still could carry plas-
mids after one round of non-selective growth. pHZ1351
(Fig. 6a) has a polylinker cloning site and can be
propagated both in Streptomyces and in E. coli.
PHZ1358 (Fig. 6b) has additionally the origin of transfer
of the conjugative plasmid RP4 for efficient conjuga-
tive transfer from E. coli to Streptomyces, and a phage λ cos
site flanked by T3 and T7 promoters for the efficient
generation of ordered cosmid libraries. It is not clear
whether sti has anything to do with the structural
stability of the plasmids as has been suggested by Zaman
et al. (1993) and Pigac et al. (1988) because there are also
structurally stable plasmids such as pIJ702 that lack this
function, and a case of instability of pIJ702 and its use
for gene disruption experiments had been reported in Sac.
erythraea (Weber et al., 1990). The extreme
segregational instability of pHZ1351 (Fig. 6a) and
PHZ1358 (Fig. 6b) is likely to be caused by a sponta-
neous deletion of the 693 bp fragment which we have
categorized between rep and sti, a region suggested to
be required for plasmid maintenance (Kieser et al.,
1982).

Successful gene replacement experiments using
PHZ1351 or pHZ1358 were also performed for muta-
genizing a peptide pathway gene in Streptomyces bygro-
scopicus 10-22 and a polyyne pathway gene in Strepto-
myces sp. FR008 in our laboratory (unpublished results)
and in other laboratories, for mutagenizing the milbe-
mycin PKS of Streptomyces griseochromogenes (P.
Dyson, personal communication) and the clavulanic
acid pathway of Streptomyces clavuligerus, a goal that
could not be achieved with many other vector plasmids
(P. Liras, personal communication).

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