Plasmid Loss and Changes Within the Chromosomal DNA of *Streptomyces reticuli*

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The sporulating wild-type strain of *Streptomyces reticuli*, which produces a melanin pigment and the macrolide leucomycin, contains plasmid DNA of 48 to 49 megadaltons. Plasmidless variants had an altered secondary metabolism and a changed antibiotic resistance pattern. By using a new colony hybridization technique developed for streptomycetes, it could be shown that plasmidless variants could be transformed with the wild-type plasmid DNA, which, however, is quickly lost from regenerated mycelium. In contrast to the wild-type strain, the plasmidless variants contain amplified nucleotide sequences within the chromosomal DNA. The number and size of these sequences vary with the strain tested. Hybridization studies revealed that the reiterated sequences are neither amplified ribosomal nor plasmid genes, but are present in small concentrations within the wild-type chromosome. Some of them share extensive homologies with each other and are located at different positions within the chromosome. It is assumed that alterations in secondary metabolism are due to changes within both the chromosomal and the extrachromosomal DNAs of *S. reticuli*.

In this paper, I present data on the loss of extrachromosomal DNA and concomitant changes within the chromosomal DNA of *S. reticuli*. I suggest that these DNA changes may affect the production of secondary metabolites and resistance to antibiotics in *S. reticuli*.

**MATERIALS AND METHODS**

**Strains.** *S. reticuli* was kindly provided by H. Zähner, University of Tübingen, Germany. *Bacillus subtilis*, *Micrococcus flavus*, and *Staphylococcus aureus* were obtained from the Deutsche Sammlung für Mikroorganismen culture collection, Göttingen, Germany.

**Media and buffers.** Minimal salt medium containing 2% Casamino Acids and tyrosine (75 μg/ml), sucrose-Casamino Acids-glycine (SCG; glycine, 0.25%) medium (18), S medium (11), and production medium (12) were used. SS medium contained (per liter): 4 g of yeast extract (Difco Laboratories), 4 g of peptone (Difco), 150 g of sucrose, 10 g of glucose, 10 ml of 5 M CaCl₂, 20 ml of 1 M MgCl₂, 10 ml of 2.5% K₂SO₄, and 20 μl of trace elements stock solution (11). NB medium contained 4 g of nutrient broth (Difco) per liter of distilled water.

TES buffer was composed of 0.03 M Tris-hydrochloride, 0.005 M EDTA, and 0.15 M sodium chloride. 1× SSC buffer contained 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0.

**Reagents.** L-Dopa (3,4-dihydroxy-L-phenylalanine) and cesium chloride were obtained from Merck AG, polyethylene glycol was from Koch Light Laboratories, ethidium bromide was from Serva, and agarose (ME and Seaplaque) was from Seakem. Ficoll (molec-
ular weight, 400,000) was purchased from Sigma Chemical Co. (α-32P)dCTP (specific activity, 400 Ci/mmoll was from New England Nuclear Corp., and nucleic acid filters (BA 85) were from Schleicher & Schuell.

Antibiotic activity. Strains of streptomycetes were grown for 24 to 80 h on plates containing production medium. Agar cylinders were laid on NB soft agar plates seeded with the indicator microorganism. Plates were incubated for 18 h and viewed for zones of growth inhibition.

Tyrosinase and β-lactamase activities. Mycelia were pregrown in production medium for 48 to 60 h. To test for intracellular enzyme activities, mycelia (1 g) were washed twice with 0.05 M NaPO4 buffer, pH 7.4, resuspended in 10 ml of the same buffer, and sonicated 10 times (30 s each) with the microtip of a Branson sonifier. The suspension was centrifuged in a JA 20 rotor, using a Beckman centrifuge at 20,000 rpm for 10 min. Proteins were precipitated with ammonium sulfate (0 to 50% and 50 to 100% saturation). To test for extracellular activities, proteins were precipitated from the culture filtrates with ammonium sulfate as described above. (NH4)2SO4 precipitates were dissolved in 1 ml of 0.05 M NaPO4 buffer, pH 7.4, containing 30% glycerol. Samples (10 to 100 μl) were taken to test for tyrosinase activities with the substrate L-dopa (3,4-dihydroxyphenylalanine) and for β-lactamaose activity with the substrate nitrocefin (Glaxo) (7, 9).

Growth conditions and isolation of DNA. A 10 ml amount of SCG medium was inoculated with spores of S. reticuli harvested from a slant or with 0.1 to 0.5 ml of a mycelium stock frozen at −20°C in culture medium containing 30% glycerol. Cultivation followed at 30°C in an orbital shaker. This preculture was diluted after 24 h in 150 ml of SCG medium and grown for another 48 h at 30°C. Mycelia were then harvested and washed in 25% sucrose. Mycelia (2 g) were suspended in 30 ml of 25% sucrose in 10 mM Tris-hydrochloride−50 mM EDTA, pH 7.4, and protoplasts were formed by the addition of lysozyme (4 mg/ml) after 10 to 20 min at 30°C. Protoplasts were lysed in the presence of proteinase K (100 μg/ml) and 0.5% sodium dodecyl sulfate (SDS). After incubation at 30 min at 30°C, the SDS concentration was raised to 1%, and the mixture was incubated for another 30 min. A 0.2 volume of 5 M NaCl was added, and, after 2 h at 0°C, the lysates were centrifuged (30 min, 5°C, 48,000 × g). Then 0.2 volume of 50% polyethylene glycol (molecular weight, 6,000) in TES buffer was added to the supernatant (2 h, 0°C). The ensuing precipitate was collected by low-speed centrifugation and dissolved in 8 ml of TES buffer. Extrachromosomal DNA was isolated after two cycles of isopycnic centrifugation in CsCl-ethidium bromide gradients, using Ti 60 (Beckman) and TV 850 (Sorvall) rotors. Ethidium bromide was removed with isoamyl alcohol from fractions containing DNA, and DNA solutions were dialyzed against 10 mM Tris-hydrochloride−1 mM EDTA, pH 7.4. If necessary, the plasmid DNA solutions were treated with RNase (20 μg/ml) at 37°C for 30 min, phenol-extracted, and precipitated with 0.1 volume of 10 M LiCl and 2.5 volumes of ethanol (2 h, −20°C). The precipitate was recovered by centrifugation, washed with 70% ethanol containing 1 M LiCl, and then washed with absolute ethanol. The pellet was redissolved in 10 mM Tris-

hydrochloride−1 mM EDTA, pH 7.4. DNA was further analyzed on agarose gels (see below).

Total DNA was purified from lysates (see above) as described earlier (8).

Isolation of radioactive RNA. S. reticuli was grown in 50 ml Tris-hydrochloride-buffered (pH 7.4) SCG medium containing only 0.05% Casamino Acids and 1 μCi of 32P. Mycelia were harvested after 4 days, protoplasm with lysozyme, and finally lysed with SDS (see above). The viscous lysate was extracted twice with a mixture (1:1) of chloroform and phenol saturated with 1 M Tris-hydrochloride, pH 8.0. DNA and RNA were precipitated from the aqueous phase with 2.5 volumes of ethanol in the presence of 1 M LiCl (−70°C, 20 min). The precipitate was collected by centrifugation and dissolved in 4 ml of 10 mM Tris-hydrochloride−1 mM EDTA, pH 7.4, and 1 ml of 10 M LiCl was added (0°C, 2 h) to precipitate rRNA and mRNA. After centrifugation, the pellet was redissolved in 7.5 ml of 50 mM Tris-hydrochloride buffer, pH 8.0, containing 1 mM EDTA and 10 mM sodium bisulfite. Then 7.29 g of KI was added and dissolved. The solution was centrifuged in a Ti 50 rotor (20°C, 47,000 rpm, 4 l h) to equilibrium, and fractions were collected from the bottom of the gradient. Labeled fractions containing alkali-labile material were pooled and purified by a second isopycnic centrifugation. RNA-containing fractions were dialyzed and analyzed on agarose gels. This RNA (specific activity, 106 cpm/μg) was used for hybridization studies.

Enzymes and substrates. Sall was isolated from Streptomyces albus as described before (2), and EcoRI and Sau3A were kindly provided by H. Mayer, GFB, Braunschweig, Germany. BamHI was purchased from Bethesda Research Laboratories, and λ DNA was isolated as described before (4). DNA polymerase I and DNase were purchased from Boehringer Mannheim Corp., and RNase, proteinase K, and lysozyme were from Merck AG.

Assays for digestion with restriction enzymes. Cleavage of DNA was performed with the restriction enzymes BamHI, EcoRI, Sall, and Sau3A as described earlier (18).

Agarose gel electrophoresis. DNA was electrophoresed in a vertical agarose gel apparatus containing 0.75 to 1.5% agarose in TEA buffer (18). Gels were stained and photographed as described before (18).

Molecular weight estimation. Molecular weights of DNA fragments were determined by coelectrophoresis of an EcoRI digest of λ DNA (4) or with an EcoRI digest of SPPI phage DNA (13).

Preparation of DNA fragments. A 100-μg amount of chromosomal DNA of a plasmidless variant (type Va or Vb) of S. reticuli (see below) was cleaved with Sall and loaded on four cylindrical gels (diameter, 1 cm) containing 0.75% low-temperature-melting Seaplaque agarose in 40 mM Tris-hydroxide, 20 mM sodium acetate, and 1 mM EDTA adjusted with acetic acid to pH 7.4. Fragments were separated by electrophoresis (24 h, 4 V/cm). Bands containing amplified DNA were cut out of stained gels, heated briefly at 75°C, loaded, and further purified on 1% Seaplaque agarose gels and then on a 1.5% agarose gel. Bands containing amplified DNA were dissolved in 1 ml of a saturated KI solution in 50 mM Tris-hydrochloride−10 mM Sodium bisulfite−1 mM EDTA (pH 7.5). Then 50 μl of an aqueous suspension of hydroxylapatite (200 mg/ml,
prewashed with 1 M KPO₄-buffer (pH 7, and water) was added. The suspension was agitated carefully at room temperature for 20 min. The hydroxyapatite was collected by centrifugation, and another 25 μl of the hydroxyapatite suspension was added to the supernatant. The adsorption and centrifugation procedures were repeated. The combined hydroxyapatite pellets were washed once with 1 ml of 50 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA containing KI (1.2 g/ml) and then once in distilled water. The DNA was eluted by suspending the hydroxyapatite in 100 μl of 0.5 M KPO₄ buffer, pH 7.5. After centrifugation (15,000 × g, 10 min) the supernatant was collected, and the pellet was extracted again. The combined supernatants were desalted by gel filtration on a small Sephadex G 50 column (50 mM Tris-HCl, 1 mM EDTA, pH 7.5). Aliquots (2 μl) of each fraction were dropped on glass fiber filters (GFB, Whatman) previously soaked in a ethidium bromide solution (4 μg/ml of TES buffer) and were analyzed under short wave UV light. DNA-containing fractions were pooled and precipitated with 1 M LiCl and 2.5 volumes of ethanol and redissolved in 10 mM Tris-hydrochloride–1 mM EDTA, pH 7.4.

**Nick translation and hybridization.** 32P-labeled plasmid DNA or DNA-fragments were prepared according to the procedure of Rigby et al. (14).

DNA fragments separated on agarose gels were transferred to nitrocellulose filters, using the Southern technique (20). Filters were preincubated for 6 h at 68°C in 6x SSC, 1× Denhardt solution (20), and 50 μg of salmon sperm DNA per ml. Hybridizations were performed in small plastic tubes (4.5 ml). The hybridization mixtures contained 2× SSC, 1× Denhardt solution, 0.1% SDS, 1 mM EDTA (pH 7.5), 50 μg of sheared, denatured salmon sperm DNA per ml, and about 0.01 μg of 32P-labeled DNA per ml (10⁶ to 5 × 10⁶ cpm). Before hybridization, the DNA was boiled for 10 min for denaturation. After hybridization at 68°C for 24 h, the filters were washed 10 times in 2× SSC–0.5% SDS at 68°C over a 50-min period. A final wash was performed in 0.1× SSC–0.5% SDS. The filters were dried under red light for 30 min. Kodak X-Omat films were used for autoradiography (−70°C, 5 to 30 h).

**Transformation with plasmid DNA.** Mycelia of plasmidless variants were grown in 50 ml of S medium containing 0.125% glycine for 30 to 36 h on a shaker. Mycelia were centrifuged, washed in 15% sucrose, suspended in 5 ml of 15% sucrose supplemented with MgCl₂, CaCl₂, K₂SO₄, and trace elements (amounts were the same as those described for SS medium), and lysozyme (1 mg/ml). Protoplasts were formed after 30 to 40 min at 30°C; they were then filtered through cotton wool, washed three times with 15% sucrose containing salts (see above), and resuspended in the same sucrose solution (2 × 10¹⁰ to 5 × 10¹⁰/ml). A 50-μl amount of this suspension was treated with 1 μg of supercoiled plasmid DNA in the presence of 20, 30, or 40% polyethylene glycol (molecular weight, 1,000) as described earlier (3). Appropriate dilutions were carefully spread on agar plates consisting of SS medium and incubated 3 to 4 days at 30°C.

**Colony hybridization.** Nitrocellulose filters pre-soaked in SS medium were firmly laid on plates containing 1× young colonies regenerated from protoplasts after treatment with plasmid DNA. After 30 min, the filters were carefully put on fresh SS medium agar plates (mycelia of the nonsporulating colonies facing the lid of the petri dish). Alternatively, regenerated colonies were streaked on filters lying on SS medium plates. Filters were incubated for 20 to 30 h at 30°C and were then placed in an empty petri dish, which was then filled with TES buffer and lysozyme (4 mg/ml). Protoplasts were obtained after 30 to 45 min at 30°C. To release and denature the DNA, filters were laid on a solution containing 1% SDS and 1 N NaOH for 30 min at 30°C. Filters were neutralized with a solution consisting of 7 ml of 1 M Tris-hydrochloride (pH 7) and 3 ml of 5 M NaCl (three changes), then briefly dipped in 96% ethanol to precipitate the DNA, and finally dried in a vacuum oven at 80°C. Hybridization studies were performed with labeled plasmid DNA as described above.

**RESULTS**

**Characterization of the wild-type and variant strains of S. reticuli.** *S. reticuli* is a sporulating strain which produces several brownish pigments, including melanin, which is synthesized in the presence of tyrosine (19). Tyrosinase activity could be identified in culture filtrates (extracellular activity) and within the mycelium (intracellular activity). The antibiotic leucomycin (and a series of related macrolides) active against several gram-positive microorganisms (*M. flavus, B. subtilis, and S. aureus*) is produced on agar plates (16). The strain was found to be resistant to the macrolide antibiotics cirramycin, oleandomycin, and chalcomycin, but sensitive to many other macrolides such as carbomycin, erythromycin, spiramycin, and tylosin. The strain is very susceptible to aminoglycosides (streptomycin and kanamycin) and tetracycline but resistant to low concentrations of chloramphenicol and rifampin and to high concentrations of nalidixic acid and penicillins. β-Lactamase activity can be identified in culture filtrates or within mycelia (Table 1).

As shown earlier this strain contains one large plasmid of 48 to 49 megadaltons (19). Variants could be isolated after treatment with acridine orange or ethidium bromide which lack covalently closed circular plasmid DNA. These variants (types Va and Vb) differ from the wild type because neither aerial mycelium and spores nor antibiotic substances active against gram-positive microorganisms are produced on agar plates. Depending on the variant tested, light yellow, brownish, or black-green but no melanoid pigments were observed. Neither intracellular nor extracellular tyrosinase activity could be identified. Like the wild type, variants of type V were prototrophs, but they were sensitive to chalcomycin, the antibiotic of the wild-type (leucomycin), rifampin, and penicillins (no β-lactamase activity) (Table 1).

**Transformation experiments.** To determine precisely the function of the extrachromosomal...
DNA of *S. reticuli*, transformation experiments were performed. Protoplasts of plasmidless variants (two independent isolates Va and Vb) were treated with purified plasmid DNA of the wild-type strain in the presence of polyethylene glycol. Protoplasts were regenerated in a newly developed medium (see above) and were then tested for the formation of aerial mycelia, spores, melanin, and antibiotic substances and for resistance to chalcomycin, rifampin, and penicillins. No transformants of these phenotypes were obtained among 10^7 to 10^9 regenerated protoplasts tested. Under similar conditions, protoplasts of *S. coelicolor* A3(2) could be transformed at a frequency of 10 to 30% with pHS1 DNA (3, 18).

To test whether uptake and replication of the wild-type plasmid occurs in variants of type V, a new colony hybridization technique developed for streptomycetes was used (see above). Mycelia arising from individual protoplasts were grown on nitrocellulose filters, laid on regeneration agar plates, and incubated for 20 to 30 h at 30°C. DNA was released from protoplasts obtained from young mycelia, denatured, and precipitated on nitrocellulose filters which were incubated with denatured 32P-labeled plasmid DNA of the wild-type strain. Autoradiographs of these filters revealed that up to 1% of the plasmidless variants (Va and Vb) hybridized after transformation to the plasmid DNA of the wild-type strain. Unfortunately, the transformed mycelium of type V variants was unstable, and the plasmid DNA was quickly lost as shown by colony hybridization or analyses of DNA from restreaked colonies (Fig. 1).

Further experiments were designed to investigate whether type V colonies contain alterations within the chromosomal DNA which could affect the stability of the plasmid DNA of the wild-type strain.

**Analyses of chromosomal DNA.** To test for a possible integration of plasmid DNA or other changes within the chromosomal DNA, total DNA of the wild-type strain and the two different type V (a and b) variants were isolated. The DNAs were cleaved with restriction enzyme BamHI, *SalI*, or *Sau3A*, and the resulting fragments were separated on agarose gels by using the cleaved plasmid of the wild-type strain as a reference (Fig. 2 to 4). Interestingly, although the digestion patterns revealed that most of the fragments of the wild-type DNA were identical to those of types Va and Vb, the DNA of type Vb colonies after cleavage with BamHI contained high concentrations of 4 fragments and about 15 to 20 fragments after cleavage with *SalI* or *Sau3A*. The numbers, sizes, and quantities of these fragments varied between the DNAs of the two different strains of type V (Fig. 2A and 4A). The amount of additional DNA represented about 5 to 15% of the total DNA as calculated from fluorescence intensities depicted on densitometer tracings (data not shown).

**Hybridization studies.** To investigate whether the DNA fragments present in high concentrations might represent amplified parts of the plasmid DNA, hybridization experiments were performed. Total DNAs of two variants (Va and Vb), of the wild-type strain, and also of the wild-type plasmid DNA were cleaved with the restriction enzyme *SalI*, separated on agarose gels, and blotted on nitrocellulose filters. These were then hybridized to 32P-labeled, denatured plasmid DNA of the wild-type strain. The results of these studies revealed that the variants Va and Vb contain within their chromosomal DNAs no integrated plasmid DNA. Depending on the variant tested, three to five fragments of the

**TABLE 1. Properties of S. reticuli strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aerial mycelium</th>
<th>Spores</th>
<th>Antibiotic (plates)</th>
<th>Melanin (tyrosinase)</th>
<th>Macrolides (chalcomycin and leucomycin)</th>
<th>Penicillins (β-lactamase)</th>
<th>Rifampin</th>
<th>Plasmid DNA</th>
<th>Chromosomal changes</th>
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<tr>
<td>Wild type</td>
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<td>Variant Va</td>
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<td>Variant Vb</td>
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**FIG. 1.** Autoradiograms of colony hybridizations. Colonies of the wild type (WT), of plasmidless variants (type Va or Vb), and of transformed, restreaked colonies (T) were grown on nitrocellulose filters. The DNA was released from these colonies (see text) and hybridized with denatured, labeled plasmid DNA isolated from the wild-type strain.
chromosomal DNA hybridized very weakly with the plasmid DNA (Fig. 2B).

Further hybridization studies were performed to show whether the amplified DNA regions are ribosomal genes. In vivo-labeled rRNA was hybridized to Southern blots of total DNA (cleaved with SalI) isolated from the variants and the wild-type strain. rRNA hybridized in all cases to two SalI fragments not identical in size to the amplified DNA regions (Fig. 2C).

Some of the amplified SalI fragments of type Vb, i.e., the double fragment 1 (1a, 2.8 kilobases [kb]; 1b, 2.25 kb), the double fragment 2 (2a, 2.0 kb; 2b, 1.8 kb), and fragment 3 (1.35 kb), were isolated from agarose gels (Fig. 3A) and purified. These fragments were then labeled by nick translation, denatured, and hybridized to nitrocellulose filters (see above) containing single-stranded SalI fragments derived from the chromosomal DNAs of different strains. Autoradiographs (Fig. 3B) revealed that the nucleotide sequences of each of the fragments (1, 2, and 3) were present within defined regions of the wild-type chromosome. However, within the cleaved chromosomal DNAs of the variants (Va and Vb), additional stretches of DNA (larger and smaller SalI fragments) shared homologies with one or the other of the three fragments. Also, the three labeled SalI fragments hybridize to each other and to each of the amplified BamHI fragments within the cleaved DNAs of plasmidless variants (Fig. 4B). The distribution and extent of homologous sequences varied according to the source of the chromosomal DNA.

**DISCUSSION**

The sporulating wild-type *S. reticuli* strain produces several secondary metabolites, contains one large plasmid, and is genetically unstable. As shown earlier (19), different variants with altered secondary metabolisms have been isolated. Most of them (class I, II, III, or IV) still contain plasmid DNA, but, depending on the
variant analyzed, this DNA may consist of a homogeneous or very heterogeneous population of large and small covalently closed circular DNA molecules (19).

To investigate the possible involvement of the wild-type plasmid in the expression of secondary metabolites, transformation experiments were performed with plasmidless variants of type V. It could be shown that these variants take up the wild-type plasmid. However, since it is unstable in variants of type V, the role of the plasmid genes for the production of melanin, the macrolide antibiotic leucomycin, the aerial mycelium, and spores and for the expression of antibiotic resistance could not be determined.

Interestingly, the plasmidless variants (Va and Vb) were found to contain alterations within their DNAs. Possibly, these affect the stability of the transformed wild-type plasmid DNA.

Detailed studies with restriction enzymes indicated the presence of reiterated sequences within the chromosomal DNA of the plasmidless variants. Some of these sequences share extensive homologies with each other, but their locations within the chromosomal DNA vary according to the strain tested. This is consistent with the model that, in addition to amplification, rearrangements and transposition of different DNA segments occur. The functions of these amplified DNA sequences, which are also present at low copy numbers within defined regions of the wild-type chromosome, are so far unknown. They represent neither amplified plasmid nor ribosomal genes. Depending on the plasmidless variant tested, a few chromosomal fragments hybridized very weakly with the plasmid DNA of the wild-type strain. This indicates the occurrence of a few homologous sequences within both the chromosomal and the extrachromosomal DNAs.

Meanwhile, it can be demonstrated (manuscript in preparation) that not only plasmidless variants but also some of the variants that contain deleted plasmids (19) possess similar reiterated sequences. None of the chromosomal alterations resulted in changes leading to auxotrophy. Therefore, it is assumed that chromosomal alterations happen within genes determining secondary metabolites and may contribute, together with loss or deletions of plasmid DNA, to observed changes in the secondary metabolism and in antibiotic resistance spectra.

Since my first report (16) on chromosomal changes in streptomycetes, repeated sequences
LITERATURE CITED


