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# MOLECULAR CLONING OF THE NOSIHEPTIDE RESISTANCE GENE FROM *STREPTOMYCES ACTUOSUS* ATCC 25421

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An 8.5 kb BamHI DNA fragment conferring resistance to nosiheptide, a peptide antibiotic of the 'thiostrepton group', was cloned from *Streptomyces actuosus* ATCC 25421 in *Streptomyces lividans* 1326. Two BamHI fragments of *S. actuosus*, the 8.5 kb fragment and an additional 3.0 kb fragment, hybridized with a thiostrepton resistance gene probe (pIJ30). The 8.5 kb fragment showed a relatively low degree of homology with the thiostrepton resistance gene. The restriction map of the nosiheptide resistance gene isolated here was significantly different from the map of the thiostrepton resistance gene previously published. 

Nosiheptide, also known as multhiomycin, is a peptide antibiotic produced by *Streptomyces actuosus* (1) and is used commercially as an animal feed additive to promote weight gain (2). Nosiheptide belongs to the 'thiostrepton group' of antibiotics which includes thiostrepton, siomycin, sporangiomycin and thiopeptin (3). Nosiheptide acts by binding to the 23S ribosomal RNA and ribosomal protein L-11 to inhibit the activities of elongation factors Tu and G (3). *S. actuosus* is resistant to the nosiheptide it produces by methylating an adenosine residue of the 23S ribosomal RNA to produce 2'-O-methyladenosine (3), similar to the mechanism of thiostrepton resistance by the thiostrepton producer, *S. azureus* (3). We describe the isolation and preliminary characterization of a DNA fragment cloned from *S. actuosus* which confers resistance to both nosiheptide and thiostrepton in *Streptomyces lividans*.

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**ABBREVIATIONS:** SSC, standard saline citrate; SDS, sodium dodecyl sulfate; aph, aminoglycoside phosphotransferase (neomycin resistance); tsr, thiostrepton resistance.

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## MATERIALS AND METHODS

*Streptomyces actuosus*, *Streptomyces azureus*, and *Streptomyces laurentii* were obtained from the American Type Culture Collection as ATCC 25421, 14921, and 31255, respectively. *Streptomyces lividans* 1326 was used as the nosiheptide and thiostrepton sensitive recipient in shot-gun cloning experiments. *Escherichia coli* JM83 was used to propagate plasmids for restriction endonuclease digestion analyses. The *Streptomyces* strains normally were grown in YEME (4) supplemented with 34% sucrose. *E. coli* was grown in LB and plasmids were introduced into *E. coli* by transformation using standard procedures (5). For strains harboring plasmids, the appropriate antibiotics were added to the media as follows (in μg/ml): thiostrepton, 50; nosiheptide, 50; neomycin, 10; ampicillin, 100; tetracycline, 15.

Chromosomal DNA, purified from *S. actuosus* according to the procedure of Kieser (6), was digested with BamHI for 60 min using 2 units of enzyme per μg of DNA. Plasmid pANT29, purified from *S. lividans* 1326, was digested with BamHI for 2 h, using 1 unit of enzyme per μg of DNA and treated for 15 min with 1 unit of calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). The DNA preparations in an insert to vector ratio of 4:1 were ligated overnight at 4°C using T4 DNA ligase (Boehringer-Mannheim) as described previously (7). Protoplasts of *S. lividans* were formed as described by Hopwood *et al.* (4). The ligated DNA was introduced into protoplasts of *S. lividans* by transformation and the protoplasts were regenerated to mycelia on R2YE medium as described previously (7). After 18 h of incubation on R2YE at 30°C, the transformants were challenged with a soft agar overlay of R2YE containing 50 μg of nosiheptide per ml. After 72 h of growth, nosiheptide-resistant colonies containing pANT29 with *S. actuosus* DNA inserts were picked over to fresh plates of YEME containing 50 μg of nosiheptide per ml.

Plasmids were obtained using the small scale preparation procedure described by Birnboim and Doly (8). Restriction analyses were carried out according to the specification of the enzyme supplier (Bethesda Research Laboratories, Gaithersburg, MD). DNA fragment sizes were determined by electrophoresis through 0.7% agarose gels. Plasmids to be used as probes were labelled by the $^{32}$P-oligolabelling procedure described by Feinberg and Vogelstein (9) using 50 μCi of [α-$^{32}$P]dCTP per μg of DNA. For hybridization analyses, chromosomal DNA was purified from *S. actuosus*, *S. laurentii*, and *S. azureus* according to the procedure of Kieser (6), and was digested with BamHI for 12 h using 2 units of enzyme per μg of DNA. Southern blots and hybridizations were performed as described by Maniatis *et al.* (5).

## RESULTS AND DISCUSSION

Construction of pANT29. Most available streptomycete plasmid vectors use the thiostrepton resistance gene from *Streptomyces azureus* as a selective marker (4). This resistance gene also confers resistance to nosiheptide. To construct a vector which could be used to clone the nosiheptide resistance gene in the absence of an active thiostrepton resistance gene, fragments of bacteriophage lambda DNA generated by restriction with EcoRV were ligated with EcoRV-digested pIJ61 (4) and this mixture was introduced into *S. lividans* 1326 by transformation. A neomycin-resistant, thiostrepton-sensitive transformant yielded plasmid pANT29 (Fig. 1) which carried the EcoRV fragment of lambda from 26821 to 28198 bp of the phage genome (10). This fragment added several restriction sites to the vector, in particular BamHI (Fig. 1).

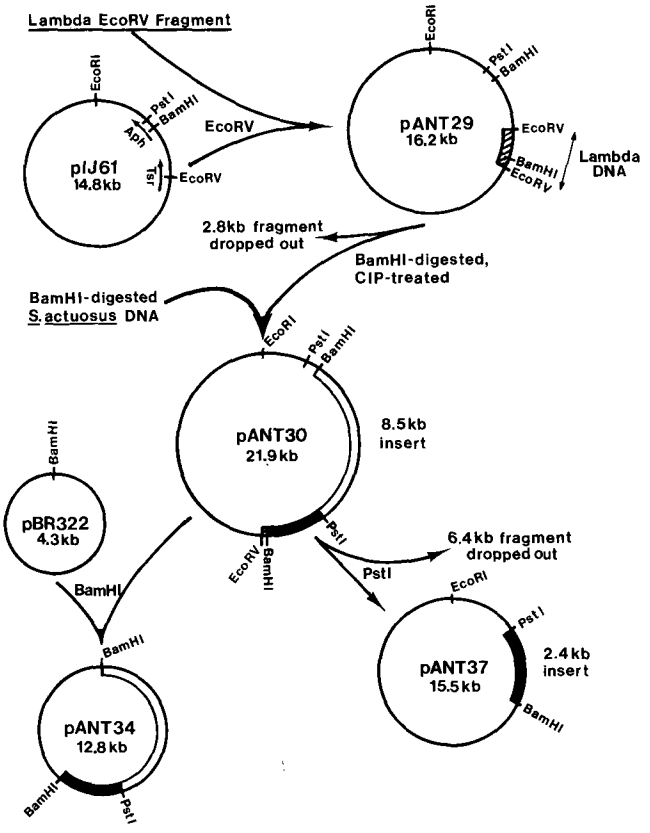


**Figure 1.** Construction of plasmids used in this study and cloning scheme for the nosiheptide resistance gene. Plasmids pANT30 and pANT37 both conferred resistance to nosiheptide and thiostrepton in *S. lividans* 1326.

Cloning of the nosiheptide resistance gene. Two nosiheptide-resistant transformants were obtained after transformation of *S. lividans* 1326 protoplasts with a BamHI-digested *S. actuosus* library in pANT29. The plasmids isolated from both of the nosiheptide-resistant *S. lividans* transformants contained an 8.5 kb BamHI insert. During the ligations, part of pANT29, including the 3' half of the thiostrepton resistance gene, was deleted (Fig. 1). When these new plasmids were digested with BamHI and probed with pIJ30 (11), the remaining part of the thiostrepton resistance gene and the 8.5 kb DNA insert from *S. actuosus* showed homology with the probe (data not shown). Plasmid pANT30, which contained the 8.5 kb BamHI *S. actuosus* DNA insert that shared homology with pIJ30, was introduced into *S. lividans* 1326 by transformation and again this plasmid conferred resistance to nosiheptide, as well as to thiostrepton. Plasmid pANT30 also restored thiostrepton and nosiheptide resistance to *S. azureus* AZ-6Y, a thiostrepton-sensitive, blocked (thiostrepton nonproducing) mutant of *S. azureus* ATCC 14921. Further subcloning of pANT30 indicated that the DNA which conferred

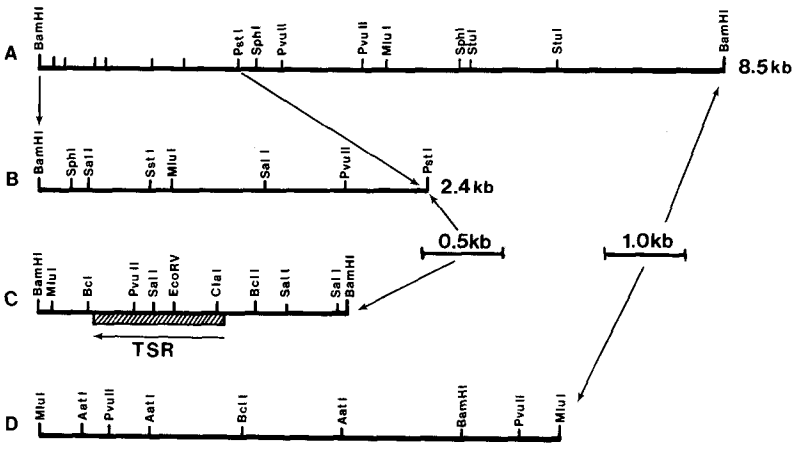


**Figure 2.** Comparison of restriction maps of the 8.5 kb DNA fragment cloned from *S. actuosus* (A), the 2.4 kb sub-clone of that fragment which confers resistance to nosiheptide (B), the thiostrepton resistance gene from *S. azureus* (C; ref. 11), and the 6.4 kb MluI DNA fragment described by Ohkishi *et al.* (15) which conferred nosiheptide resistance on *S. lividans* (D). Restriction endonucleases which did not cleave the 8.5 kb fragment were: BclI, BglII ClaI, EcoRI, EcoRV, HindIII, HpaI, KpnI, XbaI, and XhoI. Please note that AatI and StuI are isoschizomers.

resistance to nosiheptide resided wholly on a 2.4 kb BamHI-PstI restriction fragment of pANT37 (Fig. 1).

Structural characterization of nosiheptide resistance gene. The 8.5 kb BamHI fragment was subcloned in the *Escherichia coli* vector pBR322 to construct pANT34 from which a detailed restriction map of the insert was determined (Fig. 2). Because thiostrepton and nosiheptide are similar in chemical structure (12) and activities (3), and are cross-resistant, we originally hypothesized that the resistance genes might have a high degree of homology. As shown in Figure 2, the restriction map of the nosiheptide resistance gene was found to be very different from that of the thiostrepton resistance gene previously published (11).

Hybridization of chromosomal DNA preparations with pIJ30 and pANT34. Although the restriction maps of the nosiheptide and thiostrepton resistance genes showed no apparent similarities, there was sequence homology demonstrated by Southern blotting of the plasmids pANT30, pANT34, pANT37 with $^{32}$P-labelled pIJ30 (data not shown). When we confirmed this by probing BamHI-digested DNA isolated from *S. actuosus* with $^{32}$P-labelled pIJ30, the expected 8.5 kb fragment, as well as an additional 3.0 kb fragment, hybridized to the probe under moderate stringency conditions (Fig. 3). When BamHI-digested DNA from *Streptomyces azureus* was probed with $^{32}$P-labelled pIJ30, DNA fragments of ca. 6.1 and 9.0 kb in length hybridized with the plasmid (Fig. 3). The thiostrepton resistance gene of *S. azureus* was

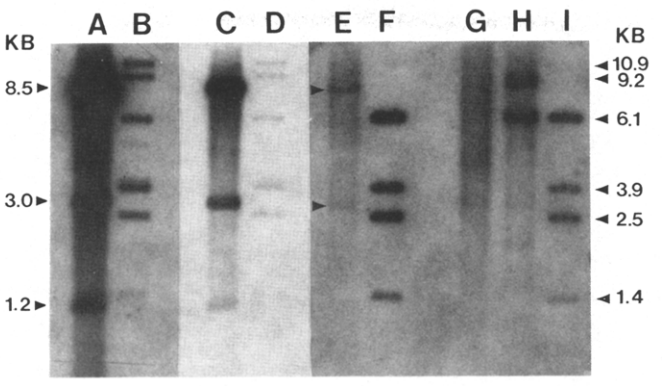


**Figure 3.** Southern blot analysis of BamHI-digested DNA isolated from *S. laurentii*, *S. azureus*, and *S. actuosus*. The Southern blots were hybridized with $^{32}$P-labelled plasmids, and washed twice for 15 min each at room temperature with 2x SSC and 0.1% SDS, followed by a third wash at 45°C with 0.4x SSC and 0.1% SDS. Lanes B, D, F, and I are standards made from digestions of pIJ941 which yield DNA fragments which contain the thiostrepton resistance gene; fragment sizes are given in the figure in kb (kilobases). Lanes A and C: *S. azureus* DNA digests probed with pANT34 and exposed to film for 17 and 3 days, respectively, showing DNA fragments of 1.2, 3.0, an 8.5 kb which hybridize with the probe. Lanes E, G, and H: DNA digests from *S. actuosus* (E), *S. laurentii* (G), and *S. azureus*, respectively, probed with pIJ30. The following amounts of BamHI-digested DNA were loaded into the respective lanes: A, C, and E, 2.5 μg of *S. actuosus* DNA; lane G, 1.0 μg of *S. laurentii* DNA; lane H, 1.0 μg of *S. azureus* DNA.

originally isolated on a 6.9 kb insert generated from an MboI partial digest of *S. azureus* chromosomal DNA which was ligated into BamHI-digested SLP1.2 to make pIJ6 (13). The MboI-BamHI junctions between the insert and vector DNA in pIJ6 were able to be cleaved with BamHI. Plasmid pIJ30 was formed by subcloning a 1.9 kb BamHI DNA fragment, containing the thiostrepton resistance gene from pIJ6, into pBR322 (11). Our data showing that pIJ30 hybridizes to 6.1 and 9.0 kb BamHI DNA fragments of *S. azureus* (Fig. 3) indicate that only one of the BamHI sites of the 1.9 kb insert in pIJ30 originated from *S. azureus* DNA.

*S. laurentii* ATCC 31255 is capable of producing 10.5 g of thiostrepton per liter in high-density fed-batch fermentations (14). Thus, it was very interesting that *S. laurentii* did not have DNA which was homologous with the thiostrepton resistance gene from *S. azureus*, especially since only moderately stringent conditions were used for these hybridizations (Fig. 3). This suggests that the thiostrepton resistance gene in *S. laurentii* has a markedly different sequence from the thiostrepton and nosiheptide resistance genes described here.

When BamHI-digested *S. actuosus* chromosomal DNA was probed with pANT34 (which contained the 8.5 kb BamHI fragment conferring nosiheptide

resistance), the 8.5 kb cloned fragment hybridized with itself strongly and also hybridized to DNA fragments of 3.0 and 1.2 kb in length (Fig. 3). The presence of hybridizing BamHI DNA fragments in *S. actuosus*, other than the 8.5 kb fragment, is intriguing because it suggests that there may be multiple copies of DNA which confer resistance to (and perhaps production of) nosiheptide. Ohkishi *et al.* (15) recently isolated a 6.4 kb MluI DNA fragment from *S. actuosus* NRRL 2954 (the same strain as ATCC 14921) which conferred nosiheptide resistance in *S. lividans* 1326. Their DNA fragment also shared homology with the thiostrepton resistance gene (15), but it had a markedly different restriction endonuclease pattern from that shown for the nosiheptide resistance-conferring 8.5 kb DNA fragment or the 2.4 kb subclone shown in Fig. 2. The restriction map of the nosiheptide resistance gene determined by Ohkishi *et al.* (15; Fig. 2D) is consistent with our data which showed that the 3.0 kb BamHI DNA fragment hybridized with both pIJ30 and pANT34 (Fig. 3). If the nosiheptide resistance gene isolated by Ohkishi *et al.* (15) is indeed present on the 3.0 kb BamHI hybridizing fragment (Fig. 3), then the entire gene would necessarily reside within the ca. 1.2 kb BamHI-MluI fragment shown at the right end of the restriction map (Fig. 2D). It is probable that the 1.2 kb BamHI fragment is DNA which hybridized to sequences flanking the resistance gene on the probe (pANT34).

The apparent redundancy of nosiheptide resistance genes in *S. actuosus* is interesting, particularly in light of recent reports showing the potential for gene duplication in streptomycetes. Recently, Henderson *et al.* (16) cloned and sequenced two protease genes from *Streptomyces griseus*. These two protease genes were homologous by Southern analysis but contained different restriction maps and had only a 54% amino acid sequence homology. This prompted them to suggest that the presence of a second gene was the result of gene duplication (16). Moreover, Stutzman-Engwall and Hutchinson (submitted to Proc. Natl. Acad. Sci.) recently found that four separate segments of DNA from *Streptomyces peucetius* ATCC 29050 hybridized to an actI (polyketide synthase) gene probe. Their data indicated that each DNA fragment contained a cluster of genes which could encode functions required for biosynthesis of anthracyclines (Stutzman-Engwall and Hutchinson, submitted). Hopwood also recently reported that four separate DNA clusters from the chlorothricin producer, *Streptomyces antibioticus*, hybridized to the actI gene probe (D.A. Hopwood, Plenary Lecture, Seventh International Symposium on the Biology of the Actinomycetes, Tokyo, Japan). It is tempting to suggest that a similar situation exists with the nosiheptide-thiostrepton resistance (and production?) genes of *S. actuosus*. We currently are attempting to clone the other pIJ34-hybridizing BamHI DNA fragments from *S. actuosus* for comparison to the nosiheptide resistance gene already cloned.

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