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Transfer of Streptomycin Biosynthesis Gene Clusters within Streptomycetes Isolated from Soil

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Streptomyces **strains isolated from soil were found to possess various numbers of genes from the streptomycin biosynthesis cluster. The strains missing genes from the cluster also lacked the ability to produce streptomycin. Two of the isolates which contain only part of the cluster are apparently recipients of a gene transfer event. The implications for the role of gene transfer in antibiotic evolution are discussed.**

Streptomyces species are mycelial, gram-positive bacteria that are readily isolated from soil and produce a diverse range of antibiotics. Streptomycin is one of the best-studied antibiotics at the biochemical and genetic levels. Streptomycin and related compounds (SARCs) are three-ringed structures principally derived from glucose-6-phosphate by a complex, branched biosynthesis pathway. The genetics of streptomycin production is well characterized in *Streptomyces griseus* N2-3-11 (10, 11), for which more than 25 clustered genes have been described (Fig. 1) that encode biosynthesis, regulatory, and transport functions.

Lateral transfer of genes between bacteria has been widely documented for traits under environmental selection: for example, antibiotic resistance (1, 9, 14) and catabolic functions (3, 15). Transfer of antibiotic biosynthesis genes has not been demonstrated, although there is indirect evidence that it occurs (4, 17, 18). If antibiotic production plays an important role in the natural environment, then selection and transfer of these antibiotic production genes might be expected.

A previous study implicated the transfer of the streptomycin resistance gene, *strA*, from an *S. griseus*-like donor to at least two streptomycetes isolated from Brazilian soil (ASB37 and ASSF15) (8, 18). The evidence for transfer was based on incongruent phylogenies derived from comparative analyses of 16S rRNA and *strA* gene sequences. This result led us to question whether other genes in the cluster had also been transferred. In this paper, we report the results of our analysis of five additional *str* genes and streptomycin production in six streptomycete isolates.

The *Streptomyces* strains analyzed in this study have been previously described (8, 18). All streptomycetes were grown on standard liquid and agar media (7) and stored as spores at −20°C. Streptomycete DNA was isolated by the method of Fisher (procedure 4) (7). One regulatory gene (*strR*) and four biosynthesis genes (*strB1*, *strF*, *strN*, and *strS*) were selected for examination in the six Brazilian isolates. These were representative of two of the three branches of the streptomycin biosynthesis pathway and dispersed throughout the cluster (Fig. 1). Genes were amplified by PCR with primers based on *S. griseus* sequences and then sequenced and/or probed to verify that they were homologous to the *S. griseus* gene. Chromosomal DNA Southern hybridization was used to confirm negative PCR results. All PCR mixtures contained 1.2 U of *Taq* enzyme (Gibco BRL), 25 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 200 nmol of deoxynucleoside triphosphates, and 30 nmol of each primer. Approximately 100 ng of genomic DNA was used in each reaction mixture. The volume was brought up to 50 μl and overlaid with mineral oil. The PCR protocol was the same for each primer pair, except for the annealing temperatures (TA). The protocol consisted of a 10-min denaturation step, followed by 35 cycles of 1 min at 94°C, 1 min at TA, and a 1-min extension at 72°C. A final incubation for 7 min at 72°C ensured completion of strand synthesis, with various annealing temperatures for the different primer pairs. Two PCR products for each primer pair were prepared for sequencing by purification of single band products by using Microcon spin column concentrators (Amicon) following the manufacturer's instructions. One microgram of DNA was then subjected to cycle sequencing with dye terminators (ABI model 373A automatic sequencer) with 3.2 pmol of the forward or reverse primer in each sequence reaction. The primer sequences are available from the authors upon request. Products for Southern blotting were transferred onto positively charged nylon membranes (Hybond N+; Amersham) and, following probing, were washed at high stringency according to the manufacturer's instructions. Probes were generated by PCR from *S. griseus* DNA or from plasmids containing genes of the streptomycin biosynthesis gene cluster of *S. griseus* as the template. Probe DNA was labelled by random-primed labelling with [γ-^{32}P]dGTP.

The gene *strB1* was amplified from all strains (TA, 62°C). Partial sequence analysis of *strB1* (244 bp from the beginning of the gene sequenced with the forward PCR primer) showed that all of the Brazilian isolates shared a very high level of identity with *S. griseus* (Table 1). Products were obtained from all strains with primers derived from *strR* (TA, 64°C), and *strR* was confirmed as the correct gene by sequencing with the forward primer (or probing in the case of ASB27). PCR amplification was attempted for *strF*, *strN*, and *strS* (TA, 62, 64, and 64°C, respectively). In the case of *strF*, ASSF15, ASB27, and ASB37 gave single products with sizes similar to those observed for ASSF13, ASSF22, ASB37, and *S. griseus* (Fig. 2B); however, probing (Fig. 2C) and sequencing demonstrated that the PCR products were not produced by the correct gene. A combination of sequencing and probing showed that the correct products were also obtained for *strN* and *strS* in ASSF13, ASSF22, and ASB33 (Table 1 and Fig. 2). The other three strains were negative for these genes. Probing of chro-

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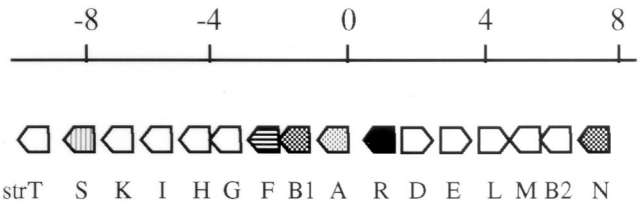

FIG. 1. Partial streptomycin biosynthesis cluster for genes flanking *strA*. (The figure is reproduced with permission from W. Piepersberg [11a].) The genes analyzed in this study are shaded. The bar represents distance in kilobases. , aminotransferase, biosynthesis; , presumed epimerase, biosynthesis; , amidinotransferase, biosynthesis; , phosphotransferase, streptomycin resistance; , regulation.

TABLE 1. Distribution of streptomycin biosynthesis genes in *Streptomyces*

Strain or species	Presence of gene[a]				
	strB1	*strR*	*strF*	*strN*	*strS*
ASSF13	+ (100)	+	+	+	+
ASSF15	+ (100)	+	−	−	−
ASSF22	+ (99.5)	+	+	+	+
ASB27	+ (99.1)	+	−	−	−
ASB33	+ (99.6)	+	+	+	+
ASB37	+ (99.1)	+	−	−	−
Streptomyces griseus	+ (100)	+	+	+	+
Streptomyces glaucescens[b]	+ (82.4)	+	+	+	+
Streptomyces bluensis[b]	+ (83.3)	+	+	+	Not known

[a] Values in parentheses represent the percentage of homology to the + gene.
[b] Sequence from database.

mosomal DNA confirmed that only these three strains had these genes. For those genes sequenced, homologies to the *S. griseus* counterparts were as high as those observed for the *strB1* genes. The results for all genes are summarized in Table 1.

Two *Escherichia coli* strains were used for streptomycin production assays: ATCC 29842 (streptomycin resistant) and ATCC 29839 (isogenic streptomycin-sensitive strain). The Brazilian isolates, *S. griseus* N2-3-11, and *Streptomyces lividans* TK23 were streaked on Trypticase soy broth agar plates and incubated for 5 days at 30°C. Duplicates were then overlaid with overnight cultures of the two *E. coli* strains in soft agar and incubated overnight at 37°C. Plates were examined for clear zones. Inhibition zones were detected around colonies of ASSF13, ASSF22, ASB33, and *S. griseus* by using *E. coli* ATCC 29839. No zones were detected for strains ASSF15, ASB27, and ASB37 and for *S. lividans*. No zones were observed on the plates overlaid with ATCC 29842 for any *Streptomyces* spp. that were examined.

The results from this study have shown that in addition to *strA*, two flanking genes in the streptomycin cluster, *strB1* and *strR*, were nearly identical in a set of phylogenetically diverse streptomycetes, including *S. griseus* and six isolates from a region of Brazil. Two of these isolates (ASS13 and ASSF22) were shown to be synonyms of the type strain of *S. griseus* from sequence analysis of 16S rRNA and tryptophan synthase genes (18). These strains produce streptomycin, possess at least six *str* genes, and most likely possess the entire *str* cluster. The remaining four strains (ASSF15, ASB27, ASB37, and ASB33) were shown not to be closely related to *S. griseus* or other SARC producers, and yet all possessed at least two additional genes from the cluster. ASSF15 and ASB37 have previously been shown to be very closely related to each other (18).

Analysis of the non-*S. griseus*-like isolates (ASSF15, ASB27, ASB37, and ASB33) suggests that they possess various parts of the streptomycin cluster. ASSF15, ASB27, and ASB37 possess *strA*, *strB1*, and *strR*, but do not appear to have *strN*, *strF*, or *strS*, nor do they produce streptomycin. The remaining strain, ASB33, does produce streptomycin and has all of the genes we examined. This could be explained by transfer of the entire gene cluster as a functional unit into this isolate. Based on the phylogenetic relationships between these strains (18), *strA*, *strB1*, and *strR* have probably transferred into ASSF15 and ASB37. Analysis of a more phylogenetically informative gene (*trpBA*) (5) has shown that ASB27 is very closely related to ASB33, and both are different from *S. griseus*, yet ASB27 was missing many genes from the cluster. A possible explanation for this result is that part of the cluster was lost from ASB27, as has been reported for hydroxystreptomycin genes in *Streptomyces glaucescens*, another SARC producer (2). ASB27 had an unstable phenotype for sporulation and pigment production (5a), which has been shown to occur via large DNA deletions in other streptomycetes (6).

Secondary metabolic pathways are thought to have arisen from adaptive extensions of essential primary pathways within ancestral organisms (13). With this model, duplication of primary metabolic genes and mutation of the copies resulted in novel proteins which then catalyzed reactions converting primary pathway intermediates into new products (16). The majority of the secondary metabolites probably originated with a small number of early successful reactions that evolved into many different pathways by incorporation of additional genes. Horizontal transfer of these "core" biosynthetic pathways followed by posttransfer refinements may explain the diversity of secondary metabolic pathways (16). For example, a number of *str* genes (*strD*, *strE*, and *strM*) have homologs in the cluster encoding avermectin production (12), which could be explained by past transfer events and subsequent divergence. The transfer of *strB1* into ASSF15 and ASB37 could also result in

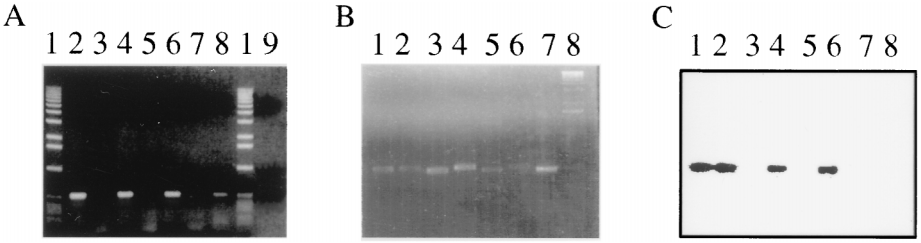

FIG. 2. PCR amplification and probing of the *strN* (A) and *strF* (B) genes. (C) Southern analysis of *strF*. (A) Lanes: 1, 1-kb ladder; 2, ASSF13; 3, ASSF15; 4, ASSF22; 5, ASB27; 6, ASB33; 7, ASB37; 8, *S. griseus*; 9, negative control. (B) Lanes: 1, *S. griseus*; 2, ASSF13; 3, ASSF15; 4, ASB22; 5, ASB27; 6, ASB33; 7, ASB37; 8, 1-kb ladder. (C) Same as for panel B.

this gene becoming involved in the production of other novel secondary metabolites. Further studies should help to clarify the roles of gene duplication, transfer, and divergence in the evolution of antibiotic production.

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