Use of \textit{rpsL} for Dominance Selection and Gene Replacement in \textit{Streptomyces roseosporus}

THOMAS J. HOSTED AND RICHARD H. BALTZ*

Lilly Research Laboratories, A Division of Eli Lilly and Company, Indianapolis, Indiana 46285

Received 19 July 1996/Accepted 22 October 1996

We developed a gene replacement system using the \textit{rpsL} gene of \textit{Streptomyces roseosporus} and demonstrated its utility by constructing a deletion in the \textit{S. roseosporus} \textit{glnA} gene. A 1.3-kb \textit{BamHI} fragment that hybridized to the \textit{Mycobacterium smegmatis} \textit{rpsL} gene was subcloned from an \textit{S. roseosporus} cosmID library and sequenced. Plasmid pRHB514 containing the \textit{rpsL} gene conferred streptomyces sensitivity (Sm\textsuperscript{s}) to the Sm\textsuperscript{r} \textit{S. roseosporus TH149}. The temperature-sensitive plasmid pRHB543 containing \textit{rpsL} and the \textit{S. roseosporus} \textit{glnA} gene disrupted with a hygromycin resistance (Hm\textsuperscript{r}) gene was introduced into \textit{S. roseosporus} TH149, and recombinants containing single and double crossovers were obtained after a temperature increase. Southern hybridization analysis revealed that single crossovers occurred in the \textit{glnA} or \textit{rpsL} genes and that double crossovers resulted in replacement of the chromosomal \textit{glnA} gene with the disrupted \textit{glnA}. Glutamine synthetase activity was undetectable in the recombinant containing the disrupted \textit{glnA} gene.

Streptomyces are commercially important microorganisms that produce secondary metabolites with diverse biological activities. Much is known about the organization and regulation of many secondary metabolic pathway genes, and these studies have been aided by gene disruption and gene replacement techniques (3). Gene replacement methods have also been used to insert genes into the chromosome to improve product yields and to produce novel products (3). Most of the procedures used for gene replacement or gene addition select for the first crossover integrating a plasmid, then screen for a second crossover that exchanges or inserts a gene and eliminates the remaining plasmid sequences. To develop a more versatile gene replacement system, we have explored use of the \textit{rpsL} gene in \textit{Streptomyces roseosporus}.

The \textit{rpsL} gene, which encodes ribosomal protein S12, has been used as a counterselectable marker since it confers dominant streptomyces sensitivity (Sm\textsuperscript{s}) in an Sm\textsuperscript{r} background (17). Gene replacement using \textit{rpsL} was first demonstrated in \textit{Escherichia coli} (24) and more recently in \textit{Mycobacterium smegmatis} and \textit{Yersinia pestis} (27, 31). \textit{E. coli} \textit{rpsL} has also been used as a counterselectable marker for gene replacement by heterologous expression in \textit{Vibrio cholerae} (30). A potential advantage of the \textit{rpsL} system in streptomyces is that Sm\textsuperscript{s} strains are not defective in primary metabolic functions and are readily isolated in most strains of interest, thus facilitating the use of the \textit{rpsL} system in many actinomycetes. We report here the cloning and use of the \textit{S. roseosporus} \textit{rpsL} gene for counterselection in \textit{S. roseosporus} to select double crossovers that replace the chromosomal \textit{glnA} gene with a disrupted \textit{glnA} gene.

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains and plasmids.}

The bacterial strains and plasmids used in this study are listed in Table 1.

\subsection*{Media and growth conditions.}

\textit{S. roseosporus} strains were grown in CSM media (13) at 29 or 39°C, fragmented into individual CFU by ultrasonic vibration (2), and grown on B agar (32). \textit{E. coli} strains were grown in TY media (26) or Cirecgrow media (Bio 101). Antibiotics were added to appropriate media for streptomyces strains at 30 \textmu g/ml for apramycin (AM), 200 \textmu g/ml for hygromycin (HM), 50 \textmu g/ml for nalidixic acid, and 50 \textmu g/ml for streptomyces (SM). For \textit{E. coli} strains, antibiotics were added at 100 \textmu g/ml for AM and HM. Auxotrophy was determined as described elsewhere (12) on CDA agar (32).

\subsection*{DNA techniques and plasmid constructions.}

Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming, and Southern hybridization analysis (12, 26). Restriction endonucleases and other enzymes were used according to the manufacturer's recommendations. DNA fragments and vectors used for subcloning and radiolabeling were isolated from 0.8% SeaKem agarose (FMC) gels following electrophoresis using GeneClean II (Bio 101). DNA was concentrated and denatured by using Microcon 30 microconcentrators (Amicon) prior to ligation or electrotransformation. \textit{S. roseosporus} genomic DNA was isolated as described previously (12). DNA sequence was determined by using a Taq Dye Deoxy Terminator Cycle sequencing kit and a model 373A DNA sequencing system (Applied Biosystems). PCR amplification was performed with a Perkin-Elmer GenAmpl 9600 using standard PCR conditions and a Touchdown PCR amplification program (9). Oligonucleotides were synthesized by Genosys (Woodlands, Tex.). Primers for PCR amplification were designed with the Oligo Primer Analysis software (National Biosciences, Inc.). Plasmid map constructions were performed with the Gene Constructor Kit version 1.2 (Textco, Inc., West Lebanon, N.H.).

Plasmids were constructed as follows. A 1.3-kb \textit{BamHI} fragment from cosmID pRHB545 that hybridized to the \textit{M. smegmatis} \textit{rpsL} gene was ligated to pBlue-script II KS\textsuperscript{+} digested with \textit{BamHI} to yield pRHB515. The 1.3-kb \textit{BamHI} fragment from pRHB515 containing the \textit{S. roseosporus} \textit{rpsL} gene was ligated to pKCI39 digested with \textit{BamHI} to yield pRHB514. A 1.46-kb fragment containing the \textit{S. roseosporus} \textit{glnA} gene was PCR amplified from chromosomal DNA with primers PR170 (5'-GCCGAGATGC GC CGCCACAC 3') and PR171 (5'-ACGCCGCGCCGCTGAGGTA 3') and ligated to pCR II (Invitrogen) to yield pRHB534. An Hm\textsuperscript{r} gene from pCAZ256 was inserted into a SacI site within the \textit{glnA} gene on pRHB534 as follows. A 1.7-kb \textit{BamHI}-to-HindIII fragment from pCAZ256 was treated with mung bean nuclease and ligated to pRHB534 previously digested with SacI and mung bean nuclease to yield pRHB537. A 687-bp fragment containing \textit{rpsL} was PCR amplified to introduce \textit{BglII} sites at the ends with primers PR115 (5'-GGGGAGATCTGCGGGAGAACTCAGGATCT-3') and PR116 (5'-GGGGAGATCTGCGAGAGACTGGATCT-3') and ligated with pCR II to yield pRHB541. A 0.69-kb BglII fragment from pRHB541 containing \textit{rpsL} was ligated with BglII-digested pKC1139 to yield pRHB538. A cosmID library of \textit{S. roseosporus} DNA was constructed by partially digesting genomic DNA with Sau3A1 and alkaline phosphatase (Boehringer Mannheim Biochemicals). DNA of approximately 40 kb was isolated and ligated to \textit{BamHI}-digested pKC1371 and packaged with a Gigapack packaging extract (Stratagene). Packaged DNA was introduced into \textit{E. coli} XL1-Blue-MRF\textsuperscript{+}, and individual clones containing cosmID DNA were stored as an ordered array in 96-well microtiter plates. Primary screening filters were prepared with a 96-well-disk blot apparatus. Twelve cultures from a row of microtiter wells were pooled, and plasmid DNA was prepared, bound to nylon filters (Bio-Rad Zeta-probe GT), and probed with a 255-bp fragment of \textit{M. smegmatis} \textit{rpsL}. DNA amplified from chromosomal DNA with primers PR99 (5'-CCGCGAAGGCTGCGAGAAGATTGCG 3') and PR100 (5'-CTTGAAACGACCCGGCCGGTTT 3') (28). Secondary screening filters were prepared in a similar manner except that DNAs from individual cosmID clones were blotted to nylon filters.
Transformation, electroporation, and conjugation. Plasmids were introduced into *E. coli* XL1-Blue MFR by electroporation using a Bio-Rad Gene Pulse electroporator. Electrocompetent *E. coli* cells were prepared as described previously (34). *E. coli* S17-1 was transformed as described elsewhere (26). Plasmids pRHB514, pRHB538, and pRHB543 were introduced into *S. roseosporus* strains by conjugation from *E. coli* S17-1 containing the appropriate plasmid and were spread on Bagar plates with appropriate antibiotics. Colonies appearing at 39°C were analyzed by Southern hybridization using a radiolabeled probe. Similar patterns of hybridization were observed with the two cosmids. A 1.3-kb fragment containing _rpsL_ hybridizing fragment was subcloned into pBluescript II KS + containing _rpsL_, _rpsG_, and part of fusA. The DNA and predicted amino acid sequences are shown in Fig. 1. The nucleotide sequence for _rpsL_, _rpsG_, and part of _fusA_ has been assigned GenBank accession number U60191.

RESULTS AND DISCUSSION

Cloning and characterization of the *S. roseosporus* _rpsL_ gene. A library of *S. roseosporus* chromosomal DNA prepared in cosmid pKC1471 was probed with the *M. smegmatis* _rpsL_ gene, and cosmids pRHB545 and pRHB546 containing _rpsL_-hybridizing sequences were identified. The cosmids were digested with _BamH_I, _EcoR_I, and _KpnI_, and DNA fragments were separated by gel electrophoresis, blotted to nylon filters, and analyzed by Southern hybridization using a radiolabeled *M. smegmatis* _rpsL_ probe. Similar patterns of hybridization were observed with the two cosmids. A 1.3-kb _BamH_I fragment that hybridized to the *M. smegmatis* _rpsL_ probe was subcloned into pBluescript II KS + and sequenced. The DNA and predicted amino acid sequences are shown in Fig. 1. Three open reading frames were identified by codon preference analysis of the region, each having the predicted percent G + C (~70:50:90) at positions 1, 2, and 3 of codons as generally observed in streptomycete coding regions (5). These open reading frames en-
code proteins highly homologous to the gene products of rpsL, rpsG, and fusA in other bacteria. The linkage relationship of these three genes is the same as that in other bacteria (14, 21, 23). A possible promoter sequence was located at nucleotide positions 97 to 126 upstream of the rpsL translational start site. The sequence TTGACC-16n-TACGCT is similar to the streptomycete E. coli-like promoters (SEP promoters) which have a consensus sequence of TTGACPy-16n-TAGPuPyT (35) and to a promoter upstream of the M. smegmatis rpsL gene by about the same distance (~190 bp) (14). A comparison of the deduced S. roseosporus S12 protein amino acid sequence to those of other S12 proteins is shown in Fig. 2. S. roseosporus S12 shows the closest similarity to M. smegmatis and Mycobacterium tuberculosis S12 (~90% amino acid identity) and to Micrococcus luteus (85% identity). The S. roseosporus S12 protein lacks a terminal amino acid found in many of the other bacteria and also lacks a 13-amino-acid region observed in the Streptococcus pneumoniae, Bacillus steaothermophilus, and Staphylococcus aureus S12 proteins. The predicted initiation codon (GTG) for rpsL is the same as that in M. luteus and is preceded 5 bp upstream by a potential ribosomal binding site (GGAG). The S. roseosporus S12 and S7 proteins appear to be translationally coupled with 2 nucleotides separating the S12 stop codon and the predicted S7 start codon. A potential ribosomal binding site (GGAG) is located 8 nucleotides upstream of the S7 initiation codon and within the S12 coding region. The S12 and S7 proteins of M. smegmatis, M. tuberculosis, Mycobacterium leprae, and Mycobacterium intracellulare also appear to be translationally coupled and contain identical potential ribosomal binding sites (GGAG) 8 nucleotides up-

FIG. 1. Organization and DNA sequence of the rpsL gene of S. roseosporus. (A) Nucleotide sequence and the predicted amino acid sequence of the S. roseosporus rpsL and rpsG genes and the 5' terminus of the fusA gene. Potential promoter and ribosomal binding sites are underlined. (B) Schematic representation of the 1.3-kb BamHI fragment. The direction of transcription is indicated (arrows).
stream of the S7 initiation codon and located within the S12 coding region (14, 20). The Mycobacterium species appear to contain overlapping stop and start codon sequences (TAATG or TGATG) for the S12 and S7 proteins, a configuration which was not observed in S. roseosporus since its S12 protein is 1 amino acid shorter. Potential translational coupling of S12 and S7 was not observed in the Haemophilus influenzae, E. coli, S. pneumoniae, B. stearothermophilus, and Staphylococcus aureus operons (data not shown).

The rpsL gene expresses dominant streptomycin sensitivity. Gene replacements using wild-type rpsL (Sm

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) for counterselection are performed in an Sm

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 background (24, 27, 31). Sm

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 maps to the rpsL gene in many different microorganisms (18). We selected spontaneous Sm

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 S. roseosporus mutants, and strain TH149 was further examined as a host for double-crossover analysis. To determine if the S. roseosporus rpsL gene would confer Sm

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, we introduced plasmid pRHB514 into TH149 by conjugation from E. coli S17-1 and transconjugants were isolated and streaked onto B agar containing SM. TH149 containing pRHB514 was Sm

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, but a small number of Sm

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 colonies grew in the patch, presumably due to homogenotization. The results indicated that the S. roseosporus rpsL gene expresses a dominant Sm

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 phenotype in an Sm

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 background, suggesting that the apparent promotor sequence upstream of rpsL on plasmid pRHB514 is functional (Fig. 1).

Selection for single and double crossovers using rpsL. Recombinants containing single or double crossovers between plasmids pRHB543 and the S. roseosporus chromosome were selected, and the frequency of single crossovers after a temperature shift to 39°C was about 10

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. To select for recombinants containing double crossovers, a temperature shift from 29 to 39°C followed by 2 to 3 days of growth in liquid culture was necessary to cure the plasmid containing the rpsL gene prior to SM selection. Therefore, a direct measurement of the double-crossover frequency was not possible. However, after 2 to 3 days of outgrowth following the temperature increase, the frequency of recombinants containing single crossovers was about 100-fold greater than the frequency of recombinants containing double crossovers. If the ratio of recombinants containing single and double crossovers remains relatively constant during growth, then the initial double-crossover frequency prior to plasmid curing was approximately 10

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To investigate the nature of the DNA insertions in recombinants containing single or double crossovers, chromosomal DNA was digested with BamHI and EcoRI and DNA fragments were separated by gel electrophoresis, blotted onto nylon filters, and analyzed by Southern hybridization using pRHB543 or an Hm

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 gene fragment as a probe. Figure 3A shows the expected DNA structures of recombinants containing single crossovers in glnA or rpsL sequences, and Fig. 3B shows the expected structure of a recombinant containing two crossovers to insert the Hm

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-disrupted glnA gene in the chromosome. Figures 3C and D show the Southern hybridization patterns of several recombinants. Strains TH172 and TH174, which contained single crossovers, had identical junction fragments of 3 and 20 kb, two pRHB543 internal Hm

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-hybridizing bands (1.8 and 1.4 kb), a 9.0-kb glnA-hybridizing fragment, but not the 1.3-kb rpsL-hybridizing fragment. This hybridization pattern is consistent with the insertion of pRHB543 into the rpsL gene in strains TH172 and TH174 (Fig. 3A, X3). Strains TH173 and TH175, which contained single crossovers, had identical junction fragments of 23 and 4.3 kb, two pRHB543 internal fragments (7.2 and 1.4 kb), a 1.3-kb rpsL-hybridizing fragment, but not the 9.0-kb glnA-hybridizing fragment. In this case, one internal fragment (1.4 kb) and one junction fragment (4.3 kb) hybridized to the Hm

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 probe. This pattern is consistent with the insertion of pRHB543 into the glnA gene (Fig. 3A, X2). Figure 3D shows that recombinant strains TH168 and TH169, which express the appropriate phenotype for a double-crossover gene replacement (Hm

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), contain identical Hm

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-hybridizing junction fragments. One fragment (4.3 kb) is identical to one Hm

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-hybridizing fragment in strains TH173 and TH175. The new junction fragment generated by the second crossover was about 7 kb. Neither TH168 or TH169 contained any plasmid sequences (Fig. 3C and D), and each contained a single intact rpsL gene identical to that in wild-type S. roseosporus. This pattern of hybridization is consistent with exchange of glnA: Hm

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 from pRHB543 into the chromosome of the recombinants. In summary, these results indicate that, although recombination between plasmid and chromosomal rpsL was observed during selection for single crossovers, it did not interfere significantly with the ability to directly select for the desired recombinants containing double crossovers in a target gene. Since recombination between partially homologous sequences in streptomycetes occurs at greatly reduced frequencies compared to homologous recombination (13), the S. roseosporus rpsL system should be even more useful in other heterologous streptomycetes and possibly other actinomycetes.

Determination of GS activity in recombinant strains. Streptomycetes species contain two GSs (10, 11). GSI, encoded by the glnA gene, is heat stable, whereas GSII, encoded by the glnII gene, is heat labile (11). Streptomycetes coelicolor glnA is regulated by a positive regulatory factor encoded by glnR (10). Biochemical studies of Streptomyces viridochromogenes showed that GSI and GSII activities are expressed under different conditions and that insertional inactivation of either glnA or glnII did not cause glutamine auxotrophy (11).

To determine if recombinant strains express GSI activity, we carried out GS assays with permeabilized S. roseosporus strains grown in CSM media with and without heat treatment. S. roseosporus A21978.6 and the Sm

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 strain TH149 expressed GS specific activities of 3.9 and 3.1, respectively, before heat treatment. Heat treatment (57°C for 30 min) of these strains caused an ~15% reduction in GS activity, similar to that in S. viridochromogenes (11). Strain TH168 containing glnA: Hm

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 had no detectable GS activity on CSM medium. It is not clear why heat-labile GSII activity was not detected. TH168 was prototrophic on CDA agar, suggesting that GSII activity was expressed on this medium. The recombinant strain TH173 containing a single-crossover insertion of plasmid pRHB543 into
the glnA gene also expressed no detectable GS activity. Since the glaA fragment in pRHB543 lacks a promoter and C-terminal glaA sequences, a single crossover should generate two nonfunctional glaA genes (Fig. 3). Strain TH172 containing pRHB543 inserted into the rpsL gene expressed normal GS activity (specific activity, 3.1), as expected.

In summary, we have demonstrated that the rpsL gene of S. roseosporus can be used as a counterselectable marker in S. roseosporus, allowing for the direct selection for recombination events that result in gene replacement. This technology should have broad applications in Streptomyces species to stably insert genes into the chromosome, to construct mutants, and to modify secondary metabolite biosynthetic pathways. This should aid both fundamental and applied research in this important genus.

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