

## Spectinomycin Resistance in *rpsE* Mutants is Recessive in *Streptomyces roseosporus*

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Received: November 26, 2004 / Accepted: March 16, 2005

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**Abstract** Eight spontaneous mutants of *Streptomyces roseosporus* resistant to spectinomycin (SpcR) were mapped to distinct transversions or deletions in the *rpsE* gene. Most of the mutations were strongly recessive to the wild type SpcS allele. This suggests that some SpcR alleles of *rpsE* may be useful in a spectinomycin based counter-selection system.

**Keywords** spectinomycin, *rpsE*, ribosomal protein S5, *Streptomyces roseosporus*, resistance

Spectinomycin is a bacteriostatic aminocyclitol antibiotic that inhibits protein synthesis. It affects ribosome function by binding to the 30S subunit and inhibiting translocation of peptidyl-tRNA from the A-site to the P-site [1, 2]. Translocation involves local pivoting of the head of the ribosome relative to the body, and is sterically hindered by the presence of the antibiotic [3]. Amino acid substitutions in ribosomal protein S5 [4], the product of the *rpsE* gene, is associated with resistance to spectinomycin (SpcR). This protein is located on the 30S subunit and interacts with 16S rRNA and other proteins. Crystallographic studies of the *Bacillus stearothermophilus* S5 protein showed that it contains an N-terminal dsRNA binding region that interacts with 16S rRNA helix H3 [4, 5].

Early experiments on ribosome morphogenesis in *E. coli* hinted that sensitivity to spectinomycin might be a

dominant phenotype. Studies demonstrating assembly of ribosomes from a pool of subunits showed an excess of SpcS strains when isogenic merodiploids were constructed by introducing an SpcS allele episomally into a SpcR background [6]. Other merodiploids constructed between SpcR and SpcS strains were unstably SpcS, and segregated SpcR derivatives more frequently than expected from spontaneous mutation [7]. It is possible that SpcS alleles could be dominant in a heterozygote through incorporation of sensitive subunits into spcR ribosomes [6, 8]. In this study, we isolated a collection of spontaneous SpcR *rpsE* mutants of *S. roseosporus*, complemented them with a cloned *rpsE* gene and showed that SpcS is dominant over SpcR.

### Cloning of the *S. roseosporus rpsE* gene

The approach used for cloning the *rpsE* gene took advantage of the synteny observed in the chromosomes of model actinomycetes [9, 10], in this case, the local order of genes around *rpsE* (Fig. 1A). Primers P949 and P950 (5'CGSGARGCSCGGVCTSAAGTTC3' and 5'CTTSAGCTTSGGVAGVCGCATGTG3') targeting *rplR* and *rplO* were used to amplify (92°C 15 seconds, 52°C 15 seconds, 72°C 45 seconds, 30 cycles) an intervening region from *S. roseosporus* UA343 (NRRL 15998). The sequenced PCR product (deposited as Genbank AY772011) was found to include *rpsE* and *rpmD*, indicating that the *rplR*·*rpsE*·*rpmD*·*rplO* contiguity was also conserved in *S. roseosporus* (Fig. 1B). The *rpsE* gene sequence was GC rich (69.3%) and the 200 residue deduced S5 peptide was

A



B

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10      20      30      40      50      60      70      80      90
TACGCACAGCGGACGTAACAGAGAGAGGTAATCCCATGGCTGGACCCAGCGCCGCGGCAGCGGTGCCGGTGGCGGCAGCGCGGGAC
      M A G P Q R R G S G A G G G E R R D >
100     110     120     130     140     150     160     170     180
CGGAAGGGCCGTGACGGTGGCGCAGCCGCCGAGAAGACCGCGTACGTCGAGCGCGTCGTCGCGATCAACCGCGTCGCCAAGGTTGTGAAG
R K G R D G G A A A E K T A Y V E R V V A I N R V A K V V K >
190     200     210     220     230     240     250     260     270
GGTGGTTCGTCGCTTACCGCTTACCGCGCTGGTGGTGGGGCAGCGGTGACGGCAGCGTTCGGTTCGGATACGGCAAGGCCAAGGAAGTT
G G R R F S F T A L V V V G D G D G T V G V G Y G K A K E V >
280     290     300     310     320     330     340     350     360
CCCGCGCCATCGCCAAGGTTGTCGAAGAGGCCAAGAAGAACTTCTCAAGGTCGCCGCGCATCCAGGGCACCATCCCTCACCCGATCAGC
P A A I A K G V E E A K K N F F K V P R I Q G T I P H P I T >
370     380     390     400     410     420     430     440     450
GGCGAGAAGCGAGCGGCGTCTCTGCTCAAGCCGGTTCGCCCGGTACCGGTGTTATCGCCGGTGGCCCGGTGCGTGCCTGCTCGAG
G E K A A G V V L L K P A S P G T G V I A G G P V R A V L E >
460     470     480     490     500     510     520     530     540
TGCGCCGGCTTACGACATCTGTGCAAGTTCGCTTGGCTCGTCCACCGCATCAACATTGTGCACGCGACCGTGGAGCCCTGAAGGGC
C A G V H D I L S K S L G S S N A I N I V H A T V E A L K G >
550     560     570     580     590     600     610     620     630
CTGCAGCGTCCCGAGGAGATCGCGCCGCGCGGTCCCGCTCGAGGACGTCGCCCGCGGCTCGTTCGTCGCGGTGCGGGAGCG
L Q R P E E I A A R R G L P L E D V A P A A L L R A R A G A >
640     650     660     670     680     690     700     710     720
GGTGCATAATGGCTCGCCTCAAGATCACGCAGACGAAGTCGTACATCGGCAGCAAGCAGAACCCCGGCACACCTGCGTTCGCTCGGGC
G A *
      M A R L K I T Q T K S Y I G S K Q N H R D T L R S L G >
730     740     750     760     770     780     790     800     810
TCAAGCGCCTGAACGACTCGGTTGTCAAGGAGGACCCCGGAGTTCGCGGCATGGTGCAGACCGTCCGCCACCTCGTGACGGTTGAGG
L K R L N D S V V K E D R P E F R G M V Q T V R H L V T V E >
820     830     840     850     860     870     880     890     900
AGGTTGACTGACATGGCGGAGAACACCCGCTGAAGGCCACAACCTCCGGCTGCCCGGGCCCAAGACCCCAAGACCCGTTGGGT
E V D *
      M A E N S P L K A H N L R P A P G A K T A K T R V G >
910     920     930     940     950     960     970     980     990
CGTGGTGGGCGTCAAGGTAAGACCGCAGGCCGTCGTTACCAAGGTACGAAGGCCCGTACCAGGTTCCGGACGCTTCGAGGTGGGC
R G E A S K G K T A G R G T K G T K A R Y Q V P D A S R W A >
    
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C

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      10      20      30      40      50      60      70
SRO  MAGPQRRSGAGGGERRDRKGRDGA-AAEKTAYVERVVAINRVAKVVKGGRRFSFTALVVVGDGDGTG
SAV  .....A.....
SFR  .....A.....
SCO  .....G.....

      80      90      100     110     120     130     140
SRO  VGYGKAKEVPAAIKGVVEAKKNFFKVPRIQGTIPHPITGEKAAGVLLKSPGTVIAGGPVRAVLEC
SAV  .....H.....
SFR  .....H.....
SCO  .....H.....Q.....

      150     160     170     180     190     200
SRO  AGVHDILSKSLGSSNAINIVHATVEALKGLQRPEEIAARRGLPLEDVAPAALLRARAGAGA
SAV  .....A.....
SFR  ..I.V.....
SCO  ..I.V.....D.Q.....
    
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**Fig. 1** Organization of *rpsE* region and characterization of *S. roseosporus rpsE* and ribosomal protein S5. (A) Organization of *rpsE* region in *Streptomyces coelicolor* and location of primers, designed on the basis of *S. coelicolor* data, that were used to amplify the *rpsE* region of *S. roseosporus*. (B) Sequence and deduced amino acid sequence of *S. roseosporus rpsE* and neighboring genes. A 5' purine rich sequence (underlined) may serve as a ribosome-binding site (RBS) for *rpsE*. The coding region extends from nucleotides 37 to 639, and overlaps the coding region for *rpmD* (639 to 821). The PCR product includes the start of the coding region for *rplO* (nucleotide 823) which is preceded by a canonical RBS (double underline). (C) Alignment of S5 from *S. roseosporus* (SRO), *Streptomyces avermitilis* (SAV) (Genbank Accession no. NP\_826120), *S. coelicolor* (SCO) (NP\_628878) and *Streptomyces fradiae* (SFR) (AY772012). Dots indicate residues identical in all four streptomycetes.

**Table 1** SpcR mutants and test for dominance

Strain <sup>a)</sup>	Allele	Mutation in S5 <sup>b)</sup>	Growth of strain without pXH006		Growth of strain with pXH006 <sup>c)</sup>	
			0 $\mu$ g/ml spc	750 $\mu$ g/ml spc	0 $\mu$ g/ml spc	750 $\mu$ g/ml spc
UA343	<i>rpsE</i>	—	+	—	(not tested)	
XH32	<i>rpsE6</i>	V43F (GTC~>TTC)	+	+	+	—
XH31	<i>rpsE5</i>	V43L (GTC~>CTC)	+	+	+	—
XH27	<i>rpsE4</i>	A44P (GCC~>CCC)	+	+	+	—
XH26	<i>rpsE8</i>	$\Delta$ V46-G49 ( $\Delta$ 12 nucleotides)	+	+	+	—
XH28	<i>rpsE2</i>	K48N (AAG~>AAC)	+	+	+	(+/-)
XH33	<i>rpsE2</i>	K48N (AAG~>AAC)	+	+	(not tested)	
XH29	<i>rpsE3</i>	K48N (AAG~>AAT)	+	+	+	(+/-)
XH34	<i>rpsE9</i>	$\Delta$ R51 ( $\Delta$ 3 nucleotides)	+	+	(not tested)	
XH35	<i>rpsE7</i>	R52P (CGC~>CCC)	+	+	+	(+/-)
XH30	<i>rpsE</i>	—	+	+	+	+

a) *S. roseosporus* UA343 is the progenitor strain. Derived strains are listed by the position of mutation. Strains XH26~XH30 were mutants isolated on AS-1 supplemented with 100  $\mu$ g/ml spectinomycin; the rest were from media with 50  $\mu$ g/ml spectinomycin. b) Mutations are indicated by the standard one letter amino acid code; e.g. "V43F" indicates that valine at position 43 was changed to phenylalanine, and "(GTC~>TTC)" indicates that this was due to a G to T transversion. c) Exconjugants of each strain with pXH006, carrying the wildtype (SpcS) *rpsE* gene, were patch tested on AS-1 containing spectinomycin and inspected for growth after incubation at 30°C for 6 days. "—" indicates no growth, "+" indicates growth comparable to UA343 on media without antibiotic. "+/-" indicates very poor growth after a delay of several days.

extremely similar to homologs found in other streptomycetes (Fig. 1C).

### Isolation of SpcR Mutants

*S. roseosporus* is sensitive to <6  $\mu$ g/ml spectinomycin (J. Penn, personal communication) in AS-1 medium [11]. SpcR colonies were recovered after 3~15 days at a frequency of  $3.4 \times 10^{-8}$  and  $2.7 \times 10^{-8}$  from spores plated on media containing 50  $\mu$ g/ml or 100  $\mu$ g/ml spectinomycin, respectively. Ten colonies selected at random were analysed (Table 1). All exhibited wildtype morphology and sporulation, and grew well on AS-1 even with 750  $\mu$ g/ml spectinomycin. Mutations in *rpsE*, including transversions and deletions, were present in nine of the ten SpcR strains (Table 1). All mutations resided in the ds16S rRNA binding region of S5, "loop 2" as delimited by residues N19 and F33 in the *Escherichia coli* protein [5, 12], or their equivalent, N41 and F55, in *S. roseosporus* (Fig. 2). The highly conserved lysines and arginines here form a positively charged  $\beta$ -ribbon that interacts with the minor groove in 16S rRNA, and the glycine-glycine (GG) residues allow a tight turn before the conserved phenylalanine at the end of the flexible region. As both spectinomycin [3] and "loop 2" interact with 16S rRNA H34 [5], it has been

SRO	F															
SRO	L P			N				$\Delta$ P								
SRO	N <sub>41</sub>	R	V	A	K	V	V	K	G	G	R	R	F	S	F <sub>55</sub>	
BSU	· <sub>19</sub>	·	·	·	·	·	·	·	·	·	·	·	·	·	R <sub>33</sub>	
ECO	· <sub>19</sub>	·	·	S	·	T	·	·	·	·	·	·	I	·	· <sub>33</sub>	
ECO	L E P															
BSU	I															

**Fig. 2** Deduced mutations in ribosomal protein S5. Residues in the loop 2 region of S5 (delimited by *E. coli* N19 and F33) from *S. roseosporus* (SRO), *E. coli* (ECO) and *B. subtilis* (BSU) are aligned in the middle of the diagram to show conserved positions. Dots indicate residues identical in all three microorganisms. Individual mutations in SRO are shown at the top, and known ECO and BSU mutations at the bottom.  $\Delta$  symbols denote a deletion, or a group of deletions (bracketed).

hypothesized that mutations contributing to greater flexibility in this region may counteract constraints resulting from binding of the antibiotic, thus conferring resistance. Co-localization of the few known SpcR mutations in other bacteria [13~16] to this region is consistent with a mechanism of resistance that involves ribosomal function. The tenth *S. roseosporus* SpcR strain, XH30, had wildtype *rpsE*. While it is possible that

mutations in 16S account for non-S5 related SpcR [1, 17], the likelihood is low here because streptomycetes have multiple rRNA operons.

### Dominance of SpeS

To test possible dominance relationships, the coding region of *rpsE* (nucleotides 40~695, Fig. 1B) was subcloned under control of the strong constitutive *ermEp\** promoter in pHM11a, an integrative *E. coli*/*Streptomyces* shuttle vector [18]. The resulting plasmid, pXH006, was introduced into *E. coli* DH10B carrying helper plasmid pUZ8002, and transferred by conjugation using standard methods [19] into several SpcR mutants. Two to six exconjugants were patch tested for sensitivity on AS-1 plates with spectinomycin. Growth was completely inhibited at 750 µg/ml spectinomycin in strains carrying *rpsE4*, *rpsE5*, *rpsE6*, or *rpsE8*, but not in strains with *rpsE2*, *rpsE3*, or *rpsE7* which showed some growth only after several days (Table 1). Notably, SpcR mutations at the N-terminal side of “loop 2” were completely recessive, whereas those at the C terminus (K48-R52) were not. Introduction of pHM11a alone did not affect growth or spectinomycin resistance. That the non-*rpsE* related resistance in strain XH30 remained unaffected by the introduction of the wildtype gene supports the specificity of this complementation test.

### Utility of *rpsE* for Counter-selection

Homologous gene replacement is a useful tool for precise molecular dissection of gene function but has a disadvantage in being laborious. To overcome this, plasmid-borne counter-selection systems based on recessive resistance phenotypes, e.g. *rpsL*-dependent streptomycin resistance, have been used [20]. The present study suggests that *rpsE* might be useful in a similar manner. In particular, investigations requiring multiple replacements at one locus may be well served by construction of an intermediate strain in which counter-selection, such as provided by *rpsE*, is directly applied to the target locus, rather than to the delivery plasmid. In this arrangement, a dominant allele for sensitivity is placed at the desired target locus and is epistatic over a recessive allele for resistance at the native locus: subsequent homologous recombination of test constructs at the target evicts the dominant allele, and expression of resistance is indicative of successful gene replacement by double crossover. Since *S. roseosporus rpsE* mutations are similar to those in *E. coli* and *B. subtilis*, SpcR based counter-selection could have a broad applicability. Conservation of *rpsE* proteins among streptomycetes further suggests that the *S. roseosporus rpsE* gene might confer a dominant sensitivity phenotype when introduced into SpcR strains

derived from other streptomycetes.

**Acknowledgments** The authors thank K. Nguyen for help with conjugation, E. Cundliffe for comments on the manuscript, and J. Davies for useful discussions.

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