Chromosomal Instability in *Streptomyces glaucescens*: Mapping of Streptomycin-sensitive Mutants

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Streptomyces glaucescens strain GLA0 (= ETH 22794) produces hydroxystreptomycin and has a high natural resistance to hydroxystreptomycin, dihydrostreptomycin and streptomycin. The wild-type strain gives rise spontaneously to streptomycin-sensitive (StrS⁻) variants at a frequency of 0.2 to 1.4%. These mutants lack streptomycin phosphotransferase activity responsible for the wild-type resistance to streptomycin group antibiotics and are unable to produce detectable amounts of hydroxystreptomycin.

Mapping experiments showed that the *strS* marker lies between the chromosomal markers *lys-2* and *ura-3* on the linkage map of *S. glaucescens*. The molecular basis for instability of this marker is as yet unknown.

INTRODUCTION

Genetic instability is a widespread phenomenon among streptomycetes. The most thoroughly investigated unstable characters are natural resistance to antibiotics (Freeman *et al.*, 1977; Freeman & Hopwood, 1978; Sermonti *et al.*, 1977, 1978, 1980; Fedorenko & Danilenko, 1980; Kirby & Lewis, 1981), aerial mycelium formation (Pogell, 1979; Redshaw *et al.*, 1979), production of pigments (Gregory & Huang, 1964*a, b*; Pogell, 1979; Hütter *et al.*, 1981; Schrempf, 1981) and the production of antibiotics (Shaw & Piwowarski, 1977; Kirby, 1978; Schrempf, 1981).

An extensive genetic analysis is available only for chloramphenicol resistance in *Streptomyces coelicolor* A3(2) (Freeman *et al.*, 1977; Sermonti *et al.*, 1977, 1978, 1980) and for erythromycin resistance in the same strain (Puzynina *et al.*, 1979). Chloramphenicol sensitivity was found to be reversible and no constant map position for the resistance gene could be established (Freeman *et al.*, 1977). According to Sermonti *et al.* (1980) two chromosomal map positions may be deduced from crossing data and the authors therefore deduced that a jumping gene might be responsible for chloramphenicol resistance in this strain. However, the interpretation of the data may be questioned (Lanfaloni *et al.*, 1980).

In the case of erythromycin resistance the marker was found to segregate chromosomally and the authors postulated, that erythromycin sensitivity is caused by transposition of genetic material (Puzynina *et al.*, 1979). In both cases, chloramphenicol and erythromycin resistance, no data about the resistance mechanism were published.

Similarly, *Streptomyces glaucescens* possesses a natural resistance towards streptomycin group aminoglycoside antibiotics, but the resistance is highly unstable. Streptomycin-sensitive variants arise with high frequency spontaneously after induction by curing agents or after other

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Table 1. *Streptomyces glaucescens* strains

Strain	Genetic markers*	Status of streptomycin resistance†	Mutagenic treatment‡
GLA0	Wild-type	<i>strS</i> ⁺	—
GLA15	<i>ura-3 met-2</i>	<i>strS</i> ⁺	—
GLA20	<i>gua-4 lys-1</i>	<i>strS</i> ⁺	—
GLA21	<i>hom-1 lys-1</i>	<i>strS</i> ⁺	—
GLA23	<i>lys-2 ura-5</i>	<i>strS</i> ⁺	—
GLA24	<i>thr-1 lys-2</i>	<i>strS-24</i>	NTG (GLA31)
GLA31	<i>lys-2</i>	<i>strS</i> ⁺	—
GLA46	<i>thr-1</i>	<i>strS-24</i>	—
GLA58	<i>nic-2</i>	<i>strS</i> ⁺	—
GLA62	<i>lys-2 ura-3</i>	<i>strS</i> ⁺	—
GLA65	<i>ura-5 met-2</i>	<i>strS</i> ⁺	—
GLA71	<i>pro-1 ade-1</i>	<i>strS</i> ⁺	—
GLA72	<i>lys-2 ade-1</i>	<i>strS</i> ⁺	—
GLA75	<i>leu-2 ura-2 ade-1</i>	<i>strS</i> ⁺	—
GLA84	<i>lys-2 pro-1</i>	<i>strS</i> ⁺	—
GLA128	<i>rif-565 met-8</i>	<i>strS-128</i>	EB (GLA565)
GLA205	Prototroph	<i>strS-205</i>	CS (GLA0)
GLA207	Prototroph	<i>strS-207</i>	AO (GLA0)
GLA212	Prototroph	<i>strS-212</i>	EB (GLA0)
GLA512	<i>his-2</i>	<i>strS</i> ⁺	—
GLA516	<i>nic-2 ura-3</i>	<i>strS</i> ⁺	—
GLA528	<i>lys-3 leu-2</i>	<i>strS</i> ⁺	—
GLA541	<i>ura-3 nic-2</i>	<i>strS-541</i>	EB (GLA516)
GLA543	<i>lys-2 ade-1</i>	<i>strS-543</i>	Spontaneous (GLA72)
GLA544	<i>nic-2</i>	<i>strS-544</i>	EB (GLA58)
GLA547	<i>ura-2 nic-2 rif-547</i>	<i>strS</i> ⁺	—
GLA565	<i>met-8 rif-565</i>	<i>strS</i> ⁺	—
GLA567	<i>met-8 rif-567</i>	<i>strS</i> ⁺	—
GLA572	<i>lys-2 thi-3 rif-572</i>	<i>strS</i> ⁺	—
GLA877	<i>rif-567 pro-1 ade-1</i>	<i>strS</i> ⁺	—

* Marker designations are according to Demerec *et al.* (1966) (see also Baumann *et al.*, 1974 and Hütter *et al.*, 1981).

† Two loci determining the degree of streptomycin resistance are distinguished (Hütter *et al.*, 1981). The wild-type strain GLA0 *strA*⁺ *strS*⁺ can grow on agar media containing up to 10 mg streptomycin l⁻¹. Strains carrying the mutant allele *strA-4* (*strS*⁺ or *strS*) exhibit a high streptomycin resistance (growth on media with ≥ 100 mg streptomycin l⁻¹), while *strA*⁺ *strS* strains are highly streptomycin sensitive (minimal inhibitory concentration 0.2 mg streptomycin l⁻¹). All strains used had the genotype *strA*⁺.

‡ NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; EB, ethidium bromide; CS, cold storage; AO, acridine orange. The strain numbers in parentheses designate the direct parent of the corresponding mutant strain. —, Designates mutational and recombinational derivatives from the stock culture collection.

mutagenic treatments (Freeman & Hopwood, 1978; Suter *et al.*, 1978; Hütter *et al.*, 1981). The natural streptomycin resistance observed in the wild-type strain is due to the presence of a streptomycin phosphotransferase. Sensitive mutant strains lack this enzyme. Simultaneously they lose the capacity to produce detectable amounts of hydroxystreptomycin, indicating either the participation of the phosphotransferase both in antibiotic production and antibiotic resistance or the presence of multiple gene defects in *strS* mutant strains (Ono *et al.*, 1983).

In this paper we report on the genetic analysis of the streptomycin-sensitive mutants. A preliminary report of the data was given in Hütter *et al.* (1981).

METHODS

Strains. All mutant strains used are derivatives of *Streptomyces glaucescens* strain GLA0 (corresponding to strain ETH 22794, Baumann *et al.*, 1974; Hütter *et al.*, 1981) and are listed in Table 1. The linkage map of the markers used is given in Fig. 1.

Table 2. Isolation of streptomycin-sensitive colonies from the wild-type and from mutant strains of *Streptomyces glaucescens*

Strain	No. of colonies tested*	StrS isolates	
		Total	%
(a) <i>Spontaneously occurring StrS mutants</i>			
GLA0	1856	8	0.43
GLA15	757	5	0.66
GLA20	218	0	—
GLA21	280	0	—
GLA23	134	1	0.75
GLA62	700	0	—
GLA65	700	0	—
GLA71	2514	0	—
GLA72	822	3	0.36
GLA75	558	8	1.43
GLA84	650	0	—
GLA512	337	0	—
GLA516	1800	0	—
GLA528	170	0	—
GLA547	880	2	0.23
GLA567	1654	0	—
GLA572	1356	0	—

Strain	No. of colonies tested†	StrS isolates	
		Total	%
(b) <i>StrS mutants after growth on media containing ethidium bromide</i>			
GLA0	9090	6819	75.01
GLA65	956	653	68.30
GLA516	1247	1156	92.70

Strain	No. of colonies tested	Auxotrophic mutants		StrS isolates	
		Total	%	Total	%
(c) <i>StrS mutants after UV irradiation</i>					
GLA0	9658	64	0.66	17	0.18
GLA65	8899	58	0.65	27	0.30
GLA516	9223	49	0.53	3	0.03

Strain	No. of colonies tested	Auxotrophic mutants		StrS isolates	
		Total	%	Total	%
(d) <i>StrS mutants after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine</i>					
GLA0	3856	34	0.88	11	0.29
GLA65	5260	37	0.70	23	0.44
GLA516	6941	59	0.85	35	0.50

* No spontaneous auxotrophic mutants could be detected (spontaneous mutant frequency $<10^{-4}$).

† Survivors on agar media containing $7 \mu\text{mol}$ ethidium bromide l^{-1} . Auxotrophic mutants arose with frequencies $<10^{-4}$.

Cultural conditions. All chemicals were of analytical grade where possible. Strains were grown on yeast extract/malt extract agar (Difco) (Pridham *et al.*, 1956/7) at 37°C . Well sporulated cultures were usually stored at 4°C . Streptomycin-sensitive mutants, however, were cold-sensitive and lost their viability quickly at low temperatures; they were therefore stored at room temperature. For segregation analysis a minimal medium of the following composition was used: glucose, 10 g; NaNO_3 , 0.3 g; K_2HPO_4 , 0.5 g; CaCl_2 , 0.01 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.04 g; Bacto-Agar (Difco), 20 g; deionized water to 1000 ml, pH adjusted to 7.5 with 1 mol HCl l^{-1} before autoclaving. Selective media were prepared from minimal medium by the addition of the necessary supplements at the following

Table 3. *Fertility of crosses involving StrS mutant strains*

Parent strains*	Selection	Fertility† with respect to	
		Parent 1	Parent 2
GLA72 × GLA58 (control)	} <i>ade⁺ nic⁺</i>	8×10^{-3}	2×10^{-3}
GLA72 × GLA544		4×10^{-4}	1×10^{-3}
GLA541 × GLA24	<i>lys⁺ nic⁺</i>	8×10^{-3}	5×10^{-3}
GLA72 × GLA516 (control)	} <i>ade⁺ ura⁺</i>	7×10^{-5}	7×10^{-7}
GLA72 × GLA541		6×10^{-5}	3×10^{-3}
GLA543 × GLA541		6×10^{-6}	6×10^{-5}

* StrS strains are underlined.

† Fertility defined as number of recombinant colonies per number of parental colonies.

concentrations: amino acids, 10^{-3} mol l⁻¹; purines and pyrimidines, 5×10^{-4} mol l⁻¹; vitamins, 5×10^{-5} mol l⁻¹. The wild-type strain is sensitive to 2 mg rifampicin l⁻¹ (Ciba-Geigy) and to 10 mg streptomycin sulphate l⁻¹ (Novo Industri, Copenhagen, Denmark). Rifamycin-resistant (Rif^r) recombinants were selected with 100 mg rifampicin l⁻¹, *strS⁺* recombinants with 2 mg streptomycin l⁻¹.

Genetic techniques. These were as described by Baumann *et al.* (1974).

Isolation of streptomycin-sensitive mutants. Streptomycin-sensitive mutants were isolated as clones unable to grow on agar media containing 2 mg streptomycin l⁻¹, after one of the following treatments: UV irradiation (dose 1.9 mJ mm⁻²), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Serva) mutagenesis (Delić *et al.*, 1970), growth on yeast extract/malt extract agar containing 6 or 7 µmol ethidium bromide (EB) l⁻¹ (Serva) (Suter, 1978; Hütter *et al.*, 1981) or acridine orange (Serva) at the same concentrations, or after cold storage (Suter *et al.*, 1978). Sensitive colonies were routinely subcloned three times.

RESULTS

Frequency of StrS mutant strains

The wild-type strain and auxotrophic *strA⁺strS⁺* mutant strains of *S. glaucescens* can grow on agar media supplemented with up to 10 µg streptomycin ml⁻¹ (Hütter *et al.*, 1981). Mutant derivatives more sensitive to streptomycin than the parent strain arise spontaneously at a frequency of approximately 0.2% (Freeman & Hopwood, 1978; Hütter *et al.*, 1981). These strains were designated StrS-types.

In a more detailed study the spontaneous frequency of StrS progeny was analysed in sixteen mutational and recombinational derivatives of *S. glaucescens*. Five of the strains investigated yielded streptomycin-sensitive variants with frequencies between 0.2 and 1.4% (Table 2a). The frequency of auxotrophic mutations was below 10⁻⁴. UV irradiation or mutagenesis with NTG did not increase the frequency of StrS mutations in selected strains above the spontaneous frequency or above the frequency of induced auxotrophic mutations (Table 2c, d). However, growth on agar media containing EB yielded up to 90% StrS variants among the survivors with the wild-type strain and with all mutant strains tested (Table 2b).

All streptomycin-sensitive mutants were initially unstable and reverted phenotypically to wild-type resistance, but after successive recloning of single spore isolates no spontaneous reversion to StrS⁺ was observed (reversion frequency <10⁻⁹).

Crosses between independent StrS derivatives

Crosses between independently isolated streptomycin-sensitive strains (StrS⁻) never yielded recombinant colonies with wild-type streptomycin resistance (data not shown). The detection level of recombinant frequency was about 10⁻⁹. Crosses exhibited normal fertility with respect to chromosomal markers and no systematic influence on fertility was observed in crosses heterozygous for StrS (Table 3).

Table 4. Analysis of a multi-factor cross of strains GLA516 *ura-3, nic-2, strS⁺* and GLA24 *thr-1, lys-2, strS-24*

Genotype*					Selection for				Crossover in intervals†
					<i>thr⁺</i> <i>ura⁺</i>	<i>thr⁺</i> <i>nic⁺</i>	<i>lys⁺</i> <i>ura⁺</i>	<i>lys⁺</i> <i>nic⁺</i>	
<i>thr</i>	<i>lys</i>	<i>ura</i>	<i>nic</i>	<i>strS</i>					
+	+	+	+	+	18	25	18	30	2,4
+	+	+	+	<i>strS</i>	57	78	36	72	2,4
+	+	+	<i>nic</i>	+	10	—	23	—	2,3
+	+	+	<i>nic</i>	<i>strS</i>	54	—	54	—	2,3
+	+	<i>ura</i>	+	+	—	21	—	23	3,4
+	+	<i>ura</i>	+	<i>strS</i>	—	9	—	7	3,4
+	<i>lys</i>	+	+	+	1	2	—	—	1,4
+	<i>lys</i>	+	+	<i>strS</i>	8	13	—	—	1,4
<i>thr</i>	+	+	+	+	—	—	12	7	1,2
<i>thr</i>	+	+	+	<i>strS</i>	—	—	5	12	1,2
<i>thr</i>	+	<i>ura</i>	+	+	—	—	—	1	1,4
Total					148	148	148	152	

* +, Wild-type allele; *strS*, streptomycin sensitivity.† The *strS* allele is not considered.

Marker frequencies based on the recombinant analysis are given. The two circles represent schematically the circular linkage map of the *strS⁺* strain GLA516 (outer circle) and the *strS* strain GLA24 (inner circle). Triangles in the diagram indicate selected alleles. The arrows designate possible locations of the *strS⁺* allele as derived from marker frequencies. Thick arrows indicate the interval highly favoured by minimizing the number of multiple crossovers for placing the *strS⁺* marker in this interval.

Mapping of *strS* alleles

Crosses were made primarily between strains carrying the *strS-24* allele and multiply marked *strS⁺* strains. Results were evaluated by tabulating the number of segregants carrying the *strS⁺* allele versus segregants carrying the *strS* allele in relation to the non-selected markers. The map location was deduced by minimizing the number of required multiple crossovers and from the

Table 5. Analysis of a multi-factor cross of strains GLA877 *rif*-567, *pro*-1, *ade*-1, *strS*⁺ and GLA46 *thr*-1, *strS*-24

<i>rif</i>	Genotype*				Selection for				Crossover in intervals†
	<i>thr</i>	<i>pro</i>	<i>ade</i>	<i>strS</i>	<i>ade</i> ⁺ <i>thr</i> ⁺	<i>pro</i> ⁺ <i>thr</i> ⁺	<i>pro</i> ⁺ <i>rif</i> ⁺	<i>ade</i> ⁺ <i>rif</i> ⁺	
+	+	+	+	+	12	8	—	—	1,2
+	+	+	+	<i>strS</i>	11	15	—	—	1,2
<i>rif</i>	+	+	+	+	18	15	21	29	2,4
<i>rif</i>	+	+	+	<i>strS</i>	22	26	14	18	2,4
+	+	<i>pro</i>	+	+	1	—	—	—	1,3
<i>rif</i>	<i>thr</i>	+	+	+	—	—	0	1	1,4
<i>rif</i>	<i>thr</i>	+	+	<i>strS</i>	—	—	18	29	1,4
<i>rif</i>	+	<i>pro</i>	+	+	10	—	—	14	3,4
<i>rif</i>	+	<i>pro</i>	+	<i>strS</i>	1	—	—	0	3,4
<i>rif</i>	+	+	<i>ade</i>	+	—	5	8	—	2,3
<i>rif</i>	+	+	<i>ade</i>	<i>strS</i>	—	7	4	—	2,3
<i>rif</i>	<i>thr</i>	<i>pro</i>	+	+	—	—	—	1	1,2,3,4
<i>rif</i>	<i>thr</i>	+	<i>ade</i>	<i>strS</i>	—	—	12	—	1,3
Total					75	76	77	92	

* +, Wild-type allele; *rif*, rifamycin resistance.† The *strS* allele is not considered.

The two circles represent the circular linkage map of the *strS*⁺ strain GLA877 (outer circle) and the *strS* strain GLA46 (inner circle). For further details of arrangement see Table 4.

marker gradient. In all cases a chromosomal location of the *strS* marker in the region between *lys*-2 and *ura*-3 on the linkage map of *S. glaucescens* was found. Two examples of crosses are presented in Tables 4 and 5. The map position of the *strS*-24 allele was confirmed by the results of several additional crosses (data not shown).

The segregation of the *strS* alleles present in other mutant strains (GLA128, GLA205, GLA207, GLA212, GLA541, GLA543 and GLA544, see Table 1) obtained after various mutagenic treatments was analysed by crossing these strains with the multiply marked *strS*⁺

Fig. 1. Linkage map of *Streptomyces glaucescens*. The linkage map shown is according to Baumann *et al.* (1974) and Hütter *et al.* (1981). Only the markers used in this work are shown (see Table 1); ---- uncertain map position in this region.

strains previously mentioned. The results obtained were comparable to those obtained with the *strS*-24 allele (data not shown). All these crosses showed normal recombination frequencies for chromosomal markers (see above).

DISCUSSION

Streptomyces glaucescens strain GLA0 produces hydroxystreptomycin and is naturally resistant to this antibiotic and the related compounds streptomycin and dihydrostreptomycin. Streptomycin sensitive (*StrS*⁻) strains arose spontaneously with frequencies between 0.2 and 1.4%. All *StrS*⁻ isolates had lost hydroxystreptomycin production as well (Ono *et al.*, 1983). The frequency of *StrS*⁻ strains could be increased to up to 90% by growth of colonies derived from single spores on agar media containing EB. Notably, the frequency of *StrS*⁻ strains was not increased above the level of other chromosomal mutations by UV irradiation which is known to increase the frequency of plasmid loss in other streptomycetes (Friend *et al.*, 1978; Hopwood, 1978). Freshly isolated *StrS*⁻ strains did revert to the wild-type resistance level at a frequency of about 10^{-5} (Freeman & Hopwood, 1978). After two or three steps of subculturing, however, stable *StrS*⁻ strains were usually isolated ($<10^{-9}$ revertants in spore suspensions). The basis of stability after successive recloning is not yet understood. The high spontaneous frequency of *StrS*⁻ strains and the increase in frequency after EB treatment suggested the possible involvement of an unstable extrachromosomal genetic element involved in hydroxystreptomycin biosynthesis and resistance. This hypothesis was supported by the observation, that crosses between independent, stable *StrS*⁻ strains never led to recombinant progeny with the wild-type level of streptomycin resistance. Controls showed that the absence of *StrS*⁺ recombinant progeny was not due to sterility in these crosses.

Despite numerous attempts, no plasmid DNA could be isolated from strain GLA0 nor any of its derivatives. Genetic mapping, however, indicated a unique chromosomal location for the *strS* mutation on the circular linkage map of *S. glaucescens* between the *lys*-2 and the *ura*-3

markers (Fig. 1). All *strS* mutant strains tested lacked hydroxystreptomycin 6-phosphotransferase activity, while cell free extracts of strain GLA0 effectively inactivate streptomycins in an ATP-dependent reaction (Ono *et al.*, 1983).

Two interpretations are likely for our data. It is conceivable that the phosphotransferase is necessary to hydroxystreptomycin production (see Ono *et al.*, 1983). This could account for the concomitant loss of both antibiotic resistance and production. Another possibility is, that the *strS* mutation affects several genes either due to polarity or to a large deletion. Although unlikely, it is still possible, that these genes are coded on a stable plasmid, which is dependent on an unstable chromosomal gene for its maintenance. Additional investigations at the DNA level are required to uncover the molecular basis of the genetic instability at the *strS*⁺ site.

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