Hydroxystreptomycin Production and Resistance in *Streptomyces glaucescens*

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The wild-type strain of *Streptomyces glaucescens* produces hydroxystreptomycin and shows an inherent natural resistance to streptomycin group aminoglycoside antibiotics. Cell free extracts of the wild-type strain were able to inactivate streptomycin, hydroxystreptomycin and dihydrostreptomycin in the presence of ATP. The phosphotransferase did not inactivate other aminoglycoside antibiotics, including spectinomycin.

Mutant strains were isolated, which were highly sensitive to streptomycin group aminoglycosides, had no measurable phosphotransferase activity and were unable to form detectable amounts of hydroxystreptomycin. This suggests either a correlation between phosphotransferase activity, streptomycin resistance and hydroxystreptomycin formation or defects in more than one gene in the *strS* mutant strains tested.

INTRODUCTION

Streptomyces glaucescens is capable of producing several antimicrobial compounds, of which hydroxystreptomycin (Hütter, 1967; Fig. 1) and the tetracenomycins (Weber *et al.*, 1979, 1981) are chemically characterized. In addition a bacteriocin-like substance, glaucescin, is formed (Schurter *et al.*, 1979; Cramer *et al.*, 1981).

Streptomyces glaucescens is inherently more resistant to streptomycin than most other streptomycetes (Freeman & Hopwood, 1978; Hütter *et al.*, 1981). The natural streptomycin resistance is a genetically unstable character. The same phenomenon has also been observed with the streptomycin producers *S. griseus* and *S. bikiniensis* (Kirby & Lewis, 1981). In *S. glaucescens* highly streptomycin-sensitive mutant strains arise spontaneously at a frequency of 0.2 to 1.4% (Hütter *et al.*, 1981; Cramer *et al.*, 1983). The frequency can be increased considerably by long-term cold storage of spores to 10% of the survivors or even higher (Suter *et al.*, 1978), and by growing the organism on agar media containing ethidium bromide, the frequency of streptomycin-sensitive variants can be increased to nearly 100%. Crossing experiments have shown that the genetically unstable streptomycin resistance is coded by a chromosomal gene (Hütter *et al.*, 1981; Cramer *et al.*, 1983).

In several streptomycetes various aminoglycoside modifying enzymes have been observed (Nimi *et al.*, 1971 *a, b*; Miller & Walker, 1969; Benveniste & Davies, 1973; Walker & Skovvaga, 1973; Dowding & Davies, 1975; Davies *et al.*, 1979; Piwowarski & Shaw, 1979), primarily phosphotransferases and acetyltransferases (Davies *et al.*, 1979). *Streptomyces glaucescens* strain GLA0 exhibits aminoglycoside 3-acetyltransferase activity (Davies *et al.*, 1979) capable of inactivating gentamicin and tobramycin, but inactive against streptomycin group antibiotics.

In this work we describe the connection between phosphotransferase activity, hydroxystreptomycin production and natural streptomycin resistance in the wild-type strain of *S. glaucescens*.

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Table 1. *Strains of Streptomyces glaucescens*

Strain number	Genetic markers*	Mutagenic treatment†
GLA0	Wild-type	None
GLA205	<i>strS</i> , <i>melC</i>	Cold storage
GLA208	<i>amf</i>	Cold storage
GLA209	<i>strS</i> , <i>melC</i>	Cold storage
GLA212	<i>strS</i> , <i>melC</i>	EB
GLA408	<i>melB</i>	UV
GLA1200	<i>strS</i>	EB
GLA1204	<i>strS</i> , <i>melC</i>	EB
GLA1208	<i>strS</i> , <i>melC</i>	EB
GLA1210	<i>melC</i>	EB
GLA1211	<i>melC</i>	EB
GLA1212	<i>strS</i> , <i>melC</i>	EB
GLA212 Str ^r	Str ^r , <i>melC</i>	AO

* Marker designations are: *amf*, aerial mycelium formation negative; *mel*, melanin negative (Hütter *et al.*, 1981), *strS*, streptomycin supersensitive (Hütter *et al.*, 1981; Crameri *et al.*, 1983).

† EB, ethidium bromide; AO, acridine orange; UV, irradiation; cold storage, for details see Crameri *et al.* (1983). Strain GLA212 Str^r was selected as a revertant of strain GLA212 on agar plates containing 6 µmol AO l⁻¹ and is streptomycin resistant (growth on media containing ≥ 5 µg streptomycin ml⁻¹).

METHODS

Strains. Wild-type *Streptomyces glaucescens* strain GLA0 (= ETH 22794; Baumann *et al.*, 1974, Hütter *et al.*, 1981) and mutant strains employed are listed in Table 1. *Staphylococcus aureus* strain 209P (= ATCC 6538P) served as test organism for hydroxystreptomycin activity. In addition a mutant derivative strain 209P Str^r, resistant to > 400 µg ml⁻¹ of streptomycin was used. Both strains are insensitive to tetracenomycins and to glaucescin. Inhibitory activity against strain 209 but not against 209 Str^r could therefore be used directly to determine production of hydroxystreptomycin, the only major aminoglycoside antibiotic produced by strain GLA0.

Cultural conditions. Stock cultures of *Streptomyces* strains were maintained as described by Crameri *et al.* (1983). Liquid cultures required for the determination of antibiotic production were prepared in a complex medium of the following composition: D-glucose, 0.5% (w/v); soluble starch, 2% (w/v); soy bean flour, 2% (w/v); proteose, peptone (Difco) 0.5% (w/v); meat extract (Lab-Lemco powder, Oxoid) 0.5% (w/v); NaCl, 0.3% (w/v) and CaCO₃, 1% (w/v). The pH was adjusted to 7.0 with 1 mol NaOH l⁻¹.

For testing aminoglycoside phosphotransferase activity, the *Streptomyces* strains were grown on three media described by Benveniste & Davies (1973). Medium A corresponds to the medium used for *S. kanamyceticus* and *S. spectabilis*, medium B to that used for *S. coelicolor* and medium C to that used for *S. fradiae*.

Staphylococcus aureus was grown for 18 h at 37 °C on slants of trypticase soy agar (Becton, Dickinson & Co., Cockeysville, Md, U.S.A.). The cells were then washed from the slants with deionized water (5 ml), centrifuged (10000 g, 5 min), resuspended in 50% (v/v) glycerol (2 ml) and stored at -70 °C.

Determination of hydroxystreptomycin and hydroxystreptomycin 6-phosphate production. Erlenmeyer flasks (500 ml with four baffles) containing medium (100 ml) were inoculated with about 10⁷ spores and incubated on a rotary shaker (120 r.p.m.) at 30 °C for 4 to 7 d. During the fermentation period samples of the cultures were centrifuged (30000 g, 15 min). Petri plates (90 mm) were prepared, filled with trypticase soy agar (12 ml) and inoculated with about 5 × 10³ indicator bacteria ml⁻¹. The culture supernatant (50 µl) was transferred into holes (diam. 8 mm; prepared with a cork borer). After 18 h incubation at 37 °C the inhibition zones were measured. The amount of antibiotic produced was quantified by comparison with standards. The relative potency of hydroxystreptomycin in comparison to streptomycin (base) was determined as 61%.

Hydroxystreptomycin 6-phosphate was determined as antibiotically active hydroxystreptomycin after dephosphorylation with alkaline phosphatase III (final concentration 1.5 units ml⁻¹) for 2 h at 37 °C.

Determination of MIC. Spores and mycelia of the wild-type strain GLA0 and of its mutant derivatives were harvested from agar slants, ground with a glass rod in a test tube and suspended in 50 mmol Tris/HCl buffer l⁻¹, pH 7.3, containing 0.001% (v/v) Triton X-100. Samples of the suspensions (about 10⁷ c.f.u.) were plated on yeast extract/malt extract agar containing a range of antibiotic concentrations, starting with 100 µg ml⁻¹ and using 1:2 dilutions down to 0.1 µg ml⁻¹. The plates were then incubated for 4 d at 30 °C. MIC values (µg ml⁻¹) for a given antibiotic are those able to inhibit completely the growth of the strain tested.

Preparation of cell free extracts. The mycelium from cultures (100 ml) in medium A was collected by centrifugation (30000 g, 15 min), washed with sterile deionized water and resuspended in 40 ml of 0.01 M Tris/HCl buffer l⁻¹, pH 7.8, containing 10 mmol MgCl₂ l⁻¹, 10 mmol NH₄Cl l⁻¹ and 5 mmol mercaptoethanol l⁻¹.

Cells were disrupted by ultrasonication according to Crameri *et al.* (1982). The resulting suspension was centrifuged for 30 min at 30000 g. DNAase I (bovine pancreas) was added to the supernatant (final concentration of 4 $\mu\text{g ml}^{-1}$). After a further centrifugation (105000 g, 4 h) to pellet the ribosomes, the supernatant was dialysed overnight against at least 10 volumes of the same buffer. Dialysed samples were stored at -20°C .

Assay for hydroxystreptomycin phosphorylating enzymes. To 250 μl of cell free extract from cells pregrown in medium A, 250 μl of the following solution were added: 156 μmol hydroxystreptomycin l^{-1} , 0.29 mmol ATP l^{-1} , 1.95 mmol Tris/HCl l^{-1} , pH 7.8, 0.78 mmol MgCl_2 l^{-1} and 0.1 mmol dithioerythritol l^{-1} . After incubation for 2 h at 30°C with gentle agitation, the reaction mixture was treated for 5 min at 100°C to stop the reaction. Residual antibiotic activity was determined as described above for hydroxystreptomycin production. The amount of inactivation was calculated from the remaining biological activity in the reaction mixture, in comparison to a control without cell free extract.

For the reactivation of inactivated hydroxystreptomycin samples (100 μl) were treated with *Escherichia coli* alkaline phosphatase III (final concentration 1.5 units ml^{-1}) for 2 h at 37°C . The reaction was stopped by heating to 100°C for 5 min, and the extent of restoration of antimicrobial activity was determined using *Staphylococcus aureus* strain 209P as test organism as described above.

Chemicals. The antibiotics and precursors used were as follows: ampicillin, novobiocin (sodium salt), oxytetracycline (hydrochloride), neomycin (sulphate), kanamycin (sulphate) and chloramphenicol (Sigma), streptomycin (sulphate) (Novo Industri, Copenhagen, Denmark), dihydrostreptomycin and erythromycin (base) (Serva, Heidelberg, F.R.G.), cephaloridin (Glaxo), hydroxystreptomycin (Abbott, Chicago, Ill, U.S.A.), spectinomycin (Upjohn, Kalamazoo, Mich., U.S.A.), gentamicin and antibiotic G-418 (Schering, Bloomfield, N.J., U.S.A.), rifampicin (Ciba-Geigy), paromomycin (Parke-Davis, Detroit, Mich., U.S.A.), spiramycin and deoxystreptamine (Kyowa Hakko, Tokyo, Japan), streptidine (H. Grisebach, Freiburg i.Br., F.R.G.).

Alkaline phosphatase type III (EC 3.1.3.1) was from Sigma, bovine pancreas DNAase (EC 3.1.4.5) from Calbiochem. Other chemicals were of highest available grades from Sigma, Merck or Fluka, Buchs SG, Switzerland.

RESULTS

Production of hydroxystreptomycin by Streptomyces glaucescens

The wild-type strain GLA0 of *S. glaucescens* was found to produce up to 88 μg hydroxystreptomycin ml^{-1} in the complex fermentation medium (Fig. 2). The optimal temperature for antibiotic production was 30°C , significantly below the optimal growth temperature which was 37°C . In media containing inorganic phosphate antibiotically inactive hydroxystreptomycin 6-phosphate (see Fig. 1) was accumulated instead of the active antibiotic (Table 2).

Natural resistance to streptomycin-type aminoglycoside antibiotics

The susceptibility of the wild-type strain GLA0 and some of its derivatives to various antibiotics was tested. The results (Table 3) show that the increased antibiotic sensitivity of strains GLA1200, GLA1204, GLA1208 and GLA1212 is specific to the streptomycin group antibiotics dihydrostreptomycin, hydroxystreptomycin and streptomycin itself. The difference in absolute resistance levels in comparison to previously published values (Freeman & Hopwood, 1978; Hütter *et al.*, 1981) has to be attributed to test differences. Previously we used spores and tested the effect on spore germination and growth. In this study we used suspensions composed primarily of mycelial fragments to compensate for bad sporulation of some mutant strains. Mycelial fragments have already proven to be much more resistant than spores in other hydroxystreptomycin producers (Benedict *et al.*, 1951) and furthermore the resistance level is highly dependent on the physiological age of the culture (Cella & Vining, 1975; Piwowarski & Shaw, 1979).

Hydroxystreptomycin phosphotransferase activity

Cell free extracts of the wild-type strain GLA0 were tested for the ability to inactivate hydroxystreptomycin by phosphorylation. Hydroxystreptomycin was inactivated by cell free extracts only in the presence of ATP (Fig. 3a). Inactivation was dependent on extract quantity and incubation time following classical saturation kinetics; 1 mol phosphate was required for the inactivation of 1 mol streptomycin (data not shown). Cell free extracts from mycelia grown

Fig. 1. Chemical structure of hydroxystreptomycin, streptomycin and dihydrostreptomycin. For hydroxystreptomycin $R_1 = \text{CHO}$ and $R_2 = \text{CH}_2\text{OH}$; for streptomycin $R_1 = \text{CHO}$ and $R_2 = \text{CH}_3$; for dihydrostreptomycin $R_1 = \text{CH}_2\text{OH}$ and $R_2 = \text{CH}_3$; APH denotes aminoglycoside phosphotransferase.

Fig. 2. Hydroxystreptomycin production in *Streptomyces glaucescens* strain GLA0 at different incubation temperatures. ▲, 26 °C; ●, 30 °C; ■, 37 °C.

Table 2. Influence of media composition on the production of hydroxystreptomycin and hydroxystreptomycin phosphate by *S. glaucescens* strain GLA0

The cultures were grown for 7 d on the media A, B and C described by Benveniste & Davies (1973). Media A and B contain inorganic phosphate, medium C does not. Cultures were harvested and antibiotic activity was tested in the supernatant. Phosphotransferase activity was tested in cell free extracts. For details see Methods. Antibiotic concentrations were determined after growth for 7 d at 30 °C. Hydroxystreptomycin was determined in untreated culture supernatants. Hydroxystreptomycin 6-phosphate was analysed as described in Methods (see Fig. 3b).

Growth medium	Hydroxystreptomycin ($\mu\text{g ml}^{-1}$)	Hydroxystreptomycin phosphate ($\mu\text{g ml}^{-1}$)	Phosphotransferase activity*
A	<1	48	100
B	<1	45	32
C	74	<1	<5

* Arbitrary units. Highest activity in extracts from medium A was taken as 100%, corresponding to total inactivation of the antibiotic under standard test conditions (see Methods).

Table 3. Susceptibility of *Streptomyces glaucescens* strain GLA0 and mutant derivatives to various antibiotics

	MIC values ($\mu\text{g ml}^{-1}$)					
	GLA 0	GLA 1210	GLA 1200	GLA 1204	GLA 1208	GLA 1212
Hydroxystreptomycin	>100	>100	3.13	3.13	3.13	3.13
Streptomycin	>100	>100	1.56	1.56	1.56	1.56
Dihydrostreptomycin	>100	>100	1.56	1.56	1.56	1.56
Kanamycin	1.56	1.56	0.78	0.39	0.78	0.78
Neomycin	12.5	6.25	3.13	1.56	3.13	3.13
Paromomycin	6.25	6.25	6.25	1.56	3.13	3.13
Spectinomycin	25	25	25	25	12.5	12.5
Gentamicin	6.25	6.25	3.13	6.25	3.13	6.25
G-418	50	25	25	6.25	12.5	12.5
Ampicillin	50	50	50	100	>100	100
Cephaloridin	25	25	25	25	25	25
Oxytetracycline	3.13	3.13	12.5	3.13	1.56	1.56
Chloramphenicol	12.5	12.5	25	25	12.5	12.5
Erythromycin	25	25	>100	50	25	25
Spiramycin	50	50	>100	50	50	50
Novobiocin	0.78	0.78	0.78	0.2	0.2	0.2
Rifampicin	3.13	3.13	6.25	3.13	3.13	3.13

Fig. 3. (a) Inactivation of hydroxystreptomycin by cell free extracts of *Streptomyces glaucescens* strain GLA0. Hydroxystreptomycin ($25 \mu\text{g ml}^{-1}$). A, Complete incubation mixture (see Methods), inactivation >99%; B, no cell free extract; C, no ATP; D, no hydroxystreptomycin. (b) Inactivation of hydroxystreptomycin by cell free extracts from *Streptomyces glaucescens* strain GLA0 and reactivation by treatment with alkaline phosphatase III. A, Hydroxystreptomycin ($25 \mu\text{g ml}^{-1}$); B, with addition of alkaline phosphatase III (control); C, with addition of cell free extract with phosphotransferase activity; D, sample C treated with alkaline phosphatase III. For details see Methods.

on medium A had the highest specific activity (100%), extracts from medium B grown cells exhibited about 30% activity, while extracts from medium C grown cells had no detectable phosphorylating activity (Table 2).

The biological activity of the inactivated hydroxystreptomycin could be restored by treating the samples with alkaline phosphatase III (Fig. 3b). Furthermore direct incorporation of ^{32}P was also detected with streptomycin and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (J. Davies, personal communication), thus hydroxystreptomycin inactivation was caused by the activity of a phosphotransferase.

or deoxystreptamin did not restore antibiotic production. Additional studies have shown, that streptomycin-sensitive mutant strains are unable to produce hydroxystreptomycin-phosphate on various media, while the wild-type strain accumulated hydroxystreptomycin-phosphate in liquid media containing inorganic phosphate (Table 2).

Hydroxystreptomycin phosphotransferase activity was only measurable in hydroxystreptomycin-resistant strains such as the wild-type strain GLA0 or the mutant strains GLA208, GLA408, GLA1210 or GLA1211. However, none of the sensitive mutant strains showed any detectable phosphotransferase activity. The hydroxystreptomycin resistant revertant strain GLA212 Str^r, derived from the sensitive mutant strain GLA212, did not exhibit any phosphotransferase activity, and no hydroxystreptomycin was detectable in the fermentation broth.

DISCUSSION

Antibiotic producing micro-organisms are usually more resistant towards their own antibiotics than non-producing strains (Demain, 1974; Vining, 1979). This has also been observed in the hydroxystreptomycin producing *S. glaucescens* strain GLA0 (Freeman & Hopwood, 1978; Hütter *et al.*, 1981). It was initially thought, that the inherent resistance was due, in the absence of modifying enzymes, to low accumulation of streptomycin-type aminoglycosides from the environment, but it was later found that the strains synthesized a specific phosphotransferase (J. Davies, personal communications; Hütter *et al.*, 1981) capable of attacking dihydrostreptomycin, hydroxystreptomycin and streptomycin, but inactive with other aminoglycosides. The ability to produce phosphotransferase activity is frequently lost in *S. glaucescens*. This loss reduced specifically the inherent resistance of the wild-type strain towards streptomycin-type aminoglycosides but did not change the susceptibility pattern towards other antibiotics. Genetic investigations showed, that the loss of phosphotransferase activity is due to chromosomal mutations of unknown nature in the *strS*⁺ gene, or in closely linked genes in the *strS*⁺ region (Hütter *et al.*, 1981; Crameri *et al.*, 1983). We therefore concluded that the activity of the (hydroxy)streptomycin phosphotransferase is the main cause for the inherent resistance of the wild-type strain GLA0 of *S. glaucescens* towards streptomycin-type aminoglycosides.

Such a correlation between the synthesis of a phosphotransferase and streptomycin susceptibility is not new for streptomycin producing streptomycetes. It has already been demonstrated for *S. bikiniensis* by Piwowarski & Shaw (1979) and was studied in *S. griseus* by Nimi *et al.* (1971 *a, b*) and Sugiyama *et al.* (1981 *a, b*) and to some extent by Hotta *et al.* (1981). We have at present no information, whether a decreased affinity of ribosomes or a reduced cell permeability (Cella & Vining, 1975; Sugiyama *et al.*, 1981 *a, b*; Yamamoto *et al.*, 1981) are additional factors contributing at least to some extent to streptomycin resistance in *S. glaucescens*. But the fact that the loss of phosphotransferase activity in the *strS* mutant strains reduces the level of resistance towards streptomycin-type aminoglycosides to that in other streptomycetes is evidence against a strong contribution of these two other factors. We rather assume, as postulated by Nimi *et al.* (1981), that externally added streptomycins are only transiently accumulated in the cell and then excreted again after phosphorylation. Accumulation data with *S. glaucescens* wild-type strain GLA0 (Hütter *et al.*, 1981) are in agreement with such an assumption.

As the phosphotransferase activity was lost completely in the mutant strain and since 1 mol ATP was sufficient to inactivate 1 mol hydroxystreptomycin, we assume that only one type of phosphotransferase was synthesized by our strain and that we are not dealing with diphosphorylations (Walker & Skorvaga, 1973). The phosphorylation of streptidine by our enzyme preparation strongly supports the assumption, that our strain contains a (hydroxy)streptomycin 6-phosphotransferase (Walker, 1975 *b*).

The high specificity of the phosphotransferase activity for streptomycin-type aminoglycosides, including hydroxystreptomycin, seems to support the hypothesis that phosphotransferase is involved in hydroxystreptomycin biosynthesis. The effect of phosphate on hydroxystreptomycin production also supports this assumption. In media containing inorganic phosphate

hydroxystreptomycin phosphate is accumulated, presumably due to phosphate repression of phosphatases (Miller & Walker, 1970; Demain & Inamine, 1970). In cells grown in media without inorganic phosphate hydroxystreptomycin production is high. A direct correlation between the production of unphosphorylated antibiotic and phosphatase levels has also been suggested by Shirafuji *et al.* (1977) based on studies with partially phosphatase defective mutant strains in the butirosin producing *Bacillus vitellinus*. The absence of detectable phosphotransferase activity in medium without inorganic phosphate might be attributed to a test artefact caused by high phosphatase levels, including ATPases. The same effect might also be responsible for the absence of detectable phosphotransferase activity in the phage resistant streptomycin producing *S. griseus* mutant mentioned in Ogawa & Perlman (1976).

Walker (1975*b*) pointed out that possibly a number of intermediates in the biosynthetic pathway between streptidine 6-phosphate and streptomycin 6-phosphate, might become dephosphorylated by a streptomycin 6-phosphate phosphatase (Walker, 1975*a*) or other phosphatases. The streptomycin 6-phosphotransferase would then be necessary to salvage these products by rephosphorylation. Absence of the enzyme would lead to accumulation of dephosphorylated intermediates. On the other hand we can not exclude the possibility, that the *strS* mutation affects several genes either due to polarity or to large deletions.

The cloning of the *strS*⁺ gene and its reintroduction into *strS* mutant strains of *S. glaucescens* will show whether such a direct correlation between phosphotransferase activity, streptomycin resistance and hydroxystreptomycin production exists or whether the *strS* mutant strains carry defects in more than one gene.

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