PRESERVATION OF *STREPTOMYCETES*

MONIKA ZIPPEL AND MARLIES NEIGENFIND

*Zentralinstitut für Mikrobiologie und experimentelle Therapie,
Akademie der Wissenschaften der DDR, Forschungszentrum für
Molekularbiologie und Medizin, Beutenbergstraße 11,
DDR-6900 Jena, German Democratic Republic*

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Three methods for preserving 16 *Streptomyces* strains were tested: lyophilization on soil and storage in frozen glycerol medium (-20°C), and in liquid nitrogen (-196°C) in the presence of dimethyl sulfoxide. The viability, stability of auxotrophic markers, antibiotic production, and resistance to their own antibiotics for a period up to one year were studied. No variations in the production of and resistance to antibiotics or accumulation of revertants of mutants were evident during the total preservation time in all three storage methods. A drastic decrease in viable counts was observed after lyophilization on soil. Viability of strains frozen in glycerol and after storage in liquid nitrogen was similar and ranged from 2.3% to 36.6%.

Storage of streptomycetes in frozen glycerol is recommended as a quick and reliable method for frequent studies in the laboratory. Storage in liquid nitrogen is recommended as a long-term preservation method.

Various methods have been suggested for the long-term preservation of streptomycetes and other bacteria. These include lyophilization (1), drying from the liquid state in vacuo (2), deep-frozen slopes (3), soil cultures (4), storage on agar slants overlaid with mineral oil (5, 6), and preservation in liquid nitrogen (7). PELL and SNEATH (8) tested the survival of bacteria after freezing them in a medium containing 15% (v/v) glycerol and subsequent storage at temperatures of 4°C or 22°C .

The storage of streptomycetes without genetic alteration and with a high viability over a long period is of practical interest. Several *Streptomyces* strains with different genetic properties were studied and responses to three preservation methods compared.

Address reprint requests to: Dr. Monika Zippel, Zentralinstitut für Mikrobiologie und experimentelle Therapie, Beutenbergstraße 11, PSF 73, 6900-JENA, DDR.

Lyophilization on soil is a time-consuming procedure, but a simple, rapid and efficient method is required for preserving large numbers of genetically marked *Streptomyces* strains. Lyophilization and storage on paper wicks was described by HOPWOOD and FERGUSON (1), but viability of streptomycetes was the only parameter tested and reported in that paper. Glycerol and dimethyl sulfoxide are the most commonly used cryoprotectants. Glycerol penetrates cells and reduces damage during freezing and thawing (9). Preservation of actinomycetes in frozen glycerol was described by WELLINGTON and WILLIAMS (10).

Preservation in liquid nitrogen was reported by BREESE and SHARP (7) for *Escherichia coli* and the viability of strains was determined after different preservation times. They used this procedure for streptomycetes and additional characteristics of strains were studied under preservation conditions.

In the present study all three preservation methods were used to examine the viability, production of antibiotics, antibiotic resistance, stability of auxotrophic markers, and formation of aerial mycelium in various *Streptomyces* strains.

MATERIALS AND METHODS

Bacterial strains. These are listed in Table 1.

Cultural media. The complete medium AL53 of ZIPPEL et al. (11) and the complete medium M79 of KLAUS et al. (12) were used. The minimal medium of HOPWOOD (13) was supplemented with individual amino acids at a concentration of 100 µg/ml to characterize auxotrophic mutants. The glycerol preservation medium of SALSER (14) contained (g/l): K_2HPO_4 , 12.6; Na-citrate, 0.9; $MgSO_4 \cdot 6H_2O$, 0.18; $(NH_4)_2SO_4$, 1.8; KH_2PO_4 , 3.6; glycerol, 88.0.

Chemicals. Dimethyl sulfoxide (Reachim, SU) was used as a cryoprotectant during liquid nitrogen preservation. LUVOS-Heilerde (type II; VEB Chemisch-Pharmazeutisches Werk Oschersleben, GDR), a sand-like soil served as the solid carrier during lyophilization, since streptomycetes are soil bacteria.

Preservation procedures. Cultures were grown on slopes of agar medium AL53 at 28°C for 10 days. Spores and mycelia were harvested and resuspended in 0.9% saline (10 ml/slope). These suspensions were used to assess all preservation procedures.

1) *Lyophilization.* Glass ampoules were filled with 2.0 g of the soil (LUVOS-Heilerde), sterilized at 180°C for 4 h and 0.9 ml of spore suspensions (sporulating strains) or mycelium suspensions (non-sporulating strains) added. After freezing at -78°C, the contents were lyophilized by drying with a freeze-drying apparatus for 20 h at 20°C and partial atmospheric pressure of 2-10 Pa. The ampoules were filled with sterilized air by a special ventilation device, the sealed.

2) *Preservation in frozen glycerol.* Spore or mycelium suspensions were mixed in vials of 5 ml capacity with the glycerol medium of SALSER (14) in a ratio of 1:1, then stored at -20°C.

3) *Preservation in liquid nitrogen.* Dimethyl sulfoxide was added to the spore

Table 1. Strains of streptomycetes examined.

Strain designation			Characteristics			
<i>Streptomyces hygroscopicus</i>						
IMET JA 6599			Tur ⁺	Tur ^R	Amy ⁺	(wild type)
CC1			Tur ⁻	Tur ^S	Amy ⁻	
R27			Tur ⁺	Tur ^R	Amy ⁺	(high-producing strain)
16LM		ts	Tur ⁻	Tur ^S	Amy ⁺	
22		ilv	Tur ⁻	Tur ^S	Amy ⁻	
48	thr	met	Tur ⁺	Tur ^R	Amy ⁺	
62	met	arg	Tur ⁺	Tur ^R	Amy ⁺	
69		ts	Tur ⁻	Tyr ^S	Amy ⁺	
87	lys	trp	Tur ⁻	Tur ^S	Amy ⁻	
<i>Streptomyces coelicolor</i>						
1098		pheA1		Str ^S	Amy ⁺	
M110	hisA1	uraA1		Str ^S	Amy ⁺	
M130	hisA1	urA1		Str ^S	Amy ⁺	
<i>Streptomyces griseus</i>						
HP			Str ⁺	Str ^R	Amy ⁻	(high-producing strain)
WS			Str ⁺	Str ^R	Amy ⁻	
LM1			Str ⁻	Str ^R	Amy ⁻	
<i>Streptomyces lividans</i> 66						
					Amy ⁺	

Test organisms used for the proof of antibiotic production: *Bacillus subtilis* ATCC 6633—for turimycin production, *Bacillus subtilis* IMET 10881—for streptomycin production.

The strains of *S. coelicolor* were a gift from the collection of the John Innes Institute, Norwich, U. K. and the wild type of *S. hygroscopicus* was obtained from the IMET -Kulturen-sammlung, Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena, GDR. We are thankful to Dr. M. ROTH (18) for the strains of *S. griseus* and the variants CC1 and R27 of *S. hygroscopicus*. We are also grateful to Dr. N. D. LOMOVSKAYA for *S. lividans* strains 66.

His, ilv, lys, met, phe, thr, trp, and ura indicate nutritional requirements for histidine, isoleucine plus valine, lysine, methionine, phenylalanine, threonine, tryptophan, and uracil.

Tur⁻ and Str⁻ indicate inability to produce turimycin and streptomycin, respectively.

Tur^R means resistance and Tur^S sensitivity to their own antibiotic of *S. hygroscopicus* strains; Str^R means resistance and Str^S sensitivity to streptomycin.

Amy⁻ indicates the inability to produce aerial mycelia and spores on complete medium AL53; ts indicates the inability to grow at 37°C. Wild type alleles are omitted.

or mycelium suspensions to give a final concentration of 5% (v/v); the ampoules were filled with 1 ml of this mixture, then stored at -196°C (15).

Before preservation and after different storage periods by all three methods the stability of auxotrophic markers, formation of aerial mycelium, antibiotic production, antibiotic resistance, and viability were tested.

Ampoules were opened and the contents resuspended in 5 ml of saline/ampoule. Strains in frozen glycerol were thawed at room temperature and tested undiluted. Liquid nitrogen suspensions were thawed at room temperature

and diluted with 2 ml of saline. Henceforth these suspensions are referred to as "rehydrated suspensions."

Viable counts. From the rehydrated suspensions ten-fold dilutions in sterile saline were prepared and aliquats were plated on agar medium AL53. After incubation at 28°C for 6 days the percentage viability and formation of aerial mycelia were determined.

Stability of auxotrophic markers. The reversion frequencies of mutants were determined by plating "rehydrated suspensions" on appropriately supplemented minimal media. The temperature-sensitive mutants were tested at 37°C.

Antibiotic production. After determining the viable count, the plates with an appropriate number of colonies were overlaid with the test organism suspended in 0.5% AL53 medium. To assay turimycin production, spores of *Bacillus subtilis* ATCC 6633 were added at a concentration of 1×10^6 spores/ml. To assay streptomycin *B. subtilis* IMET 10881 was used. After incubating at 37°C overnight, turimycin- and streptomycin-producing colonies were detected by their inhibition zones.

Determination of antibiotic resistance. Antibiotic resistance was determined for the strains of *Streptomyces hygroscopicus* and *Streptomyces griseus* because of their importance as antibiotic producers. The spore or mycelium suspension (1 ml) was mixed with 2 ml of liquid medium AL53 (*S. hygroscopicus*) and medium M79 (*S. griseus*), respectively, and incubated in a rotary shaker (240 rpm, 5 cm stroke) for 24 h. Medium M79 was used because the strains of *S. griseus* need some conditions for rapid growth in the overnight cultures that are different from those of *S. hygroscopicus*. From these cultures 1 ml was added to a glass tube with 10 ml of overlay agar medium (0.5% AL53) and poured out on the basic layer of AL53 medium. Wells were punched into the agar and antibiotic solutions filled in at the following concentrations: turimycin, 10; 50; 100, and 250 µg/ml, streptomycin, 10; 100; 500, and 1,000 µg/ml with sterile distilled water as a control. The inhibition zones were measured after incubation at 28°C for 1–3 days. In addition, minimal inhibitory concentration for the variant CC1 and the mutants 22 and 87 were determined with respect to colony-forming ability as described by ROTH et al. (16).

RESULTS

Stability of auxotrophic markers and formation of aerial mycelium

There was no accumulation of revertants of *Streptomyces* mutants in any of the preservation procedures during the whole storage time. The strain *Streptomyces coelicolor* 1098 was already unstable for the marker phenylalanine, as were the mutants M110 and M130 for histidine, but the number of revertants did not increase after preservation.

The ability of the strains tested to form aerial mycelia was unaffected by all three preservation systems.

Production of antibiotics

For all three preservation methods qualitative assessment of antibiotic production showed that strains of *S. hygroscopicus* IMET JA 6599, R27, 48, and 62 retained their productivity while the variant CC1 and the mutants 16LM, 22, 69, and 87 did not regain their ability to produce turimycin. Similarly streptomycin-producing mutants of *S. griseus* did not change during the storage period.

Antibiotic resistance

All strains of *S. hygroscopicus* producing turimycin were resistant to their own antibiotic; the non-producing mutants 16LM and 69 were sensitive and no variations occurred during preservation. Using the method described, the non-producing strains CC1, 22, and 87 were found to be resistant to turimycin. However, by a more exact method reported by ROTH et al. (16) the strains CC1, 22, and 87 were tested and found to be sensitive to turimycin. During the total storage time no variation of streptomycin resistance of *S. coelicolor* and *S. griseus* was found.

Viability

Lyophilization on soil led to a significant loss of viability. After 2 or 7 days there was a drastic decrease in viability of all strains. This decrease proceeded slowly during the subsequent preservation time (Tables 2 and 3). After one year, the viable counts of the strains preserved in frozen glycerol ranged for *S. hygroscopicus* strains from 4.0% to 36.6% (Table 2) and for the other three *Streptomyces* species from 2.3% to 19.0% (Table 3). These results did not depend on whether the strains were able to form aerial mycelia (*S. hygroscopicus* R27 and mutant 48, Table 2) or not (*S. hygroscopicus* CC1 and mutant 87, Table 2).

DISCUSSION

Viability of strains depends on the preservation method. Lyophilization on soil without protectant is not recommended for preservation of streptomycetes. The stresses during lyophilization on soil are very great whether spores or hyphal fragments are preserved. The subsequent long-term storage has only a little influence on viability. A further disadvantage of lyophilization is the time-consuming procedure of the the preparation of ampoules.

Unlike lyophilization on soil, preservation in frozen glycerol represents a simple, cheap and effective method. Damage during frozen storage is probably caused by a rise in the concentration of electrolytes within the cell, since water is removed as ice (9). Glycerol as a protective agent reduces the death rate of cells and concentrations of 10–15% (v/v) were tested successfully for storage of several bacterial species on glass beads (17). BARBOUR and PRIEST (18) recommended preservation of lactobacilli in 20% (v/v) glycerol. WELLINGTON and WILLIAMS (10) reported that 10% (v/v) glycerol was sufficient for preservation of actinomycetes. Higher concentrations were detrimental, but several freezing and thawing cycles had

Table 2. Viability of *Streptomyces hygroscopicus* strains after lyophilization (L) or storage in glycerol medium (G) and liquid nitrogen (N).

Strains	Viable count before preservation	Type of preservation	2 days	1 week	5 weeks	3 months	6 months	12 months
				(% viability retained after storage) (n.t. means not tested)				
JA 6599	1.6×10^7	L	36.0	0.1	1.7	0.4	0.2	0.08
		G	106.2	93.7	112.0	58.0	81.0	35.0
		N	48.7	n.t.	30.0	36.0	29.0	32.5
CC1	1.2×10^8	L	0.8	0.2	0.02	0.01	0.002	0.0007
		G	67.5	12.5	6.1	2.8	4.3	9.2
		N	20.0	21.6	7.6	10.0	20.0	10.8
R27	1.5×10^6	L	2.1	0.6	0.1	0.02	0.1	0.01
		G	113.0	93.3	107.0	20.6	8.7	6.5
		N	120.0	93.3	150.0	113.0	106.6	100.0
16LM	4.6×10^7	L	96.0	1.3	2.8	2.0	2.0	1.6
		G	108.7	12.2	34.8	20.8	28.3	20.4
		N	119.5	6.9	16.0	21.0	7.0	14.5
22	1.8×10^7	L	0.9	n.t.	0.07	0.02	0.01	0.04
		G	46.0	6.7	26.0	19.8	26.0	10.5
		N	30.0	16.0	19.0	33.0	12.0	10.0
48	1.3×10^6	L	5.6	1.8	0.6	0.4	0.2	0.07
		G	131.0	100.0	55.4	41.5	7.7	36.6
		N	17.0	35.0	42.0	24.0	27.0	33.1
62	4.5×10^6	L	n.t.	0.02	0.004	0.004	0.003	0.004
		G	57.8	44.4	12.2	3.6	3.8	4.0
		N	4.0	5.0	n.t.	n.t.	8.0	3.3
69	1.6×10^8	L	30.0	3.1	2.6	1.8	1.4	1.1
		G	112.5	36.2	53.8	14.4	15.0	9.3
		N	49.0	32.0	n.t.	8.0	18.0	8.8
87	4.5×10^6	L	1.1	2.1	0.01	0.007	0.02	0.005
		G	24.0	35.0	15.3	44.4	40.0	14.0
		N	16.0	n.t.	50.0	40.0	26.0	14.0

only a small effect on the viability of strains. This fact is of particular relevance to the preservation of streptomycetes in frozen glycerol because our method allows the use of parts of the inoculum of one ampoule several times without genetic alteration.

Storage in liquid nitrogen requires special equipment and is limited by the number of strains which can be stored, but the damage to the cells is small. FELTHAM et al. (17) recommended dimethyl sulfoxide as a cryoprotectant at a concentration of 5% (v/v); the viability of strains was similar to that in frozen glycerol and did not depend on spores of hyphal fragments being preserved.

The explanation of viabilities of more than 100% could be due to the clumping

Table 3. Viability of *Streptomyces* strains after lyophilization (L) or storage in glycerol medium (G) and liquid nitrogen (N).

Strains	Viable count before preservation	Type of preser- vation	2 days	1 week	5 weeks	3 months	6 months	12 months
				(% viability retained after storage)				
				(n.t. means not tested)				
<i>S. coelicolor</i>								
1098	4.0×10^7	L	40.0	n.t.	0.5	0.4	0.6	0.3
		G	47.5	n.t.	25.0	21.5	13.0	11.5
		N	80.0	n.t.	10.5	10.0	12.0	14.5
M110	3.5×10^7	L	0.9	n.t.	0.002	0.003	0.0007	0.0006
		G	114.0	21.1	42.8	4.0	19.1	6.8
		N	126.0	n.t.	40.0	21.4	18.0	25.7
M130	3.4×10^7	L	n.t.	0.0006	0.001	0.001	0.001	0.0008
		G	15.8	1.8	4.4	0.8	1.3	2.3
		N	7.6	12.3	5.0	4.7	2.0	2.7
<i>S. lividans</i>								
66	1.1×10^7	L	16.0	0.9	0.8	0.2	0.09	0.003
		G	84.5	47.2	34.5	10.9	12.7	19.0
		N	46.4	17.3	15.4	5.4	18.2	12.7
<i>S. griseus</i>								
HP	1.1×10^6	L	5.4	0.6	0.07	0.02	0.05	0.003
		G	136.0	29.0	34.5	29.0	10.0	11.8
		N	90.0	40.0	21.8	19.1	16.4	15.4
WS	1.2×10^5	L	1.7	0.4	0.8	0.2	0.1	0.03
		G	13.3	12.5	15.0	n.t.	20.0	6.2
		N	15.0	12.5	21.0	13.3	10.0	26.6
LM1	3.2×10^7	L	7.0	n.t.	0.2	0.02	0.01	0.002
		G	62.5	n.t.	46.0	13.1	11.0	12.2
		N	75.0	31.0	40.6	50.0	30.3	20.6

of spores and especially hyphal fragments of streptomycetes when they are washed from the agar surface. After lyophilization or freezing and thawing, the clumps could be dispersed easily during spreading on agar plates and so we get higher viabilities. Furthermore, unhomogeneous distribution of the hydrophobic spores and mycelial fragments could give different numbers of colony-forming units per ampoule.

HOPWOOD and FERGUSON(1) lyophilized streptomycetes in a gelatin-glucose-peptone medium without apparent loss of viability. In a similar way *Streptomyces* strains have been preserved by us in a mixture of glucose and gelatin (5% w/v) and stored at -20°C over some years and no genetic alterations or significant decrease of viability have been evident. Preservation should be in frozen glycerol, glucose-gelatin medium, and storage in liquid nitrogen. The highest viability was observed after storage in liquid nitrogen. Different tests can be used to determine antibiotic

resistance. The results show that our procedure provided a rough estimation. However, for simultaneous investigation of several properties of 16 *Streptomyces* strains it was the only practicable one. In addition, a more exact method (16) was used only for uncertain cases of *S. hygroscopicus* strains (CC1, 22, and 87). In summary, no variation of antibiotic resistance was evident for the three *Streptomyces* species during the total storage time.

Concerning the stability of plasmid DNA, BREESE and SHARP (7) reported that no loss occurred in *E. coli* strains following storage in liquid nitrogen. We found that all three preservation procedures have no effect on the stability of the plasmid SCP2 which can be readily isolated from strain *S. coelicolor* 1098.

In summary, we found that none of the three preservation types influenced the stability of auxotrophic markers, the ability to form aerial mycelia and to produce antibiotics, or the antibiotic resistance of 16 *Streptomyces* strains tested during storage times up to one year. Storage in frozen glycerol medium or in liquid nitrogen gives greater viabilities than lyophilization without protectant on soil. Therefore, we recommend preservation in frozen glycerol medium and in liquid nitrogen as useful methods for maintaining *Streptomyces* strains.

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