Biosynthesis of Tetracycline

Strain Improvement and Taxonomy of Productive Streptomyces

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ABSTRACT, After irradiating a streptomyces isolated from soil with UV-light, a mutant was obtained which was no longer capable of chlorinating the tetracycline nucleus. The high amount of tetracycline produced (95% of the total activity of tetracycline compounds) was not influenced by the concentration of chloride ions in the medium. Taxonomic characterization indicates that the strain thus obtained designated *Streptomyces* sp. 1717 (NCIB 9692), is different from *Streptomyces aureofaciens*.

Tetracycline (TC) is produced by *Streptomyces aureofaciens* (Doerschuk *et al.*, 1959), *Streptomyces rimosus* (Perlman *et al.*, 1960) and other streptomycetes (Waksman & Lechevalier, 1962). Many chlortetracycline (CTC) producers synthesize TC in the presence of inhibitors of chlorination. Strains with lowered ability to chlorinate which produce TC even in complex media with chlorides were obtained by mutagenic treatment (Petty 1961).

In this paper we describe the strain improvement and taxonomic characteristics of the TC producer obtained from a collection of antagonistic streptomycetes isolated from natural sources (Plachý & Nečásek, 1959).

MATERIALS AND METHODS

Strain. The original strain of streptomyces isolated from the soil sample produced CTC with traces of TC. A variant producing about 1000 μg CTC/ml was prepared from a selection of this strain. A strain producing about 2000 μg CTC/ml was obtained from this variant by repeated cultivation on agar-media with CTC (Katagiri, 1954). This strain was used for experiments with mutagens. The streptomyces species described here is included in the collection of the Research Institute of Antibiotics and Biotransformations, Roztoky near Prague, under the number 1717 and is registered in the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, under the number NCIB 9692.

Cultivation. Media used in the experiments were described by Herold and coworkers (1956). Sporulation medium (in %, w/v): Sucrose 0.3, dextrin 1.5, urea 0.01, NaCl 0.05, K_2HPO_4 0.05, $MnSO_4 . 7 H_2O$ 0.05, $FeSO_4 . 7 H_2O$ 0.001, peptone 0.5, beef extract 0.1, agar Difco 3. Sterilization 40 min at 120°, pH 6.9—7.1 after sterilization. Fermentation medium (in % w/v): sucrose 3, soyabean meal 2, molasses 0.2, corn steep liquor (50% dry) 0.5, NaCl 0.5, $CaCO_3$ 0.4, $(NH_4)_2SO_4$ 0.2. Sterilization 40 min at 120°, pH 6.6—6.8 after sterilization.

Submerged culture was carried out in round flasks (vol. 500 ml) with 80 ml of medium on a shaker (O_2 transfer 0.52 ml/ml medium/h) at 28°. A 24-hour culture, inoculated with a loop of spores and cultivated in the same medium, was used

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as inoculum. A 5% inoculum was used for production flasks. The production medium contained 2 μg of benzyl thiocyanate/ml. This compound, which stimulated the production of tetracyclines (Pecák *et al.*, 1958) was added to the medium before inoculation. Fermentation was carried out in 3 parallel flasks. The TC content was determined after 72 and 96 h of cultivation. The results shown represent the mean of maxima obtained in the three flasks.

Strain improvement. Spores growing on the surface of agar slants were suspended in 10 ml distilled water which was then filtered through cotton-wool and twice washed with distilled water. 7 ml of this suspension was irradiated during constant stirring for 10 sec in a Petri dish (diameter 70 mm). The UV-lamp, Phillips TUV 30 W, was 20 cm from the surface of the suspension. The survival rate was about 20% under these conditions, when cultivated after irradiation on a control sporulation-medium. Most of the irradiated suspension was seeded on a sporulation-medium containing 1200 μg CTC/ml in Petri dishes. This concentration of CTC caused the death of only 5% of spores.

Taxonomy. Gottlieb's (1963) and Shirling's and Gottlieb's (1966) methods were used for taxonomic studies. The experiments were carried out at 28° C on complete dehydrated media prepared for International Streptomyces Project (ISP) in Difco Laboratories, Detroit, USA. The following strains were used for taxonomic comparison: *Streptomyces aureofaciens* NRRL 2209 (Duggar, 1949), obtained from the Northern Utilization Research and Development Division, Peoria, Illinois, and *Streptomyces aureofaciens* ATCC 12416c (Minieri *et al.*, 1956) obtained from the American Type Culture Collection, Washington.

Analytical methods. Tetracycline was determined spectrophotometrically according to Levine and co-workers (1949). The proportion of CTC and TC was examined chromatographically according to Urx and co-workers (1963).

RESULTS AND DISCUSSION

A collection of 58 isolates was prepared after UV irradiation of spore suspension (25% survival) and evaluated by cultivation on a shaker. Fig. 1 shows the distribution of the productive activity of these isolates in groups of 10%. The isolates produced 80—90% of CTC and 20—10% of TC. Only one isolate produced 95% of TC.

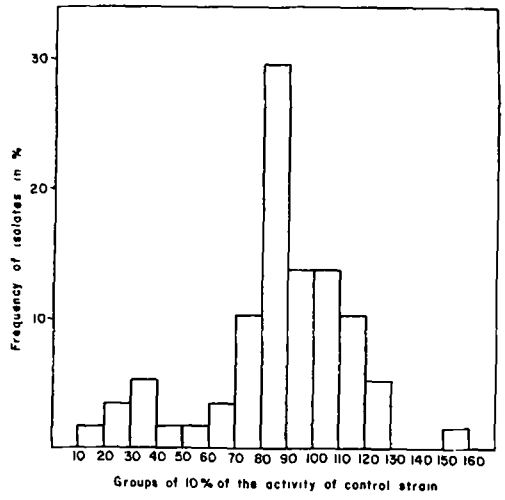

Fig. 1. Polygone of the production activity of isolates.

Table 1. Influence of sodium chloride on the production of TC by *Streptomyces* sp. 1717

% NaCl	Tetracycline compounds (total activity, μg TC/ml)	% TC
0	2810	95
0.5	2950	95
1.0	2225	95

Composition of fermentation medium as described in "Materials and Methods" except concentration of sodium chloride which varied.

The evaluation of this isolate, called strain 1717, showed that it produced more than 95% of TC with traces of CTC, independent of the concentration of NaCl (Table 1). The benzyl thiocyanate used as an ingredient of the medium did not influence the percentage of TC production (Table 2).

Table 2. Influence of benzyl thiocyanate on the production of TC by *Streptomyces* sp. 1717

Benzyl thiocyanate (μg/ml)	Tetracycline compounds (total activity, μg TC/ml)	% TC
0	1503	95
2	2920	95

Járai (1965) studied the genetic basis of chlorination ability in different mutants of *Streptomyces aureofaciens*. Since this characteristic is located in the chromosome, the mutants, which have lost their chlorination ability, are very stable and do not segregate in strains producing CTC. Our results confirm this conclusion. The strain obtained by mutagenic treatment was stable and produced TC independent of medium composition.

Table 3. Morphological characteristics of *Streptomyces* sp. 1717

Sporophores	Short, closed spirals, single hooks and loops
Shape of spores	Cylindrical
Size of spores (μ)	Diameter 1.4 × 0.7; limits 1.1—1.8 × 0.6—0.8
Surface of spores	Magn. 8000× Smooth Magn. 49000× Parallel fibrils

When studying the taxonomic characteristics of the strain, we found sporophores in the shape of short, closed spirals, single hooks and loops in a well-sporulating culture of *Streptomyces* sp. 1717. This strain thus belongs to the morphologicals section "Spira" according to the classification of Pridham and associates (1958).

Table 4. Culture characteristics of *Streptomyces* sp. 1717

Medium	Growth	Sporulation	Colour of sporulated aerial-mycelium	Soluble pigment	Colour of substrate mycelium
Yeast extract-malt extract agar	Fine	Slight	Grey-violet Oc 7t	0	Brown Oc 5r
Oatmeal agar	Slight	Slight	Grey-green--violet Coo 7s	0	Grey-brown Coo 6s
Starch agar with salts	Fine	Medium	Grey-violet Oc 7t	0	Creamy Coo 7b
Glycerol-asparagine agar	Medium	Poor	White Coo 7a	0	Light grey Coo 7b
Asparagine-meat extract glucose agar	Slight	Slight	Grey-green--violet Coo 7s	0	Yellow-brown Coo 5s
Emerson's agar	Slight	No sporulation	—	Light yellow--orange Coo 3m	Brown-green Coo 4s

Note: Colours are determined according to Prauser's scale (1964).

It belongs to the series "Griseus" of the section "Spira" of the above-mentioned classification according to the colour of ripe sporulating aerial mycelium (Table 3). The substrate mycelium of the 1717 strain on different media had the following colour: greyish, cream, brown, grey-brown, yellow-brown, brown-red or brown-green (Tabe 3). The spores formed on the aerial mycelium were cylindrical with a smooth surface. An ornamentation of parallel fibrils was seen only under high magnification (Table 4). When we tested the 1717 strain on different sugar sources (Pridham & Gottlieb, 1948), we found that it grew on sucrose, D-glucose, D-fructose and raffinose but did not grow on L-arabinose, D-xylose, inositol, D-mannitol, rhamnose and cellulose. This strain did not synthesize any melanoid pigment, did not reduce nitrates, but liquefied gelatin and peptonized milk without any visible change of pH and hydrolysed starch. The taxonomic characteristic of the 1717 strain and the results of comparative taxonomic experiments with *Streptomyces aureofaciens* NRRL 2209 and *Streptomyces aureofaciens* ATCC 12416c (Table 5) show that *Streptomyces* sp. 1717 is not identical with the *Streptomyces aureofaciens* originally described by Duggar (1948). A complete taxonomic study of the strain *Streptomyces* sp. 1717 will be published later.

Table 5. Characteristics of the strains tested

Test	*Streptomyces* sp. 1717	*Streptomyces aureofaciens*	
		NRRL 2209	ATCC 12416c
Sporophores*	Short closed spirals, some hooks and loops (Spira-Section)	Hooks and loops (Retinaculum-Apertum Section)	
Spores			
surface	Parallel fibres	"Texture"	
shape	Cylindrical	Boxy with corners rounded up to ovoidal	
size (μ)	1.1—1.8 × 0.6—0.8	0.9—1.4 × 0.6—0.9	0.9—1.6 × 0.6—0.9
Colour en masse spores*	Grey-green-violet	Light grey, grey	Light grey with brown margins
Colour of vegetative mycelium	Grey-brown	Greenish	Light grey and green violet
Soluble pigment	None	Light yellow-green	
C-utilization			
Raffinose	++	−	±
D-Xylose	−	+	±
L-Arabinose	−	++	+
Growth character	Sharp margins	Veil margins	
Litmus milk			
Coagulation	±	+	+
Peptonization	+++	+	±
Shift in pH	No shift	To acid side	To alkaline side
Gelatin liquefaction	+	−	−

* On oatmeal agar.

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