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USE OF CHEMOSTAT FOR SELECTION OF STREPTOMYCES HYGROSCOPICUS MUTANTS ALTERED IN REGULATION OF MALTOSE UTILIZATION

M. Roth, M. Neigenfind, E. J. Bormann, and D. Noack

(Central Institute of Microbiology and Experimental Therapy, Academy of Sciences of G.D.R., DDR-6900 Jena, G.D.R.)

SUMMARY

Streptomyces hygroscopicus mutants showing altered fermentation kinetics were isolated using a selection procedure in a chemostat. Several mutants were obtained which differed in their capacity to produce the macrolide antibiotic turimycin.

INTRODUCTION

The biosynthesis of the macrolide antibiotic turimycin by both wild-type and improved strains of Streptomyces hygroscopicus occurs during a short period only. After about two days of cultivation in starch-containing complex media increasing concentrations of NH_3 and corresponding medium alkalisation were observed. This was accompanied by an enhanced activity of intracellular alanine dehydrogenase and an increase in the alanine concentration in the medium. Since the level of pyruvate in the mycelium decreased, a lack of this essential precursor of turimycin may explain the cessation of antibiotic biosynthesis at that time (Gräfe et al., 1974).

From preliminary growth experiments using unbuffered mineral salts medium with different combinations of carbon sources and casamino acids we concluded that in S. hygroscopicus the α-glucosidase is probably repressed by amino acids as it has been shown in S. venezuelae (Chatterjee and Vining, 1981). If S. hygroscopicus strains were grown on maltose and casamino acids, an alkalization of the medium occurred. This was caused by deamination of amino acids and their use as a carbon source in the presence of maltose. In contrast, an acidification was observed in the

cultures if the maltose was replaced by glucose.

From these findings one can conclude that, in complex media containing amino acids, glucose, and starch, the amylase-generated maltose and maltotriose are not utilized after exhaustion of glucose. As it has been demonstrated by Gräfe et al. (1974), increasing concentrations of NH_3 lead to induction of alanine dehydrogenase and formation of alanine from pyruvate. Consequently, alanine accumulates because of the inhibition of protein synthesis by turimycin and induction of alanine dehydrogenase by alanine itself.

In order to improve turimycin production by S. hygroscopicus it was our aim to overcome this physiological situation by enhancement of carbohydrate utilization. Therefore we intended, as a first step, to select mutants whose α-glucosidase activity is less susceptible or even not subject to catabolite repression or inhibition by amino acids. Besides this property, it was important that the mutants should produce at least the same amounts of turimycin as the improved parental strain.

The technique of continuous culture in chemostat has been found to be very effective for selection of mutants of E. coli altered in the regulation of intracellular enzymes (Sikyta, 1984). We used this approach successfully to obtain the desired mutants and therefore showed that this method can also be applied to filamentous microorganisms such as streptomycetes.

MATERIALS AND METHODS

Organism

The improved turimycin-producing strain Streptomyces hygroscopicus JA6599/PR1 from the collection of the Central Institute of Microbiology and Experimental Therapy, Jena, G.D.R., was used in this study.

Culture conditions

Continuous culture of S. hygroscopicus in chemostat was carried out as described previously (Roth and Noack, 1982). Mineral salts media M1 and M2 were used. Dilution rate of the chemostat was 0.2 h^{-1}, the temperature was 30°C. Samples taken from the chemostat were plated on MSA medium. Mutants which had been initially selected on this agar medium were tested with respect to maltose utilization by cultivation on 20 ml medium MS in 100 ml flasks on a rotary

shaker (240 rev. per min., 5 cm stroke) at 28 °C.
Turimycin production by these clones was examined by overlaying of colonies grown for 6 d at 28 °C on AL53 agar with AL53 soft agar inoculated with Bacillus subtilis ATCC6633. Inhibition zones around producing colonies were detectable after 8 h incubation at 37 °C.
Turimycin production by the mutants was tested quantitatively in submerged cultures on 20 ml AL59aII medium in 100 ml flasks on a rotary shaker. Antibiotic concentrations in the cultures were determined at 48, 72, and 96 h after inoculation by means of the usual agar diffusion bioassay.

Media

M1 (g/l): KH_2PO_4, 2.72; $Na_2HPO_4 \cdot 2H_2O$, 3.56; NaCl, 5.0; Na_2SO_4, 1.0; $MgCl_2 \cdot 2H_2O$, 0.04; $FeCl_3$, 0.005; $MnCl_2$, 0.004; NH_4Cl, 0.4;maltose, 2.0; vitamin-free casamino acids (Difco), 0.15 (growth-limiting nutrient); pH 6.8.

M2: Composition as M1 with the modification that NH_4Cl was the only source of nitrogen in growth-limiting concentration (0.08 g/l).

MS (g/l): NaCl, 4.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.5; $MgCl_2 \cdot 6H_2O$, 0.04 ; maltose, 5.0; vitamin-free casamino acids (Difco), 5.0; solution of trace elements (Okanishi et al., 1974), 2 ml; pH 6.8.

MSA: Composition as MS and in addition (g/l): neutral red, 0.006; bromothymol blue, 0.006; agar (Serva), 20.0. The colour of MSA is red at acidic pH below 7.0 and green above 7.2.

AL53 agar (Roth and Noack, 1982). Soft agar contained 7.5 g agar per 1.

AL59aII (g/l): potato starch, 40; soja meal, 20; glucose, 5; molasses, 5; dehydrated yeast, 5; $CaCO_3$, 2; pH 6.8.

RESULTS AND DISCUSSION

In order to increase the probability of isolating mutants altered in the regulation of α-glucosidase a directed selection was carried out in chemostat culture. The parental strain S. hygroscopicus JA6599/PR1 was grown in a chemostat using medium M1. After the population had reached the steady state a single addition of the mutagen nitrosomethylurea at a concentration of 0.6 mg per ml culture was made to induce mutations. In medium M1 (strong limitation

by amino acids and excess of maltose) those mutants which can utilize the maltose even in presence of amino acids should attain a growth advantage in competition with the parental strain growing solely on amino acids. In the course of the selection procedure additional growth advantage for the mutants was provided by shifts to medium M2 (ammonium chloride limitation, maltose in excess, no amino acids) and vice versa to M1 (see Tab. 1). During shifts between the two media the parental strain would have to switch between utilization of amino acids or of maltose as a carbon source whereas the mutants should continue to grow without disturbance.

Several times during the selection procedure samples from the chemostat were plated on MSA medium supplemented with pH indicator dyes. After 5 d incubation red colonies acidifying the medium were detectable among the majority of green colonies representing the parental phenotype. As there is a strong correlation between aerial mycelium formation and turimycin production (Roth and Noack, 1982) only red colonies with aerial mycelium (Amy^+) were picked because we wanted to select mutants producing high amounts of the antibiotic. These primarily isolated clones were subsequently tested on AL53 agar for their ability to produce turimycin. Their maltose utilization was examined by submerged cultivation in medium MS containing maltose and casamino acids. Clones causing a decrease in the pH of the test medium and producing turimycin (Tur^+) on agar medium were chosen for further characterization.

The results of the selection procedure are summarized in Table 1. Mutations affecting differentiation processes such as aerial mycelium formation and antibiotic production were also induced by the mutagen. In agreement with earlier results (Roth and Noack, 1982) the proportion of Amy^+ clones decreased with time in the chemostat population. Therefore it was only possible during a short period of the selection procedure to isolate mutants altered in the regulation of α-glucosidase but which still produced turimycin. Thus the maximum number of mutants was obtained from the sample withdrawn from chemostat after 61 generations of culture

under selection pressure, from which 7 stable mutants were detected among 945 colonies tested on MSA medium. Taking into account that we selected mutants with a combination of two different characters, the high proportion of stable mutants (0.74 %) obtained shows the effectiveness of the selection pressure applied. Less than 20 % of the primarily picked clones proved to be stable mutants which were Amy^+ Tur^+. This can probably be attributed to heterocaryosis within the mycelium of the isolates. After more than 150 generations of continuous culture all the colonies which appeared on MSA medium were red but they were unable to produce turimycin.

Table 1: Results of the selection procedure in chemostat

Time of sampling (generations after mutagenic treatment)	Medium shifts	Amy^+ clones in the population (%)	Number of primarily selected clones (red on MSA, Amy^+)	Number of stable mutants which were Amy^+ Tur^+
0	M1	98.8	0	0
26		83.7	3	0
47	M2	37.5	12	1
61	M1	11.2	16	7
74	M2	2.0	11	1
122		0.6	4	0
143		1.1	3	0

For a first characterization of the mutants their turimycin production was examined in submerged cultures in the complex medium AL59aII. Differences in productivity were observed ranging from production of only very low levels of antibiotic to concentrations that were higher than those obtained with the parental strain JA6599/PR1. The mutants can be divided into 5 groups if one considers both antibiotic production and shifts of pH in the cultures during fermentation (Tab. 2). The 6 mutants out of the first four groups acidified the complete medium in a more or less pronounced manner. In cultures of the last group a rapid pH-increase and early cessation of turimycin biosynthesis was observed. The clones M30, M39, and M36 are of special interest because of their prolonged turimycin production

at acidic pH values. Indeed, isolation of strains of this particular phenotype was the original aim of our selection procedure. Therefore these mutants will be investigated in more detail with respect to regulation of enzymes of starch catabolism.

Our results demonstrate that the application of a suitable selection pressure in a chemostat is an effective tool to obtain mutants of antibiotic-producing streptomycetes which show preferred utilization of particular nutrients.

Table 2: Turimycin biosynthesis by mutants of S. hygroscopicus in submerged cultures

Mutant	48 h		72 h		96 h	
	Turimycin (µg/ml)	pH	Turimycin (µg/ml)	pH	Turimycin (µg/ml)	pH
JA6599/PR1	791	6.8	1608	7.2	1237	7.9
M30	401	6.4	1045	6.0	1248	5.8
M39	677	6.5	1036	6.0	1358	5.7
M36	766	6.7	1389	6.3	1641	6.4
M35	645	6.6	1133	6.0	1045	6.4
M40	722	6.5	1105	6.0	1046	6.6
M50	0	7.0	18	6.4	0	6.0
M34	268	7.8	289	7.9	285	8.1
M44	351	8.0	365	7.8	252	8.1
M48	402	7.9	469	7.8	328	8.1

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