Genetic Stability of Differentiated Functions in *Streptomyces hygroscopicus* in Relation to Conditions of Continuous Culture

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The genetic stability of the capacity of an improved strain of *Streptomyces hygroscopicus* to produce the macrolide antibiotic turimycin was investigated during long-term continuous culture. Dilution rate, growth-limiting substrate and culture temperature were varied. Certain culture conditions resulted in the stable propagation of the inoculated turimycin-producing population. Other conditions led to segregation of the initial population. Turimycin non-producing phenotypes appeared, and in each case the simultaneous loss of ability to form aerial mycelium was observed. The non-differentiating clones were found to be stable, without any reversion to the parental phenotype, indicating that a loss of genetic information probably took place.

INTRODUCTION

An outstanding feature of streptomycetes is the instability or variability of morphological and physiological characters connected with differentiation e.g. the ability to produce antibiotics and pigments, the capacity to form aerial mycelium and spores, and antibiotic resistance. Spontaneous degeneration of different *Streptomyces* species with respect to their capacity for antibiotic production has been observed upon repeated transfer on solid and in liquid media (Williams & McCoy, 1953; Perlman *et al.*, 1954; Reusser *et al.*, 1961; Miyoshi *et al.*, 1980), during continuous antibiotic fermentation or chemostat culture (Bartlett & Gerhardt, 1959; Reusser, 1961; Sala & Westlake, 1966; Shu, 1966) and during industrial scale manipulations (Miyoshi *et al.*, 1980). In some cases the resulting antibiotic non-producing variants were not able to form aerial mycelium. Spontaneous variants of *Streptomyces roseoflavus* var. *roseofungini* lacking aerial mycelium were found during submerged batch culture (Salekh *et al.*, 1978, 1979). The instability of naturally occurring resistance to antibiotics in streptomycetes was reported by Freeman & Hopwood (1978) and Fedorenko & Danilenko (1980). The basis for most of the instabilities mentioned seems to be at the genetic level, at least in those cases where no reversion of degenerate variants to the parent phenotype was observed.

Very little is known about factors and conditions influencing the genetic stability of differentiated functions in streptomycetes in spite of the industrial significance of strain degeneration. Therefore, it was the aim of our investigations to establish an experimental method allowing examination of the influence of different culture conditions on the genetic stability of *Streptomyces* strains. A time-unlimited culture under different constant and reproducible conditions can be realized in the chemostat. For this reason continuous culture

in a chemostat should be a favourable tool for studying genetic degeneration or segregation processes. In a fundamental study on *Streptomyces aureofaciens*, Sikyta *et al.* (1961) showed that under substrate limitation in the chemostat the physiological growth parameters of filamentous streptomycetes are quite similar to those of unicellular micro-organisms (Herbert *et al.*, 1956) provided that the growing mycelium is continuously fractionated.

This paper deals with long-term investigations of the genetic stability of *Streptomyces hygroscopicus* during continuous culture. The colony-forming units of the chemostat populations were tested for their capacity to produce turimycin, a macrolide antibiotic related to the leucomycin group (Knöll *et al.*, 1971). The results of experiments on the action of acridine orange and ethidium bromide on the antibiotic activity of *S. hygroscopicus* suggested that extrachromosomal DNA might be involved in the regulation of turimycin biosynthesis (Kähler & Noack, 1974). Thus, it may be that the characteristic growth parameters of chemostat cultures, such as dilution rate (growth rate), growth-limiting substrate and cultivation temperature, influence the genetic stability and stable inheritance of plasmid DNA and consequently the stability of antibiotic production.

METHODS

Organism. *Streptomyces hygroscopicus* JA6599/NG60-93 from the collection of the Central Institute of Microbiology and Experimental Therapy, Jena, G.D.R., was used in this study. It is a mutant producing about ten times more turimycin than the wild-type strain *S. hygroscopicus* JA6599. All experiments were started with spores from lyophilized stocks to obtain a uniform inoculum.

Media. AL53 agar was used for both the propagation of the strain on slants and the plating of samples from chemostat cultures. This contained (g l⁻¹): sucrose, 3; dextrin, 15; urea, 0.1; yeast extract (Difco), 1; peptone (Difco), 5; NaCl, 0.5; KH₂PO₄, 0.5; FeSO₄, 0.01; agar, 15 (pH 7.0). Continuous culture was carried out in a mineral salts medium containing (g l⁻¹): KH₂PO₄, 2.72; Na₂HPO₄·2H₂O, 3.56; NaCl, 5.1; Na₂SO₄, 1.07; MgCl₂·6H₂O, 0.041; FeCl₃, 0.005; MnCl₂, 0.004; vitamin-free Casamino acids (Difco), 0.1; NH₄Cl, 0.535 for glucose limitation and 0.08 for NH₄Cl limitation; glucose, 0.5 for glucose limitation and 2.5 for NH₄Cl limitation (pH 6.8). Glucose was added to the medium after separate autoclaving for 35 min at 120 °C.

Preparation of inocula for continuous cultures. Spores from a lyophilized stock were transferred to a slant of AL53 agar. After 8 d incubation at 28 °C the spores formed were spread on new slants and spores harvested from these slants were inoculated into 100 ml mineral salts medium (10⁶ ml⁻¹). This preculture was grown in a 450 ml Fernbach flask on a reciprocal shaker (frequency 130 strokes min⁻¹) for 15 h at 28 °C.

Continuous culture in the chemostat. For continuous culture a glass vessel of 250 ml working volume was used. The level of the culture fluid was kept constant by means of an overflow tube. The flow rate of the medium was adjusted by means of a peristaltic pump. The aeration rate was 0.1 l air h⁻¹ (ml medium)⁻¹. To keep the size of pieces of growing mycelium to a minimum the culture was stirred by a flat glass blade (15 × 8 mm) which was fixed horizontally at the end of the stirrer shaft operating at 3000 rev. min⁻¹. The continuous culture vessel was inoculated with 100 ml preculture, and the continuous flow of medium at a low rate (25 ml h⁻¹), aeration and stirring were started immediately. The flow rate was adjusted to the required final rate when the working volume of the culture vessel was reached.

Assay methods for turimycin. In culture filtrates the content of turimycin was determined by an agar diffusion assay method using *Bacillus subtilis* ATCC 6633 as the test organism. The turimycin production of single colony-forming units from chemostat populations was tested on solid medium by means of a mechanized assay method (Knöll *et al.*, 1981). Samples withdrawn from continuous cultures were diluted and plated on AL53 agar for single colonies. Plates were incubated for 12 d at 28 °C. From 250 randomly selected colonies per sample, mycelium was transferred by an inoculation apparatus to microculture bowls filled with 1 ml AL53 agar. Incubation was carried out for 6 d at 28 °C in special boxes containing 64 microcultures. Thereafter, microcultures were placed on an agar layer to allow diffusion of turimycin through the open bottoms of the microculture bowls. This agar layer was overlaid by soft agar inoculated with *Bacillus megaterium* TUA1 (Möllmann, unpublished). After 8 h incubation at 37 °C the inhibition zones were measured. The percentage of microcultures forming inhibition zones (turimycin-producing) provided a qualitative measure and the mean diameter of these inhibition zones provided a quantitative measure of antibiotic production.

In competition experiments with Tur⁺ and Tur⁻ strains, turimycin production of single colonies (incubated for 6 d) was tested by overlaying with soft agar inoculated with *Bacillus megaterium* TUA1. Inhibition zones around Tur⁺ colonies could be detected after 8 h incubation at 37 °C.

RESULTS

Growth of S. hygroscopicus in the chemostat

The main problem in obtaining a continuous culture of *S. hygroscopicus* was the fractionation of growing mycelium. To obtain a culture suitable for studies of genetic segregation processes, this fractionation had to provide a population of mycelial pieces of minimal size containing a minimal number of chromosomes. High-speed stirring (3000 rev. min⁻¹) by a glass blade stirrer was found to be sufficient for this purpose. In the steady state the resulting continuous culture consisted of small mycelium pellets, each of which contained 20–30 branching tips and had an overall mycelial length of about 100–200 µm. The mean numbers of colony-forming units under the different culture conditions are given in Table 1.

The synthetic mineral salts medium for chemostat culture guaranteed unambiguous and reproducible limitation conditions and prevented the formation of turimycin during continuous culture (if turimycin had been present, it could have influenced the genetic segregation processes under study by causing a selection advantage or disadvantage to one genotype). Because of the known inhibitory effect of inorganic phosphate on turimycin production in *S. hygroscopicus* (Gersch *et al.*, 1979), a phosphate buffer system was used as the basis of the mineral salts medium. Only traces of turimycin were detected in filtrates of the continuous culture under both glucose and ammonium chloride limitation, independent of temperature and dilution rate. The maximum specific growth rate of *S. hygroscopicus* NG60-93 in the mineral salts medium was 0.35 h⁻¹, determined by protein measurement (by the Lowry method) in batch culture with high-speed stirring.

Stability of turimycin production in relation to culture conditions in the chemostat

In each set of experiments two chemostats operating at dilution rates (*D*) of 0.1 h⁻¹ and 0.3 h⁻¹ with the same medium and at the same temperature were used in parallel. In separate experiments the culture temperature or the growth-limiting substrate of the medium was changed (Table 1). Each experiment was carried out at least twice.

Glucose limitation at 30 °C. Under these conditions at *D* = 0.3 h⁻¹ (designated culture condition I), turimycin production was stable throughout the culture period of 550 h (240 generations). Both the percentage of turimycin-producing microcultures and the mean diameter of inhibition zones remained constant at the initial levels (Fig. 1). In contrast, at *D* = 0.1 h⁻¹ (condition II), a gradual loss of antibiotic production of the colony-forming units of the chemostat population was observed. The mean diameter of inhibition zones of turimycin-producing microcultures characterizing the efficiency of antibiotic production decreased to a minimum value after continuous culture for 480 h (70 generations) continuous culture (Fig. 1). An increasing number of microcultures appeared which were not able to produce turimycin (Tur⁻). Moreover, all these clones also lost the ability to form aerial mycelium and spores (Amy⁻). From a number of such microcultures four successive transfers to fresh AL53 agar were carried out. In some of these clones antibiotic and aerial mycelium formation were restored upon repeated transfers. The other clones were stably Tur⁻ Amy⁻ and did not show any reversion to the parental phenotype (Tur⁺ Amy⁺), not even after separation of the genomes by protoplast formation and regeneration.

Glucose limitation at 37 °C. During culture for more than 1000 h (condition III, 432 generations; condition IV, 144 generations) at 37 °C the chemostat populations were stable with respect to turimycin and aerial mycelium formation, irrespective of the dilution rate. There was no loss of antibiotic activity like that observed at *D* = 0.1 h⁻¹ and 30 °C (condition II).

Ammonium chloride limitation at 30 °C. In contrast to the stability of turimycin production under condition I, the population in an ammonium chloride-limited chemostat at *D* = 0.3 h⁻¹ (condition V) segregated the Tur⁺ Amy⁺ and Tur⁻ Amy⁻ phenotypes. However, the segregation kinetics (Fig. 2) differed significantly from that found under condition II (Fig.

Table 1. *Growth conditions for continuous cultures used in the investigation of the genetic stability of turimycin production by S. hygroscopicus*

Culture condition	Growth-limiting substrate	Temperature (°C)	Dilution rate, <i>D</i> (h ⁻¹)	No. of colony-forming units ml ⁻¹	Genetic segregation
I	Glucose	30	0.3	1.5 × 10 ⁶	None
II	Glucose	30	0.1	2.5 × 10 ⁶	Observed
III	Glucose	37	0.3	3.1 × 10 ⁶	None
IV	Glucose	37	0.1	3.7 × 10 ⁶	None
V	NH ₄ Cl	30	0.3	1.0 × 10 ⁶	Observed
VI	NH ₄ Cl	30	0.1	2.0 × 10 ⁶	None

Fig. 1. Dependence of turimycin production by *S. hygroscopicus* NG60-93 on culture time in the chemostat with glucose limitation at 30 °C. Samples from continuous cultures were spread on AL53 agar medium. From the colonies obtained about 250 per sample were randomly selected and transferred to microculture bowls filled with AL53 agar. Turimycin production by microcultures was tested quantitatively (mean diameter of measurable inhibition zones; ○, ●) and qualitatively (percentage of turimycin-producing microcultures; □, ■) by a bioassay. Open symbols, *D* = 0.3 h⁻¹; filled symbols, *D* = 0.1 h⁻¹.

Fig. 2. Dependence of turimycin production by *S. hygroscopicus* NG60-93 on culture time in the chemostat with ammonium chloride limitation at 30 °C. Details and symbols as in Fig. 1.

Fig. 3. Competition between the $\text{Tur}^+ \text{Amy}^+$ strain *S. hygroscopicus* NG60-93 and the $\text{Tur}^- \text{Amy}^-$ derivative CC1 in continuous cultures with glucose (○, ●) and ammonium chloride limitation (□, ■) at 30 °C. The proportions of $\text{Tur}^+ \text{Amy}^+$ colony-forming units were determined by plating samples from the continuous cultures. Open symbols, $D = 0.3\text{ h}^{-1}$; filled symbols, $D = 0.1\text{ h}^{-1}$.

1). After about 200 h (86 generations) a few $\text{Tur}^- \text{Amy}^-$ colony-forming units appeared, and during further culture the proportion of those with the Tur^+ phenotype gradually decreased to 5% after 840 h (364 generations). However, the remaining turimycin-producing colony-forming units of the chemostat population were not altered with respect to the efficiency of antibiotic production based on the mean diameter of inhibition zones. Stable $\text{Tur}^- \text{Amy}^-$ clones showing no reversion to the parental $\text{Tur}^+ \text{Amy}^+$ phenotype were isolated.

At $D = 0.1\text{ h}^{-1}$ (condition VI), segregation into different phenotypes was not observed. Turimycin production was unchanged during continuous culture for 984 h (142 generations) (Fig. 2).

Competition experiments with Tur^+ and Tur^- strains

To obtain more information for interpretation of the segregation kinetics, competition experiments were carried out. About half the volume of a continuous culture of the $\text{Tur}^+ \text{Amy}^+$ strain NG60-93 was replaced by a culture of the $\text{Tur}^- \text{Amy}^-$ derivative CC1 (isolated in an experiment under condition II) grown under the same conditions in a chemostat. Samples from the mixed chemostat populations were plated on AL53 agar to determine the proportion of $\text{Tur}^+ \text{Amy}^+$ colony-forming units (Fig. 3). The parental strain NG60-93 was outgrown by the non-differentiating derivative CC1 under all conditions tested. With glucose limitation at $D = 0.1\text{ h}^{-1}$ a particularly rapid decrease of the $\text{Tur}^+ \text{Amy}^+$ colony-forming units within the population was observed. Under conditions I, V and VI the proportion of $\text{Tur}^+ \text{Amy}^+$ colonies decreased only after an initial increase.

These results indicate that an automatic enrichment of Tur^+ phenotypes within a heterogeneous population cannot be expected, not even with conditions I and VI, which were found to be suitable for maintaining a homogeneous $\text{Tur}^+ \text{Amy}^+$ population.

DISCUSSION

Our results show that the stability of turimycin production by *S. hygroscopicus* during continuous culture depends on the growth rate, growth-limiting substrate and temperature. On the one hand, it is possible to obtain reproducible culture conditions resulting in the propagation of a homogeneous $\text{Tur}^+ \text{Amy}^+$ population without strain degeneration. On the other hand, there are some culture conditions which lead to segregation of the initial strain into two different phenotypes.

The appearance of $\text{Tur}^- \text{Amy}^-$ phenotypes in our test procedure indicates that changes took place at the genetic level. This is further supported by the stability of the $\text{Tur}^- \text{Amy}^-$ clones. It can be assumed that in those cases where the $\text{Tur}^+ \text{Amy}^+$ phenotype was restored during repeated transfers on solid medium the mycelial colony-forming units from the chemostat were genetically inhomogeneous.

There are two possible explanations for the changes in genotype – either spontaneous mutation(s) in genes responsible for antibiotic biosynthesis or irreversible loss of genetic information. Our experimental results support the latter. First, spontaneous mutations are in general point mutations. Mutation and back-mutation occur at comparable frequencies. However, revertants from $\text{Tur}^- \text{Amy}^-$ clones were never found. Secondly, the copy-error hypothesis of mutation predicts that the mutation rate should be proportional to the rate of gene replication, and consequently to the growth rate. Experimental results concerning spontaneous and caffeine-induced mutations in continuous culture supported this hypothesis (Kubitschek & Bendigkeit, 1961, 1964). However, our experiments with glucose limitation at 30 °C (Fig. 1) showed the opposite result: at low dilution rate ($D = 0.1 \text{ h}^{-1}$) $\text{Tur}^- \text{Amy}^-$ phenotypes appeared, but a high dilution rate ($D = 0.3 \text{ h}^{-1}$) this segregation was not observed. Thirdly, the loss of turimycin production was associated in each case with loss of the ability to form aerial mycelium. Spontaneous mutations in structural or regulatory genes involved in antibiotic biosynthesis should not cause the simultaneous loss of aerial mycelium formation.

Considering earlier findings (Kähler & Noack, 1974) we suppose that the loss of genetic information responsible for differentiation might be caused by curing of extrachromosomal DNA. However, definite identification and physical characterization of this hypothetical plasmid DNA has not yet been possible due to insufficient reproducibility of banding in CsCl–ethidium bromide gradient centrifugation (Zippel, unpublished). Several authors have suggested the involvement of plasmids in genetic and metabolic processes in streptomycetes (see Hopwood, 1978; Akagawa *et al.*, 1979), but evidence by physical characterization of plasmid DNA has been obtained in only a few cases (Kirby & Hopwood, 1977; Okanishi & Umezawa, 1978; Yagisawa *et al.*, 1978; Akagawa *et al.*, 1979; Hayakawa *et al.*, 1979; Omura *et al.*, 1979). Besides plasmid loss, the deletion of chromosomal DNA as a consequence of transposition of transposons or IS elements might also have caused the appearance of non-differentiating derivatives.

The kinetics of segregation of *S. hygroscopicus* into different genotypes observed during continuous culture (conditions II and V) could result from two processes: (i) the appearance of $\text{Tur}^- \text{Amy}^-$ clones by loss of genetic information (segregation) and (ii) subsequent enrichment of the non-differentiating phenotype due to a selection advantage caused by altered growth parameters such as substrate saturation constants and yield coefficients. From the competition experiments at 30 °C (Fig. 3), we conclude that Tur^- clones appearing in an originally homogeneous Tur^+ population should outgrow the Tur^+ strain. Therefore, stability of turimycin production (conditions I and VI) reflects a lack of genetic segregation. Limitation of energy source (condition II; Fig. 1) seems to cause a continuous ‘wash-out’ of genetic information from the growing mycelium, as indicated by the gradual decrease in turimycin production of the Tur^+ phenotypes (measured as the mean diameter of inhibition zones). This might be interpreted on the basis of the supposed plasmid loss by reduced effectiveness of plasmid replication because of limitation of precursors for DNA synthesis. However, in the second case of genetic segregation (condition V; Fig. 2) the remaining Tur^+ colony-forming units were not impaired in turimycin production. Consequently, general loss of genetic information did not occur.

An increase in temperature (conditions III and IV) seems to promote genetic stability with respect to differentiation in *S. hygroscopicus*. A similar effect on degeneration of the bicyclomycin-producing *S. sapronensis* was reported by Miyoshi *et al.* (1980).

In our studies we observed lower numbers of colony-forming units at $D = 0.3 \text{ h}^{-1}$ than at

$D = 0.1 \text{ h}^{-1}$ (Table 1). This can be attributed to the smaller diameter of hyphae at the lower dilution rate (Riesenberg & Bergter, 1979) leading to an increased response to shear forces. Alterations of mycelial morphology dependent upon the limiting substrate and dilution rate of a continuous culture (Riesenberg & Bergter, 1979) could influence genetic segregation processes as well as the outcome of competition between Tur^+ and Tur^- phenotypes.

We conclude that continuous culture is a useful tool for studying genetic stability and segregation in relation to differentiation in *Streptomyces*. It was possible to isolate non-differentiating variants under suitable conditions in the chemostat without application of mutagens or plasmid-curing dyes which could cause mutations in, for example, structural genes of antibiotic biosynthesis. The availability of such variants permits genetic, physiological and biochemical investigation of the regulation of differentiation processes. In addition, the possibility of maintaining a genetically homogeneous population with respect to antibiotic production under defined conditions in the chemostat might be useful to the microbial technologist confronted with degeneration of industrial strains of *Streptomyces*.

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