Antibiotic GE37468A: A New Inhibitor of Bacterial Protein Synthesis

III. Strain and Fermentation Study

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GE37468A is a new thiazolyl peptide antibiotic acting on Elongation Factor Tu, produced by fermentation of *Streptomyces* sp. ATCC 55365. The low (<10 mg/liter) and poorly reproducible antibiotic productivity of the original soil isolate hampered the development of a scalable fermentation process. A tenfold increase of GE37468A productivity was achieved by selection of spontaneous morphochromatic variants, which appeared at high frequency in the wild type population. The higher producing ATCC 55365/O/5 colony was tyrosinase defective and blocked in the aerial mycelium differentiation (Amy$^-$Mel$^-$ phenotype). The fermentation process of this stable and purified strain was rapidly optimized to achieve a further fourfold improvement in a fed-batch process.

GE37468A is a new thiazolyl peptide antibiotic discovered in a screening program for inhibitors of bacterial protein synthesis acting on Elongation Factor Tu[1,2]. It is structurally and functionally related to the GE2270A and the amythiamicin class of EF-Tu inhibitors[3~5]. GE37468 A is highly active *in vitro* against Gram-positive bacteria and protects mice against *Staphylococcus aureus* infection. Comparative studies in cell-free *Escherichia coli* and rat liver systems showed that the inhibition is selective for prokaryotic protein synthesis. GE37468A was obtained by fermentation of *Streptomyces* sp. ATCC 55365[1]. The antibiotic productivity of the soil isolate was low (<10 mg/liter) and poorly reproducible. In this paper we describe studies on strain selection and fermentation aimed at improving GE37468A yield.

Materials and Methods

Microorganism

Morphological, physiological and chemotaxonomic characteristics of the original soil isolate *Streptomyces* sp. ATCC 55365 were previously reported[1]. Colonial and morphological characters of the selected variants were determined with standard methods[6,7]. Colour determination was made according to MAERZ and PAUL[8]. For Scanning Electron Microscopy (SEM) agar cylinders, carrying the 14 days-old mycelium, were collected from solid culture (ISP#3 medium) in Petri dishes. After 4% OsO_4 (Sigma) vapour fixation, the samples were air dried and affixed to SEM stubs for coating with gold under vacuum according to the protocol of PETROLINI *et al.*[9,10]. Examinations were carried out at 20 kV with a Stereoscan 250MK2 electron microscope (Cambridge Instr., Ltd., Cambridge, U. K.). All photographs were taken on Kodak Plus-X film.

Melanin production was followed spectrophotometrically at 480 nm after the addition of L-tyrosine or (3,4-dihydroxyphenyl)-L-alanine (L-DOPA) to the culture filtrates in the conditions described[11,12]. Inhibition of melanin production by adding different concentrations of the tyrosinase inhibitor hydroquinone (Sigma) was tested in the same experimental system[11,12] as well as in the agar diffusion test described by TOMITA *et al.*[13].

Isolation of Spontaneous Variants

After ten days of growth on ISP#3 at 28°C, spores of *Streptomyces* sp. ATCC 55365 were gently removed from the agar surface and collected in 1% Tween solution. After centrifugation at $1200 \times g$ for 3 minutes, supernatant was collected and centrifuged at $15{,}000 \times g$ for 20 minutes. The pellet was resuspended in the Tween-80 solution at the final concentration of 10^8 spores/ml. Spores were plated on different solid media (ISP#1, ISP#2, ISP#3, ISP#4, ISP#5, ISP#6, ISP#7, BENNETT's, Hickey & Tresner, R2YE, Calcium malate, Czapek-glucose, Glucose-asparagine, Nutrient, Soil extract, Potato)[6,7,14]. After four days of incubation at 28°C, colonies were overlayed with 10^6 cells/ml of *Bacillus subtilis* ATCC 6633 in Soft Nutrient Agar (Difco). After overnight incubation at 37°C, active colonies were picked up and transferred to fresh solid medium. After two weeks, morphochromatic variants were selected by stereoscopic and microscopic observations.

Flask Fermentation

A culture of *Streptomyces* sp. ATCC 55365 as well as single colonies selected as described above, grown on ISP#3 for four days, were used to inoculate 50-ml Erlenmeyer flasks containing 10 ml of seed medium (glucose 2%, yeast extract 0.5%, meat extract 0.5%, peptone 0.5%, hydrolysed casein 0.3%, NaCl 0.15%). After 48 hours of incubation, 5 ml were inoculated in 500-ml Erlenmeyer flasks containing 100 ml of production media. Unless otherwise stated, the basal production medium was AF/MS (glucose 2%, yeast extract 0.2%, soybean meal 0.8%, NaCl 0.1%, $CaCO_3$ 0.4%) or its modified version in which glucose was replaced by 2% sorbitol (AF/MS/S) or 3% dextrin (AF/MS/D). For the screening of nitrogen sources, each of them was tested at 0.5% in a basal synthetic medium (glucose 1%, KH_2PO_4 0.05%, $MgSO_4$ 0.05% and $CaCO_3$ 0.02%). Seed and production flasks were incubated at 28°C on a rotatory shaker (200 rpm). Biomass was measured as packed mycelium volume (PMV) after centrifugation at $1200 \times g$ for 10 minutes in a 10-ml graduated conical glass tube. Antibiotic production was followed by HPLC as previously described[1].

Bioreactor Fermentations

Aliquots of 2 ml of 55365/O/5 frozen mycelium were used to inoculate six 500-ml Erlenmeyer flasks containing 100 ml of AF/MS medium. After 72 hours, the cultures were transferred to the fermenters Chemap Braun CF 3000, containing 14 liters of the following production medium: AF/MS/S, AF/MS/D, or Medium C (glucose 1%, soluble starch 3.5%, hydrolysed casein 0.5%, yeast extract 0.8%, meat extract 0.35%, soybean meal 0.35%, $CaCO_3$ 0.4% and vitamin B12 0.1 mg/liter)[15]. Fermentations were carried out at 28°C, stirred at 900 rpm with an aeration rate of 0.5 standard litres of air per volume per minute. In some experiments a feeding of olive oil (Sagra, Lucca, Italy) composed of 8~13% unsaturated, 70~78% monounsaturated and 12~18% saturated fatty acids, was introduced in the first 24 hours of fermentation at a rate of 1 ml/liter/hour.

Table 1. Effect of various carbon sources to the growth and to the antibiotic productivity of *Streptomyces* sp. ATCC 55365.

Carbon source*	Growth (PMV%)	GE37468A productivity (mg/liter)
Glucose	28	7.9
Fructose	27	4.5
Galactose	30	9.0
Mannose	30	7.0
Rhamnose	24	6.1
Arabinose	23	5.0
Lactose	23	7.3
Maltose	25	2.6
Sucrose	25	1.5
Dextrin	30	8.4
Glycerol	25	2.6
Mannitol	30	3.9
Sorbitol	14	7.7
Inositol	30	7.7
Lard oil	33	4.4
Methyl oleate	30	1.6

* Each carbon source (2%) replaced glucose in AF/MS medium.

Results

Fermentation of the Original Soil Isolate

Different carbon and nitrogen sources were screened in flask fermentation to improve the production medium. *Streptomyces* sp. ATCC 55365 grew well on a variety of carbon substrates (pentose, hexoses, polysaccharides, polyols and fats; see Table 1) and utilized several organic and inorganic nitrogen sources (peptone, alburexN, Pharmamedia, corn steep liquor, hydrolysed casein, fish meal, meat extract, yeast extract, soybean meal, arginine, ammonium chloride, ammonium sulfate, ammonium phosphate and potassium nitrate; data not shown), but the antibiotic productivity in different fermentation media was generally lower than 10 mg/liter and poorly reproducible from one fermentation to another. In these conditions any further study of fermentation development and scaling-up was strongly hampered.

Different Phenotypes are Present in the Original Soil Isolate

The wild type population showed biochemical and morphological heterogeneity. From an overlay screening with *B. subtilis*, only 3% of the first thousand of analysed colonies were active, and 23 of them were reconfirmed

Fig. 1. GE37468A productivity of twenty-three colonies (sixteen brown and seven white) selected by an overlay screenig with *B. subtilis*.

Each value is the mean of three replicates in AF/MS/D.

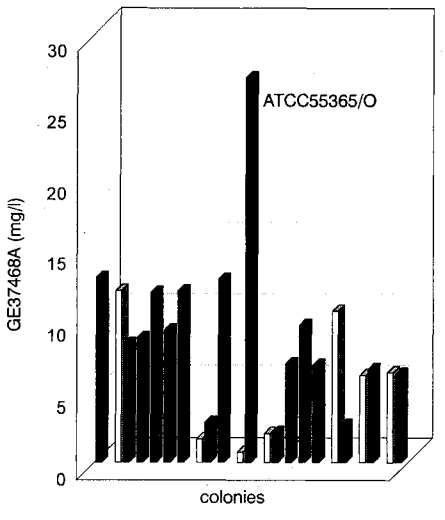


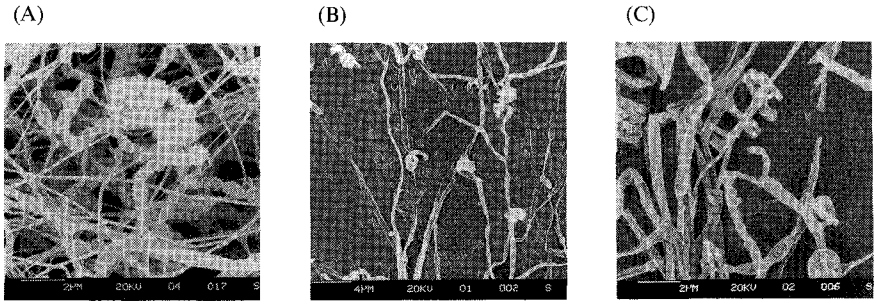
Table 2. Characteristics of the different *Streptomyces* sp. ATCC 55365 phenotypes cultured in the most representative solid media.

Medium	Soil isolate (Amy^+Mel^+)	Brown colonies (Amy^dMel^+)	White colonies (Amy^-Mel^-)
Oat meal-ISP#3	Vmy Maple *11-E-4* Amy White Spo+	Vmy Maple *11-E-4* Amy Scarce White Spo−	Vmy Topaz *12-I-8* Amy Absent Spo−
BENNETT's	Vmy Cream *9-G-2* Amy Scarce White	Vmy Cream *9-G-2* Amy Absent	Vmy Cream *9-G-2* Amy Absent
Peptone yeast extract iron-ISP#6	Vmy Grey *32-A-1* Amy Absent Mel+	Vmy Grey *32-A-1* Amy Absent mel+	Vmy Ivory *9-D-2* Amy Absent Mel−
Tyrosine-ISP#7	Vmy LogCabin *15-A-5* Amy Scarce White Mel+TH+	Vmy LogCabin *15-A-5* Amy Absent Mel+TH+	Vmy Ivory *9-B-2* Amy Absent Mel−TH−
R2YE	Vmy LogCabin *15-A-5* Amy Absent Mel+	Vmy LogCabin *15-A-5* Amy Absent Mel+	Vmy Ivory *9-B-2* Amy Absent Mel−

Vmy: vegetative mycelium; Amy: aerial mycelium; Spo+/−: presence/absence of spores; Mel+/−: presence/absence of melanin; TH +/−: tyrosine hydrolysis reaction positive/negative.
Colour codes (*e.g.*, *11-E-4*) from MAERZ and PAUL[8].

Fig. 2. Scanning electron micrographs of the aerial mycelium in *Streptomyces* sp. ATCC 55365 grown on ISP #3 agar for 14 days.

Long spirals of spiny spores in the original soil isolate (A); not sporulating primitive spirals and loops in Amy^dMel^+ phenotype (B); not sporulating and poorly differentiated aerial mycelium in Amy^-Mel^- phenotype (C).



in fermentation as producers of GE37468A. The range of antibiotic productivity was 7.95 ± 5.48 mg/liter (Fig. 1).

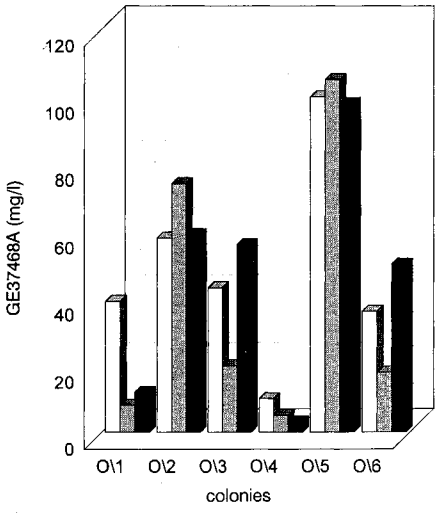
Sixteen of these producing colonies showed colonial and morphological characteristics similar to the wild type ones which were previously described[1], except for a partial mycelium deficiency (Amy^d) and a block in sporulation and for a more intense brown pigment production. The other seven colonies were pigment-defective and almost completely blocked in the formation of aerial mycelium (Amy^-) (Table 2).

Scanning electron microscopy showed that the wild-type aerial mycelium was rich with compact and medium-long spirals with 3 to 8 turns (*ca.* 2 μm diameter) as terminal ends of branched hyphae (the *Spira* section according to the classification of PRIDHAM *et al.*[16]). These spirals were formed by chains of spiny spores of *ca.* 0.7×1 μm (Fig. 2A). Such formations were not detectable in the "brown" colonies, which indeed, produced primitive spirals of $1 \sim 2$ turns and loops (the *Retinaculi aperti* section) but no spores (Fig. 2B). In the white colonies, the differentiation of the aerial mycelium appeared almost completely blocked (Fig. 2C).

The brown pigment was identified as melanin according to the test described by MIKAMI *et al.*[12]. The addition of the tyrosinase inhibitor hydroquinone to the brown colonies reversibly blocked the production of melanin according to MIKAMI *et al.*[12] and the hydrolysis of tyrosine in ISP#7 medium. The addition of tyrosine or L-DOPA to the white colonies did not induce melanin production. Since tyrosinase is the sole enzyme involved in the conversion of tyrosine to melanin[13], these Mel^- colonies appeared defective in the tyrosinase activity.

The two observed phenotypes showed a different stability in the progeny: the "brown" Mel^+Amy^d colonies segregated into Amy^-Mel^- variants with a frequency of $0.1 \sim 1\%$. These last colonies were more stable and reversion was observed at a frequency $\geq 0.01\%$.

Fig. 3. GE37468A productivity in three replicates of six sub-colonies of *Streptomyces* sp. ATCC 55365/O.



Medium was AF/MS/D.

Fig. 4. Fermentation of *Streptomyces* sp. ATCC 55365/O/5 in AF/MS/D at 20 liter fermenter scale.

pH (●), biomass (■), GE37468A (□), oxygen (▲).

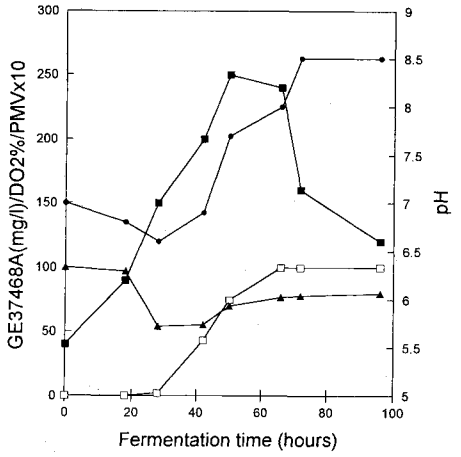
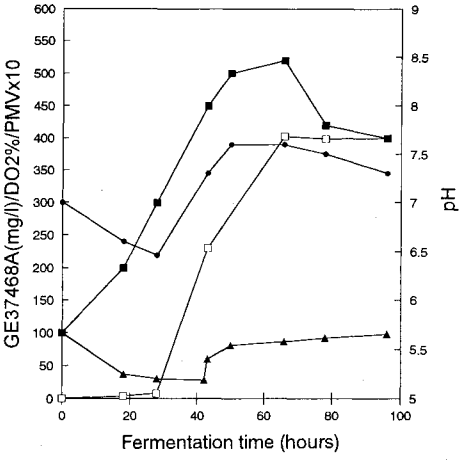


Fig. 5. Fermentation of *Streptomyces* sp. ATCC 55365/O/5 in Medium C with a feeding of olive oil (1 ml/liter/hour in the first 24 hours after inoculation) at 20 liter fermenter scale.

pH (●), biomass (■), GE37468A (□), oxygen (▲).



Selection of a Higher Producing Variant

As shown in Fig. 1, one colony (55365/O) gave productivity significantly over the mean value: 26 mg/liter. This colony had un unstable Mel^+Amy^d phenotype with a 0.4% frequency of segregating into Amy^-Mel^- variants in the progeny. Moreover it tended to lose its productivity when subcultured. Thus the original colony was divided in six sectors and few cells of each sector were replicated and then fermented. As reported in Fig. 3, the GE37468A productivity significantly differed among the sub-colonies. All these sub-colonies had a Mel^+Amy^d phenotype, except the higher producing one called 55365/O/5 which was an Amy^-Mel^- spontaneous mutant. The colonies Mel^+Amy^d were unstable both in morphology and in antibiotic productivity, whereas 55365/O/5 gave a reproducible productivity of 97 ± 11.3 mg/liter in different replicates and after many subculturing steps. No revertant of the Amy^-Mel^- phenotype was observed from 5,000 colonies analysed and during the following scaling-up of 55365/O/5 fermentation.

Fermentation of the High Producing Variant

Approaches of medium optimization, which previously failed with the wild type strain, rapidly increased ATCC 55365/O/5 productivity. Fig. 4 shows the time course of 55365/O/5 at 20 liter fermenter scale in the same medium used in flask fermentation. The production of GE37468A started after 30 hours of fermentation, in the middle of the exponential growth phase, and reached its maximum (*ca.* 100 mg/liter) at 68 hours, at the end of stationary phase. From a screening of different fermentation media previously developed for chemically similar molecules (L. GASTALDO, unpublished data), the most encouraging result was achieved with the Medium C, which was developed for the fermentation of the GE2270 producing *Planobispora rosea* ATCC 53773[15]. In this medium *Streptomyces* ATCC 55365/O/5 gave 225 mg/liter GE37468A after 96 hours of fermentation at 20 liter scale. When the use of this medium was coupled with an early feeding of an extra-carbon source as olive oil (1 ml/liter/hour in the first 24 hours of fermentation), biomass production was doubled, and antibiotic production was increased to 400 mg/liter after 72 hours of fermentation (Fig. 5).

Discussion

One of the characteristic of *Streptomyces* species is the phenomenon of genetic instability, which manifests itself as an extraordinarily high spontaneous mutation rate (0.1 to 10%) affecting specific traits such as sporulation, aerial mycelium differentiation, pigment accumulation and antibiotic resistance and production. The majority of these instabilities are the result of extensive chromosomal deletions which are frequently accompanied by intense DNA amplifications that apparently occurs in the absence of selective pressure in species-specific hot spots in the genome[17]. Strain degeneration and fermentation variability, which represent major obstacles in developing a scalable process for antibiotic production, are industrially critical facets of genetic instability in streptomycetes[18]. *Streptomyces* sp. ATCC 55365, a soil isolate, gave a poor and unreproducible GE37468A production and was morphologically unstable. The selection of morphochromatic spontaneous phenotypes led to the isolation of the stable high yielding variant ATCC 55365/O/5 producing ten-fold more than the parental strain. It was a tyrosinase-defective mutant blocked in the differentiation of aerial mycelium. It is plausible that also in this newly isolated *Streptomyces* sp., combined events of chromosomal deletions and amplifications caused the irreversible loss of melanin production and the alteration of the morphological cycle associated with an increased antibiotic production. The fermentation process of this stable and purified strain was rapidly optimized and a further four-fold improvement was obtained in a fed-batch process. It is worth noting that the best medium so far experimented for the production of GE37468A is the one previously developed for the fermentation of a different actinomyces species, *Planobispora rosea*, which produces the structurally related antibiotic GE2270A[15]. The feeding of olive oil, which was used as an extra-carbon source with the practical means of avoiding catabolite repression, gave also the advantage to control foam formation during the exponential growth of the culture. Considering the lipophilic properties of GE37468A and its prevalent intracellular accumulation, an oily carbon source may also act as an excellent product extractor by changing the membrane permeability to the antibiotic[19,20].

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