GARDIMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

I. DESCRIPTION OF THE PRODUCER STRAIN AND FERMENTATION STUDIES

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Two strains of *Actinoplanes* have been isolated that produce a new peptide antibiotic named gardimycin. A detailed taxonomical study of such strains indicates that they differ between themselves and from all the described species of *Actinoplanes*. For this reason they are considered to be new species; for which the names *A. garbadinensis* nov. sp. and *A. liguriae* nov. sp. are proposed. The type strain of *A. garbadinensis* is A/10889 (=ATCC 31049), the type strain of *A. liguriae* is A/6353 (=ATCC 31048). Studies on the medium and fermentation conditions are reported.

In our search for new antimicrobial substances produced by species of the genus *Actinoplanes* we have found a new family of antibiotics which are of considerable interest because of their high degree of activity in experimental infections in mice coupled with low acute toxicity. The most active compound of the family has been called gardimycin*.

This report describes the taxonomic studies of the two producing organisms and the fermentation of gardimycin. In companion papers, the extraction and chemical characterization, the biological properties and the mechanism of action of gardimycin will be described.

Material and Methods

Growth of the organism:

The organism was kept in the lyophilized form. The seed, the flask and the jar fermentor cultures were prepared as described by PARENTI *et al.*[1]

Assay of gardimycin:

Antibiotic activity was usually and most reliably followed by a microbial-paper disc-agar diffusion assay, with *Sarcina lutea* ATCC 9341 as the test organism.

The bacterium was grown overnight on a slant. One hundred ml of Penassay Difco agar medium were inoculated with 0.3 ml of a bacterial suspension with a concentration of 350 Klett units. Petri dishes of 9 cm diameter, containing 13 ml of inoculated medium and the 9 mm diameter paper discs were used. The diameter of the inhibition zone was read after overnight incubation at 37°C. An antibiotic preparation of $\geq$90 % chemical purity served as reference standard and the antibiotic potency of the broth was expressed as μg equivalent of the standard. The reference sample was dissolved in sterile 0.02 M phosphate buffer, pH 7.2. The plot of the log of dose *vs* the mm of the inhibition zone showed a linear relation to exist in the range of 20$\sim$1,000 μg/ml.

The linearity of the standard and unknown curves and the parallelism between them were determined according to standard statistical methods.

* From *Garden*: both producing-strains were isolated from samples of garden soil. *A. garbadinensis* from locality "Garbady" in India. *A. liguriae* from the Italian region "Liguria".

Media composition (g/liter):

V Medium: meat extract 3; tryptone 5; yeast extract 5; dextrose 1; starch 24; $CaCO_3$ 4; brought to 1,000 ml with distilled H_2O.

E Medium: meat extract 4; peptone 4; NaCl 2.5; yeast extract 1; soybean meal 10; dextrose 50; $CaCO_3$ 5; brought to 1,000 ml with tap H_2O.

The pH was adjusted to 7.6 with NaOH before sterilization, and was 6.3 after sterilization.

Results and Discussion

Description of the Producing Strains

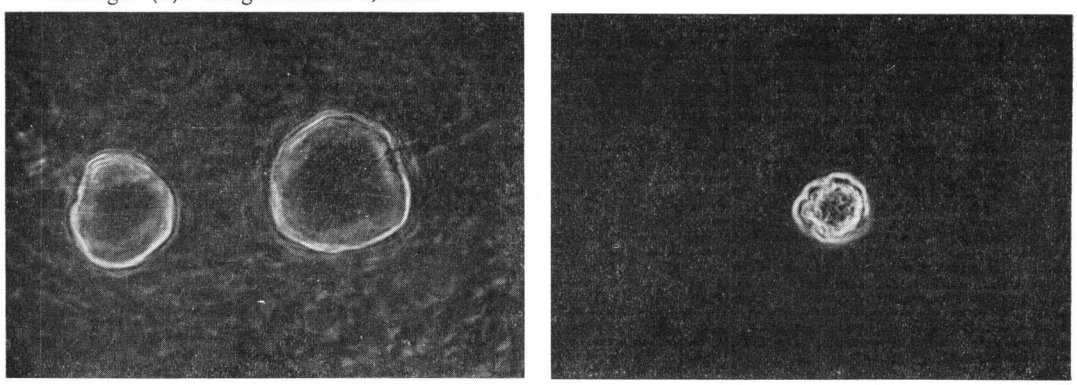
Two strains of our collection of *Actinoplanes* have been found to produce gardimycin and were given our code numbers A/6353 and A/10889.

Morphological properties:

Strain A/6353 grows well on several agar media. On oat-meal agar the colonies, about 5 mm in diameter, have indented contours, slight radial furrows and a central depression. Secondary mycelium is always absent. Sporangia form abundantly on oat-meal agar, glycerol asparagine agar and CZAPEK glucose agar showing different shapes and sizes, depending on the medium. On oat-meal agar, they have regular contours, a shape varying from spherical to oval, and are 15~25 μ in diameter (Fig. 1/A). The spores are motile and spherical with a diameter of 1.5~2 μ. A yellow-amber soluble pigment is produced in several media.

Strain A/10889 grows well on various nutrient agars. The surface is opaque and usually

Fig. 1. Sporangia of strain A/6353 grown on oat-meal-agar (A) and strain A/10889 grown on soil-agar (B). Magnification 1,200×.



(A) (B)

Table 1. A comparison of some characteristics of strains A/6353 and A/10889.

	Strains	
	A/10889	A/6353
Sporangia	formed only on calcium malate agar. Size: 7~12 mμ	formed abundantly on several agars; but variable in size and shape. On oat meal agar the size is 15~25 mμ.
Spores	subspherical (1~1.5 μm)	spherical (1.5~2 mμ)
Secondary mycelium	rudimentary	always absent
Soluble pigment	absent	yellow-amber pigment present on some agars

Table 2. Culture properties on various media.

Culture medium	A/10889	A/6353
Medium No. 2 (Yeast extract-malt agar)	Abundant growth, wrinkled surface, amber	Abundant growth, slightly wrinkled, light orange to light amber
Medium No. 3 (Oatmeal agar)	Moderate growth, smooth, opaque, cream to orange at edges	Abundant growth, with smooth and thin surface, light orange. Some sporangia, light yellow soluble pigment
Medium No. 4 (Inorganic salts-starch agar)	Moderate growth, smooth surface, deep orange	Abundant growth with smooth surface, orange. Some sporangia. Canary yellow soluble pigment
Medium No. 5 (Glycerol-asparagine agar)	Moderate growth, smooth surface, orange	Abundant growth with smooth surface, light orange. Abundant production of pigment. Canary yellow soluble pigment
Medium No. 6 (Peptone-yeast extract-iron agar)	Scanty growth, rough surface, dark brown with brown pigment	Moderate growth with smooth surface, orange
Medium No. 7 (Tyrosine agar)	Abundant growth, crusty surface, coffee-colored. Faintly brown pigment	Abundant growth with smooth surface, rose amber. Good production of sporangia. Rose amber soluble pigment
Oatmeal agar (according to WAKSMAN)	Abundant growth, crusty surface, orange. Traces of rudimentary aerial mycelium	Abundant growth, with smooth surface, light orange to topaz yellow. Good production of big sporangia. Yellow soluble pigment.
HICKEY and TRESNER's agar	Abundant growth, wrinkled surface, light brown	Abundant growth with smooth surface, rose amber. Abundant production of sporangia
CZAPEK glucose agar	Poor growth, smooth surface, light orange. Traces of rudimentary aerial mycelium	Abundant growth with smooth and thin surface, orange. Abundant production of sporangia
Glucose asparagine agar	Abundant growth, crusty surface, deep orange	Abundant growth with smooth surface, orange Some sporangia
Nutrient agar	Moderate growth, crusty surface, orange to light brown	Abundant growth with smooth surface, orange
Potato agar	Abundant growth, wrinkled surface, orange to light brown. Traces of rudimentary aerial mycelium	Abundant growth, with smooth surface, amber
BENNETT's agar	Abundant growth, wrinkled surface light orange	Abundant growth with crusty surface, light orange
Calcium malate agar	Moderate growth, crusty surface, cream to light orange	Scarce growth with smooth surface, colorless. Some sporangia
Skim milk agar	Abundant growth, wrinkled surface, deep orange	Abundant growth with slightly crusty surface, deep orange. Yellow soluble pigment
CZAPEK agar	Poor growth, crusty surface, light orange	Scarce growth, light orange. Moderate production of sporangia
Egg agar	Abundant growth, smooth surface, orange	Abundant growth, with smooth surface, light cream. Moderate production of sporangia
Peptone glucose agar	Very scanty growth, smooth surface, hyaline	Abundant growth, with wrinkled surface, deep orange
Agar	Very scanty growth, thin and smooth, hyaline	Very scanty growth thin and smooth. Colorless
LOEFFLER serum	Very scanty growth, smooth surface, light orange to amber	Scanty growth, light orange
Potato	Scanty growth, wrinkled surface, orange	Scanty growth, wrinkled, light orange

rough to wrinkled. Secondary mycelium is usually absent, although in some media rudiments are observed.

On microscopic examination the vegetative mycelium is slightly branched, with a diameter of $\sim 1\,\mu$. The sporangia from only on calcium malate agar and are globose with an irregular surface, often lobate, with diameter ranging from 7 to 12 μ (Fig. 1/B). After rupture of the sporangial wall sporangial release is observed. The subspherical spores are motile ($1.0 \sim 1.5\,\mu$ diameter).

A comparison of some morphological characteristics of the two strains is shown in Table 1.

Culture properties

Table 2 reports the culture characteristics of *Actinoplanes* A/6353 and *Actinoplanes* A/10889, cultivated on various standard media suggested by SHIRLING and GOTTLIEB[2] and otherm edia recommended by WAKSMAN[3]. and determined after 6~14 days of incubation at 30°C. The optimum temperature for colony development ranges between 28°C and 37°C for both strains.

Table 3. Carbon source utilization of strains A/6353 and A/10889.

Carbon source		Utilization*	
		A/10889	A/6353
C_5	Arabinose	+	+
	Xylose	+	+
C_6	Glucose	+	+
	Fructose	+	+
	Mannose	+	+
	Mannitol	+	−
	Inositol	−	+
	Rhamnose	+	+
$(C_6)_2$	Sucrose	+	−
	Lactose	+	−
$(C_6)_3$	Raffinose	−	−
$(C_6)_n$	Cellulose	−	−
	Starch	+	+
Special	Salicin	+	−

* + = utilization − = no growth.

Carbon utilization

Table 3 reports the utilization of carbon sources. Both strains readily utilize those carbohydrate metabolized *via* the pentose-phosphate cycle (xylose, arabinose), as do all the *Actinoplanes* so far analyzed, as well as the simple carbohydrates which can be metabolized to glucose-6-phosphate and fructose-6-phosphate (fructose, mannose, rhamnose). However A/6353 does not utilize the alcohol sugar mannitol and A/10889 is unable to use inositol, in this being similar to most *Actinoplanes* species. Strain A/6353 shows a surprising inability to hydrolyze the glucosidic bonds of lactose, salicin and sucrose, the latter being utilized by all the other *Actinoplanes* species so far described in the literature. It grows, however, on starch. Neither strain hydrolyzes the galactose-α (1, 6)-glucosidic bond of raffinose, an inability shared with all the other described species of *Actinoplanes*, except *A. filippinensis*[1]. Neither strain is cellulosolytic. A/10889 hydrolyzes the salicyl alcohol-β-glucoside (salicin).

Physiological properties

Table 4 reports the physiological characteristics of the two strains. They are clearly different in their physiological characteristics. In particular: A/6353, like *A. brasiliensis*[1] but unlike the other described species[1], does not hydrolyze tyrosine; it also fails to produce melanin and H_2S. It is unable to reduce nitrate, a feature that we have found in only 12 strains out of 100 extensively analyzed. All the described species however do reduce nitrate under the conditions of the assay.

A/10889 does not hydrolyze casein and litmus milk, in this being similar to *A. deccanensis*[1].

Table 4. Physiological characteristics of strains A/6353 and A/10889.

Test	A/10889	A/6353
Starch hydrolysis	positive	positive
H_2S formation	〃	negative
Melanin production	〃	〃
Tyrosine hydrolysis	〃	〃
Casein hydrolysis	negative	positive
Ca-malate hydrolysis	〃	negative
Litmus milk coagulation	〃	〃
Litmus milk peptonization	〃	〃
Nitrate reduction	positive	〃
Gelatin liquefaction	〃	〃

Table 5. Time course of fermentation of gardimycin in a 20-liter jar fermentor.
Strain: A/10889. Medium: E.

Hours after inoculation	pH	pmv*	Gardimycin μg/ml
48	6.7	10	—
72	7.2	12	64
96	7.7	16	240
120	7.5	20	270

* pmv=Growth measured as percent packed mycelium volume.

Table 6. Effect of nutrient variation on antibiotic yield in flasks.
Strains A/6353.

Medium N.	Composition of medium, in %(w/v)				Antibiotic yield μg/ml
	DDS	CSL	SBM	PM	
1	1	1	0	0	70
2	1	0	1	0	<30
3	1	0	0	1	70
4	0	1	1	0	350
5	0	1	0	1	<30
6	0	0	1	1	270
7	2	0	0	0	<30
8	0	2	0	0	250
9	0	0	2	0	200
10	0	0	0	2	<30

Each medium contains 0.5 % $CaCO_3$, 0.5 % glucose and 0.1 % K_2HPO_4.
DDS=distiller-dried solubles; CSL=corn-steep liquor; SBM=soya bean meal; PM=peanut meal.

The two strains A/6353 and A/10889 are both ascribed to the genus *Actinoplanes* because of their global sporangia, motile spores and colony morphology. However, they are clearly different from each other on the basis of their growth patterns on different agars, size of sporangia, production or non-production of soluble pigment, presence or absence of rudimentary secondary mycelium, carbon utilization pattern, and physiological characteristics. The two strains are also easily distinguishable from all the *Actinoplanes* species previously known with which cultural comparisons were made. For these reasons, strain A/6353 and strain A/10889 are considered to be new species of *Actinoplanes* and have been given the names *Actinoplanes liguriae* nov. sp. and *Actinoplanes garbadinensis* nov. sp., respectively. The type strain of *A. garbadinensis* is A/10889 (=ATCC 31049), the type strain of *A. linguriae* is A/6353 (=ATCC 31048).

Production of Gardimycin

A typical time-course of fermentation of A/10889 in a 20-liter fermentor containing 10 liters of medium E is shown in Table 5. The growth is rapid in the first 48 hours of fermentation and continues, although at reduced rate, up to 120 hours. The antibiotic production is delayed, with respect to growth, its rate being maximal between 72 and 96 hours.

The effect of nutrient variation on antibiotic yield was studied in flask fermentors, with strain A/6353. These results are shown in Table 6. The yield was maximal in the presence of corn-steep liquor and/or soya bean meal. Distiller-dried solubles and peanut meal inhibited antibiotic production even when supplied together with soya bean or

Table 7. Effect of initial glucose concentration on growth and antibiotic yield at 144 hours in several media.

Strain: A/6353.

Medium[1] N.	Glucose g/liter	pmv	pH	Antibiotic μg/ml
4	18	15	8.0	300
	40	15	6.0	50
	63	14	5.5	<30
8	18	15	8.1	320
	40	17	5.6	70
	63	14	5.5	<30
9	18	17	8.8	140
	40	17	7.6	80
	63	30	6.4	70
E	25	14	8.6	250
	50	20	6.6	140

[1] The medium number refers to the medium with the composition reported in Table 6.

corn-steep liquor.

The effect of initial glucose concentration on antibiotic yield is shown in Table 7.

An inhibition of antibiotic production was observed at initial glucose concentrations higher than 20 g/liter; however, no inhibition of growth was observed. In soya bean meal medium, a stimulation was evident at the highest glucose concentration.

However, in jar fermentors, the highest and most reproducible yields were obtained with medium E. A study of the effect of the initial glucose concentration of the medium E in jar fermentors clearly showed higher antibiotic yields at 25 g/liter than at 50 g/liter (Table 7), although growth was more abundant at the higher glucose concentration.

References

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