TEICHOMYCINS, NEW ANTIBIOTICS FROM *ACTINOPLANES TEICHOMYCETICUS* NOV. SP.

I. DESCRIPTION OF THE PRODUCER STRAIN, FERMENTATION STUDIES AND BIOLOGICAL PROPERTIES

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A soil isolate of *Actinoplanes* that produces the chemically unrelated new antibiotics teichomycins A_1 and A_2 has been proposed as a new species named *Actinoplanes teichomyceticus* nov. sp. (ATCC 31121). Studies of medium and fermentation conditions indicated that the highest antibiotic titers, *ca* 900 u/ml, are obtained in a medium containing 1% (w/v) glucose, 1% cotton seed meal, 1% malt extract, and 0.4% yeast extract. Both teichomycin A_1 and teichomycin A_2 are highly active against gram-positive bacteria. Teichomycin A_1 shows some activity against gram-negative bacteria. Both antibiotics cured mice experimentally infected with sensitive bacteria and showed low acute toxicity. Of the two antibiotics teichomycin A_2 is the more active.

In the course of our screening program for antibiotic-producing strains of *Actinomycetales* of the family *Actinoplanaceae*, we have isolated a strain of *Actinoplanes* that produces two new antibacterial agents, teichomycin A_1 and teichomycin A_2, that are highly active against gram-positive pathogenic bacteria, both *in vitro* and *in vivo*. In the present paper we wish to report on the taxonomy of this strain (A 8327 of our collection), on the fermentation of the teichomycins and on their biological properties. In a companion paper partial chemical characterizations of the two agents are presented[1].

Materials and Methods

Organism

Strain A 8327 was isolated from a soil sample collected at Nimodi Village, Indore (India) and was deposited in the American Type Culture Collection (ATCC) as accession number 31121. The strain was kept in lyophilized form and cultured on slants of oatmeal agar.

Determination of Growth Characteristics

To examine the growth characteristics, the organism was cultivated on the various standard media suggested by SHIRLING and GOTTLIEB[4], with the addition of several media recommended by WAKSMAN[5]. The inoculated media were incubated and examined after 1 and 2 weeks of growth. Color designations were made, when necessary, by the method of MAERZ and PAUL[3]. The ability of the organism to utilize different carbon sources was investigated by the method described by SHIRLING and GOTTLIEB[4].

The effect of temperature on growth was determined by incubating the inoculated oatmeal agar slants at temperatures ranging from 15 to 50°C.

Assay of Teichomycin A_1

Antibiotic activity was followed by a microbial paper-disc-agar diffusion assay, using *Staphylococcus aureus* ATCC 6538 as the test organism. The bacterium was grown overnight on a slant of Penassay medium.

The microbial assay was performed on Difco Noble agar medium inoculated with 0.3% (v/v) of an aqueous suspension of *S. aureus* which contained 10^6 cells/ml.

Petri dishes, 9 cm in diameter, were filled with 10 ml of inoculated medium and 9-mm diameter paper discs were used. The diameter of the inhibition zone was read with an optical device, after 18-hour incubation at 37°C. The potency of the fermentation broths was determined against a standard curve of teichomycin A_2 containing no teichomycin A_1, 1 mg of which was considered to contain 10^3 arbitrary antibiotic units.

The diameter of the inhibition zones was proportional to the log of the antibiotic concentration in the range of 15~250 μg/ml. The linearity of the unknown and standard curves, their parallelism and the concentration of the unknown sample were calculated by usual statistical procedures, using a computer.

Determination of Antimicrobial Spectrum and Experimental Infection

The minimal inhibitory concentration (MIC values) in liquid media and the effectiveness of the compounds in treating experimentally infected mice were assessed as previously described[1].

Media Composition

Difco Noble agar (g/liter): Difco Bacto peptone 5, yeast extract 3, beef extract 1.5, NaCl 70, Difco Bacto dextrose 1, KH_2PO_4 1.32, K_2HPO_4 3.68, Difco Noble Agar 10, distilled H_2O to 1,000 ml. The medium was supplemented just before use, with 10% (v/v) of a molar solution of KH_2PO_4 pH 4.2.

T_2 medium (g/liter): yeast extract 4, malt extract 10, glucose 5, Noble agar 10, distilled H_2O to 1,000 ml. The solution was titrated to pH 7.3 with NaOH before sterilization. The pH after sterilization was 6.0~6.2.

Results

Description of the Producing Strain

Macroscopic Examination

The colonies produced on oatmeal agar medium are 5~6 mm in diameter and have well defined and regular contours and a central dome-like protuberance with a pale orange color. A light orange to deep orange vegetative mycelium is produced on most media. However, on potato, HICKEY and TRESNER, skim milk and tyrosine agars the color of the vegetative mycelium is light brown. Soluble pigments are generally absent. In some media only a light brown soluble pigment is produced (Table 1). A well developed powdery aerial mycelium made up of long hyphae is found on some media.

Microscopic Examination

Sporangia, abundantly produced on most media, are found mainly on the dome of the colony. The sporangia are spherical to oval, with regular contours and have diameters ranging from 15 to 25 μ.

Sporangiophores are straight, about 15 μ long and 2 μ in diameter.

The highly motile spores are spherical to oval, with diameters of 1.5~2 μ.

The vegetative mycelium is composed of thin, twisted and branched hyphae 0.5~1 μ in diameter.

Carbon Utilization

Strain A 8327 utilizes the pentoses arabinose and xylose as sole carbon sources. It grows on the hexoses glucose, fructose, mannose and mannitol but not on rhamnose or on the cyclitol, inositol. It is able to hydrolyze the glycosidic bonds of sucrose and starch but not those of raffinose and cellulose. Lactose and salicin are poorly utilized.

Table 1.

Culture medium	Cultural characteristics
HICKEY and TRESNER's agar	Abundant growth with smooth surface, light brown 14/B/9. Traces of whitish aerial mycelium. Moderate production of sporangia. Brown soluble pigment
BENNETT's agar	Abundant growth with smooth surface, light orange 9/B/7. Moderate production of pale pink aerial mycelium
CZAPEK glucose agar	Abundant growth with smooth surface, light orange 9/H/7. Production of white-rose aerial mycelium. Some sparse sporangia
CZAPEK sucrose agar	Abundant growth with smooth surface, pale orange 9/B/7. Moderate production of sporangia
Glucose asparagine agar	Moderate growth with smooth surface, orange 10/D/12
Potato agar	Abundant growth with smooth and thin surface, light hazel-brown. Abundant production of light pink sporangia. Light hazel-brown soluble pigment
Nutrient agar	Poor growth with smooth and thin surface, orange 10/D/11
Egg albumin agar	Abundant growth with smooth surface, light pinkish orange 9/B/5. Abundant production of sporangia
Peptone-glucose agar	Abundant growth with wrinkled surface, deep orange 10/C/12. Light amber soluble pigment
Potato plug	Poor growth, with wrinkled surface, light orange
LOEFFLER blood serum	Some sparse colony, light orange with grayish brown soluble pigment
Medium ISP n. 2	Abundant growth with slightly wrinkled surface, light pinkish orange 9/B/6. Abundant sporangia, light pink 9/A/5. Amber soluble pigment
Medium ISP n. 3 (Oatmeal agar 20%)	Abundant growth with smooth surface, pale orange 9/B/5. Abundant sporangia, light pink 9/A/5
Medium ISP n. 4 (Starch agar)	Abundant growth with smooth surface, deep orange 9/H/10. Scarce production of sporangia
Medium ISP n. 5 (Glycerol asparagine agar)	Abundant growth with thin and smooth surface, pale orange 9/B/7. Moderate production of aerial mycelium, light pink
Medium ISP n. 6 (Peptone-yeast extract iron agar)	Scarce growth
Medium ISP n. 7	Moderate growth with smooth and thin surface, pinkish brown 12/A/9. Production of scant and whitish aerial mycelium. Pinkish-brown soluble pigment 12/A/9
Oatmeal agar (60%)	Abundant growth with smooth surface, pale orange 9/B/5 to light orange brown (12/B/8) depending on age. Abundant sporangia, light pink 9/B/5, light hazel-brown soluble pigment
Skim milk agar	Abundant growth with smooth surface, burnt amber 15/A/12. Deep amber brown soluble pigment 7/E/12
Calcium-malate agar	Poor growth, with smooth and thin surface, pale orange 9/B/5. Moderate production of sporangia
Gelatin	Amber soluble pigment
Nitrate broth	Amber soluble pigment

Sensitivity to Temperature

The optimum growth temperature ranges from 28 to 37°C. No growth is observed at 50°C, moderate growth at 20° and 42°C and minimal growth at 15°C.

Physiologic Characteristics

The physiologic characteristics of strain A 8327 are as follows: positive for H_2S formation, melanin production, casein hydrolysis, litmus milk peptonization, nitrate reduction and gelatine liquefaction; negative for tyrosine hydrolysis, Ca-malate hydrolysis and litmus milk coagulation.

Sporangiogenesis

Strain A 8327 readily forms sporangia on potato agar, egg albumin agar, medium ISP 2, oatmeal

agar and soil extract agar. Sporangia formation can be increased by incorporation of tea infusion in the agar media.

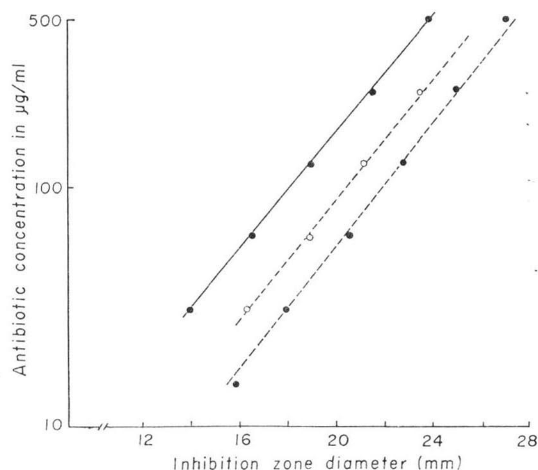
Antibiotic Susceptibility Pattern

Strain A 8327 was tested in BENNETT's medium for antibiotic susceptibility giving the following MIC values (μg/ml): penicillin (6), ampicillin (3), chloramphenicol (6), tetracycline (1.5), novobiocin (6), neomycin (6), streptomycin (3) and rifampicin (6). It showed low susceptibility to bacitracin (16) and erythromycin (16) and was insensitive to lincomycin (250).

Fermentation Studies

The assay procedure used to measure the antibiotic potency of fermentation samples detects both teichomycin A_1 and teichomycin A_2, since both are coproduced by strain A 8327 and since the test organism (*S. aureus*) is sensitive to both antibiotics. However, when samples of pure teichomycin A_1 and teichomycin A_2 were compared, teichomycin A_1 had approximately 1/3 the activity of the same weight of teichomycin A_2 (Fig. 1). Moreover, the assay measures the total activity of A_1+A_2 only if the inhibitory activities are additive. That such is indeed the case is shown in Fig. 1, where the titration curve of a 1 : 1 (w/w) mixture of pure A_1 and pure A_2 is compared with those of single pure compounds. In fact, 100 μg of the mixture/ml, 66 μg A_2/ml and 200 μg A_1/ml gave inhibition zones of 20.5, 20.6 and 20.6 mm, respectively.

Fig. 1. Microbiological, paper-disc-agar diffusion assay of teichomycin A_1 (—•—), teichomycin A_2 (---•---) and a 1: 1 (w/w) combination thereof (---○---).
The test organism used was *S. aureus* ATCC 6538.



Furthermore, it was shown by extraction and chromatography of the extract that the quantity of teichomycin A_1 synthesized in most media does not exceed, on a weight basis, 20~25% of the sum of A_1 and A_2. So the contribution of the activity of teichomycin A_1 to the total antibiotic activity (A_1+A_2) as determined by this test accounted for no more than 8~10 %. As a consequence, the assay procedure used essentially determined the concentration of teichomycin A_2 in the broth sample.

In order to study the effect of different sources of carbon and nitrogen on growth and antibiotic formation, a simple basic medium which would afford measurable antibiotic production had to be devised.

This was done by cultivating a large number of colonies on agar media of different compositions and determining the antibiotic potency of each colony by observing the sizes of the inhibition zones after covering the plates with a suspension of *S. aureus* followed by overnight incubation at 37°C. Consistently larger inhibition zones were produced by all colonies when cultured on the T_2-medium.

A good potency was also obtained when the strain was cultured in a liquid medium of the same composition except that no agar was added. All further work was done with liquid media. The experiments were carried out in 500-ml indented flasks containing 100 ml of fermentation broth, at

28°C on a rotary shaker at 200 rpm.

To assess whether any major alterations in the A_1/A_2 ratio were introduced by the different fermentation conditions, the total antibiotic activity was also extracted and chromatographed in a system that clearly separates the two activities[2]. Acetate does not support growth, nor does glyceraldehyde. Three-carbon sources that enter below the aldolase reaction into the E. M. pathway (glycerol, glyceric acid, pyruvate) and four carbon sources that feed into the KREBS cycle (glutamate, aspartate) supported good growth but allowed minimal antibiotic synthesis (<20 units/ml). Starch, lactose and fructose supported growth and supported only low levels of antibiotic formation (100~150 units/ml). The disaccharide sucrose; the hexoses glucose, mannitol, mannose and galactose; gluconate; the pentoses, xylose and arabinose; and the cyclitol, inositol, supported both biomass production and good antibiotic titers (250~500 units/ml). Ribose supported growth only sparingly with no measurable antibiotic titer. From these experiments, glucose appeared to be the best carbon source for antibiotic production giving titers ranging from 400 to 500 units/ml. The effect of different nitrogen sources added to the T_2-glucose medium was studied both in flasks and in jar fermentors. The following N-sources, at 0.5% (w/v) levels, supported good growth: Pharmamedia, soy bean meal, distiller dry solubles, peanut meal, cotton seed meal, glutamate, $NaNO_3$. Very little growth was observed when urea was used. The antibiotic titer was maximum with cotton seed meal (900 units/ml), intermediate with the other complex N-sources (300~600 units/ml) and no antibiotic was detectable with glutamate, $NaNO_3$ and urea.

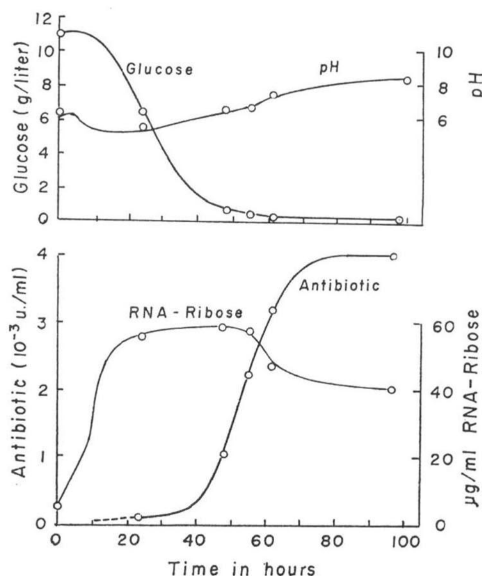
T_2-medium, supplemented with glucose and cotton seed meal was selected from these preliminary experiments and the effects of varying their initial concentrations on the antibiotic production were studied in 4-liter jar fermentors. The best medium was found to contain 10 g/liter of both glucose and cotton seed meal (Table 2).

Table 2. Effect of initial glucose and cotton seed meal concentrations on antibiotic titer

Initial glucose concentration (g/liter)	Initial Proflo* concentration (g/liter)	Antibiotic potency (units/ml)
0	1	0
1	1	930
2	1	640
1	5	350
1	10	930
1	15	780
1	20	350

* Proflo is a trade name for the cotton seed meal sold by Traders Protein Division of Traders Oil Mill Co.

Fig. 2. Time-course of fermentation of A/8327/44, a natural variant of the wild type A/8327.
The fermentation was run in a 10-liter jar fermentor filled with 4 liters of medium; aeration was 1 v/v per minute, agitation 800 rpm. Foam was controlled with automatic addition of Dow-Corning Antifoam. The fermentor was placed in a water-bath at 28°C. Upper frame: pH and glucose consumption; lower frame: antibiotic synthesis and growth, measured as RNA-ribose.



The time-course of the fermentation with this medium is shown in Fig. 2. The synthesis of antibiotic starts when the growth phase has ended and when glucose is no longer detectable in the medium. It continues more or less linearly until 80 hours, when it begins to level off. The pH of the medium decreased during the growth phase to pH 5 then rose during the antibiotic production phase.

Biological Properties

Both teichomycin A_1 and teichomycin A_2 are very active against gram-positive pathogenic bacteria (Table 3). Teichomycin A_1 but not teichomycin A_2 shows also substantial activity against several gram-negative bacteria. Neither substance shows cross-resistance to macrolides, aminoglycosides, tetracycline, penicillins, cephalosporins, rifampicin, and chloramphenicol. Teichomycin A_2 but not teichomycin A_1 has partial cross-resistance with vancomycin.

Teichomycin A_1 and teichomycin A_2 have a very low ED_{50}, when administered subcutaneously to mice challenged with a killing inoculum of sensitive bacteria (Table 4). The ED_{50} of teichomycin A_1 is of the same order of magnitude as that of erythromycin, lincomycin, vancomycin determined in parallel experiments. The ED_{50} of teichomycin A_2 is one to two orders of magnitude lower.

Teichomycin A_1 injected intravenously into mice (500 mg/kg) did not cause any death. Teichomycin A_2 had a LD_{50} of 275 mg/kg when administered intravenously, and an LD_{50} greater than 1,000 mg/kg when given orally or intraperitoneally.

Table 3. Activity of teichomycin A_1 and teichomycin A_2 in dilution test

Test organism	MIC values (μg/ml)	
	Teichomycin A_1	Teichomycin A_2
Staphylococcus aureus Tour	0.05	0.1
Streptococcus pyogenes C203	0.5	0.05
Streptococcus pneumoniae UC41	0.5	0.05
Proteus vulgaris X 194 ATCC 881	20	>100
Escherichia coli SKF 12140	10	>100
Pseudomonas aeruginosa ATCC 10145	>100	>100
Candida albicans SKF 2270	>100	>100
Trichophyton mentagrophytes I Pasteur	>100	>100
Mycobacterium tuberculosis H37RV ARC 9360	>100	>100
Mycoplasma gallisepticum	>100	>100

Table 4. Biological activities *in vivo* of teichomycin A_1 and teichomycin A_2 in comparison with known antibiotics

Infecting organism	ED_{50} (mg/kg); mice				
	Teichomycin A_1	Teichomycin A_2	Vancomycin	Erythromycin	Lincomycin
Staphylococcus aureus Tour	N.D.	0.76 (0.81~0.71)	10.8 (12.5~11.7)	34.5 (38.3~31)	56.6 (67.9~47.3)
Streptococcus pneumoniae UC41	11.8 (12.8~10.3)	0.13 (0.15~0.12)	N.D.	9.33 (11.1~7.83)	160
S. pyogenes C203	2.5	0.09 (2.8~2.2)	1.13 (1.23~1.02)	3.79 (4.56~3.12)	5.36 (7.0~4.1)

N.D.=not determined. The drugs were administered s.c. The confidence limits are reported in parenthesis.

Discussion

The colony morphology, pigmentation and the presence of motile spores bearing globose sporangia carried at the end of a sporangiophore places strain A 8327 in the genus *Actinoplanes*.

However, the presence of a well-developed although sterile aerial mycelium, the pattern of utilization of carbon sources and the pattern of physiologic characteristics make it impossible to assign strain A 8327 to any of previously described species. For these reasons, strain A 8327 is recognized as a new species of the genus *Actinoplanes*, for which the specific epithet *teichomyceticus* is proposed. More specifically, *A. teichomyceticus* nov. sp. can be described and identified by the following characteristics:

a) it bears large (more than 15 μm diameter), globose sporangia (like those of *A. armeniacus*[6], *A. filippinensis*[7], *A. liguriae*[8], *A. utahensis*[9]), which have regular contours (unlike *A. utahensis*);
b) it has a well-developed aerial mycelium, like *A. armeniacus*, which however bears conidiospores;
c) it fails to grow, under the conditions of the test, on inositol, rhamnose, raffinose and cellulose;
d) it grows, although weakly, on salicin;
e) it does not hydrolyze tyrosine and calcium malate; it does not coagulate litmus milk but peptonizes it.

Although the fermentation studies here reported were aimed at the practical goal of increasing the antibiotic titer and thus reducing the critical cost of supplying a quantity of pure antibiotic sufficient for the drug evaluation program, some general observations can be made.

1) The inability of the strain to grow on acetate as the only carbon source indicates either that A 8327 is not able to take up acetate or that it lacks the acetylCoA synthetase enzyme or the glyoxylate cycle or both.

2) Although glucose is the best carbon-source for antibiotic production, the antibiotic accumulates only when the glucose concentration of the medium is reduced to barely detectable levels. Whether this behavior is a direct catabolite repression or an indirect effect of change in medium pH associated with glucose consumption is open to question.

3) The ability of strain A 8327 to grow on either three-carbon or four-carbon compounds as sole carbon sources (besides yeast and malt extract) speaks for the presence of anaplerotic pathways that accomplish gluconeogenesis. Strain A 8327 must possess PEP carboxykinase, the malate enzyme, phospho-enolpyruvate synthetase, phospho-enolpyruvate carboxylase and fructose-diphosphatase.

Preliminary chemical studies on teichomycin A_2 show that the antibiotic contains D-mannose and D-glucosamine[2]. It is conceivable that these carbohydrates are made from the cell pool of sugar phosphates. Furthermore, the bulk of the antibiotic molecule is made up of analogs of tyrosine and thus may stem from the same pathway which starts with the condensation of PEP and the sugar phosphate erythrose-4-phosphate. The inability of the three-carbon and four-carbon substrates to support antibiotic synthesis, in contrast with the five-carbon and six-carbon substrates as well as oligo and polymeric carbohydrates, may reflect the existence of different levels of the pool of sugar-phosphates, low with the former substrates and high with the latter ones. Alternatively, the operation of gluconeogenesis may induce a cellular situation that indirectly controls the activity (or synthesis) of the enzymes of the antibiotic synthetic pathway.

All these hypotheses must be kept in mind in defining a rational strain-improvement program through mutation and selection.

The restricted antibacterial spectrum, the absence of activity on bacteria lacking cell walls including the L form of sensitive *S. aureus*, the cross-resistance with vancomycin and the chemical composition[2] suggest that teichomycin A_2 belongs to the class of glycopeptide antibiotics active as cell wall inhibitors such as vancomycin, ristomycin, ristocetin, actinoidins, LLAV290 and mannopeptins[2]. Teichomycin A_2 seems to be the most active member of this group in curing experimental infections in mice. Teichomycin A_2 is the first glycopeptide antibiotic isolated from a member of the genus *Actinoplanes*.

Teichomycin A_1 also is inactive on cell wall lacking bacteria thus suggesting it acts by inhibiting cell wall synthesis. The chemical composition and the physico-chemical properties[2] indicate that it may belong to the class of glycolipid antibiotics such as moenomycins, diumycins, prasinomycins and macarbomycin[2]. Teichomycin A_1 is the first example of this type antibiotic isolated from a member of the genus *Actinoplanes*.

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