PURIFICATION AND PARTIAL CHARACTERIZATION OF AN ANTIBIOTIC PRODUCED BY *MYXOCOCCUS CORALLOIDES*

J. M. ARIAS, C. RODRIGUEZ and E. MONTOYA

Department of Microbiology, Faculty of Sciences, University of Granada, Granada, Spain

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A strain of *Myxococcus coralloides* producing an antibiotic capable of inhibiting growth of Gram-positive bacteria was isolated. Antibiotic production occurred during vegetative growth but not during myxospore formation. The antibiotic was extracted from the growth medium with chloroform and purified by adsorption on silicic acid and by preparative silica gel thin-layer chromatography. The purified antibiotic showed a resistance to heat, acid, alkali and proteolytic enzymes. Chromatographic and electrophoretic behavior as well as infrared, ultraviolet and mass spectra are presented.

Although antibiotic production by myxobacteria was reported by OXFORD in 1947[14], there has been little work in this field and much of it has been contradictory[6,7,12,13,21]. NOREN[12] reported that *Chondrococcus coralloides* produced a substance, or substances, which showed marked antibiotic activity against *Staphylococcus aureus* and certain growth inhibitory effect on *Enterobacter aerogenes*.

PETERSON *et al.*[15] demonstrated clearly for the first time antibiotic production by myxobacteria; they showed that some strains of *Sorangium* produce an antibiotic (myxin) at the end of the logarithmic phase of growth; they were able to purify the antibiotic, determine its chemical structure and mode of action[3,8,9]. VAKS *et al.*[20] have isolated a strain of *Myxococcus xanthus* which produces an antibiotic at the end of the exponential growth. The antibiotic was purified and a large-scale production procedure was reported[11,17]; the antibiotic had bactericidal action.

As part of a systematic search for antibiotic production by myxobacteria we isolated a strain of *Myxococcus coralloides* which had ability to produce an antibiotic active against Gram-positive bacteria. Its production was influenced by the composition of culture media and the optimal conditions for antibiotic production have been studied[1]. In this paper the isolation and purification of the antibiotic as well as its partial characterization are described.

Materials and Methods

Organisms and Media

A strain of *Myxococcus coralloides* isolated in our laboratory according to method of SINGH[19] and classified according to BERGEY's Manual[4] was used in the studies reported here. A mutant which grows dispersed, referred to as *M. coralloides* D, was employed for studies relating antibiotic production and the physiological state.

Yeast agar medium containing 1% baker's yeast plus 2% agar, pH 7.2, was used for conservation and preparation of inocula.

Maximum antibiotic production was achieved in the following medium: 0.5% Casitone (Difco), 0.1% $MgSO_4$, and 1% glucose in 0.01 M potassium phosphate buffer, pH 6.

A strain of *S. aureus* from our departmental stocks, was used as the test organism. For quantitative assay of antibiotic activity, antibiotic media 1 and 2 (BBL) were used.

Antibiotic Production

In preliminary experiments 250-ml Erlenmeyer flasks, containing 40 ml of the optimal antibiotic production medium cited above, were inoculated with 1 ml of a dense suspension of 4~6-day old culture of *M. coralloides*. Incubation was at 28°C with gyratory shaking (200 rpm). Maximum antibiotic production occurred between 4 and 6 days. The cells formed a ring which clung to the flask. It was usually possible to decant the liquid to recover the supernatant fluid from which the antibiotic was extracted with chloroform.

Larger scale antibiotic production was carried out in 6-liter flasks containing 4 liters of medium with forced air at 2.4 liters air/liter culture/min.

Antibiotic assay

Antibiotic activity against *S. aureus* was determined by the paper disk assay method. Plates 100 mm in diameter were used; each plate contained 5 ml of antibiotic medium 2, which had been overlayed with antibiotic medium 1 (2.5 ml) containing 2.7×10^8 *S. aureus* cells. Units of antibiotic were determined from a standard curve of inhibition zone as a function of the quantity of antibiotic absorbed into a disk of 5.5 mm in diameter. One unit of the antibiotic is defined as the concentration that causes a zone of inhibition of 9 mm.

Antibiotic activity of paper chromatographic strips was tested by bioautography. Chromatograms and electrophoregrams were cut out in long strips of 1~2 cm width and placed on Nunc-bio plates.

For all other experiments the antibiotic was transferred into distilled water, by adding the water directly to a concentrated solution of the antibiotic and removing the remaining chloroform under reduced pressure.

Chromatographic and Analytical Procedures

Paper chromatography was performed by descending technique using Whatman No. 1 paper. Preparative thin-layer chromatography was carried out on 1.5-mm thick (20×20 cm) silica gel plates. The plates were dried at room temperature, activated at 110°C for 20 minutes, cooled and used immediately. Column (2×30 cm) chromatography was carried out on silicic acid (SIL-R), 100~300 mesh, which had been activated at 110°C for 30 minutes, cooled, stirred with chloroform, packed and then washed with two volumes of chloroform before addition of the antibiotic sample.

Electrophoresis was carried out at different pHs: in 1% borate buffer, pH 9, with 300 V for 3 hours; at pH 6.5 in a buffer of pyridine - acetic acid - water (25: 1: 475, v/v/v), with 300 V for 3 hours; at pH 4 in a buffer of pyridine - acetic acid - water (3: 1: 487, v/v/v), with 350 V for 4 hours; and at pH 2.9 in a 0.25 N acetic acid buffer with 350 V for 4 hours. An V-inverted electrophoretic tank, the right size for Whatman No. 1 paper strips 31 by 30 cm, was used according to the method of LOPEZ-GORGE[10].

Visible and ultraviolet absorption spectra were measured in a Beckman spectrophotometer model DB GT with quartz cell of 10-mm path length. The mass spectrum was determined with a Hewlett-Packard model 5930. The infrared spectrum was obtained with a Beckman IR-20.

Chemicals

The silicic acid, pronase and papain were purchased from Sigma Chemical Co., St. Louis, Mo. The spray detection reagents used were ninhydrin, DRAGENDORFF, dinitrophenylhydrazine and bismuth subnitrate (Merck), ammonium molybdate, and hydroxylamine hydrochloride (Fluka).

Results

The Relationship between Antibiotic Production and Physiological State of *M. coralloides*

M. coralloides produced an antibiotic which accumulated in the culture medium during growth. The highest activity was obtained in 4~6 days of incubation. However, it was not possible to establish a relationship between the growth of bacteria and antibiotic production because clumping

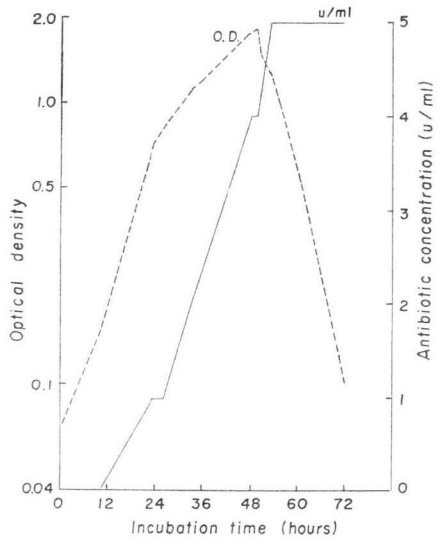
occurs when a liquid growth medium is used.

Antibiotic production as a function of growth was measured using a mutant of *M. coralloides* able to grow in a dispersed manner (Fig. 1). The antibiotic was first detected after 24 hours and increased at a linear rate for the next 36 hours, yielding the maximum of activity approximately 5 hours after the beginning of culture lysis. The levels of antibiotic remained constant thereafter. The doubling time of the cell mass was 6 hours. The pH increased from 6 to 8 during the growth phase.

In order to induce the formation of myxospores, cells of *M. coralloides* D from a logarithmic phase culture were harvested, washed and resuspended in fresh medium containing 0.5 M glycerol, which is known to induce myxospore formation[5]. No antibiotic activity was detected during the differentiation from vegetative bacilli to myxospores.

Fig. 1. Antibiotic production as a function of growth of *M. coralloides* D.

Nephelometer flasks (100 ml) containing 20 ml of medium (Materials and Methods) were inoculated with 1 ml of a 24-hour culture of *M. coralloides* D. Flasks were incubated at 28°C with gyratory shaking (200 rpm). Antibiotic activity was determined by the paper disk technique, using *S. aureus* as the test organism. Turbidity was measured at 650 nm.



Isolation and Purification of the Antibiotic

The cell-free supernatant obtained by centrifugation was concentrated ten-fold under reduced pressure at 50°C; the pH decreased to 6.5 in this step. The concentrated supernatant was treated with 2 volumes of ethanol at 4°C for 24 hours. The resulting precipitate was removed by centrifugation. The ethanol was evaporated off vacuum. The antibiotic was extracted from the supernatant at pH 9 (NaOH) by chloroform treatment (3 times, using 0.3 vols. of solvent in each step). The chloroform extracts were combined. No antibiotic activity was detected in the aqueous phase. The $CHCl_3$ extract was dried (Na_2SO_4) and chromatographed on silicic acid. The activity was eluted from the column with 5% ethanol in chloroform (3 ml/min flow rate); the active fractions in elution volume 402~435 ml were concentrated to a smaller volume, which was contaminated with a yellow pigment.

Preparative thin-layer chromatography was performed by applying the concentrated antibiotic onto the plate and developing with 5% ethanol in chloroform; the Rf of the active zone was 0.28 and was separated from some visible impurities located near the solvent front. The active spot was eluted and new TLC was carried out developing with ethanol - chloroform (1 : 9, v/v). When the TLC plated were examined under ultraviolet light a single spot was revealed which corresponded precisely with the position of antibiotic activity.

The whole purification process is summarized in Fig. 2.

Physical-chemical Properties

Preliminary tests showed that there was no loss of activity of an aqueous solution of the antibiotic at pH 7 and 60°C after 60 minutes of treatment, or at 100°C and 120°C after 20 minutes of treatment.

The results obtained from the effect of pH on the antibiotic stability at room temperature using different buffer systems (citrate, phosphate, tris-HCl) indicated that the antibiotic was stable for at least 3 days at alkaline and neutral pH values. On the contrary, a loss of activity occurred at acid pH values which increased with the time of treatment.

The test of thermostability at different pH values (Table 1) showed that the antibiotic activity was stable at all pH values at 60°C, but unstable at extreme pH values at 100°C and 120°C. The antibiotic was stable when frozen and thawed in aqueous solution.

The antibiotic remained stable for 60 minutes when treated with the proteolytic enzymes, papain and pronase.

By paper chromatography the antibiotic migrated as a single zone in several solvent systems. During electrophoresis at pH 6.5 and pH 9, the antibiotic (6 and 9 cm respectively) migrated to the anode as a single active zone; at pH 4 and pH 2.9 the antibiotic remained immobile. Also the antibiotic migrated as a single ultraviolet-absorbing spot on silica gel TLC in various solvent systems (Table 2).

In all the above-mentioned cases the ultraviolet-absorbing spot corresponded exactly with the

Fig. 2. Scheme of extraction and purification of of *M. coralloides* antibiotic.

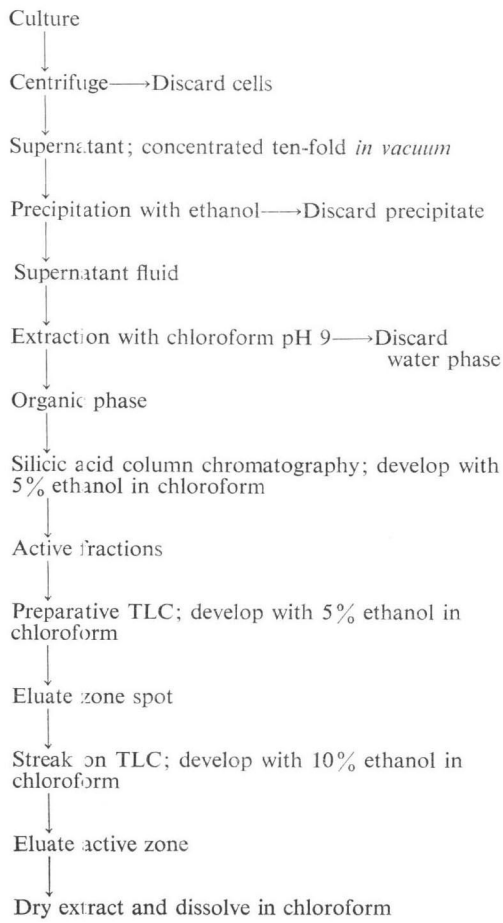


Table 1. Thermostability of antibiotic at different pH values.* 10 is the control unit potency per ml measured against *S. aureus*.

	Time (minutes)	Temperatures		
		60°C	100°C	120°C
pH 3	10	10	10	0
	20	10	1	0
	30	10	1	0
pH 7	10	10	10	10
	20	10	10	10
	30	10	5	5
pH 9	10	10	5	0
	20	10	1	0
	30	10	0	0

* Activity was measured by paper disk technique; pH adjustment was made with citrate (pH 3), phosphate (pH 7) and tris-HCl (pH 9). All buffers at 0.05 N.

Table 2. Thin-layer chromatography of *M. coralloides* antibiotic on silica gel.

Solvent system	Rf
Ethanol - chloroform (5: 95, v/v)	0.28
Ethanol - chloroform (10: 90, v/v)	0.50
Ethanol - chloroform (20: 80, v/v)	0.87
Ethyl acetate - isopropanol - water (65: 23: 12, v/v/v)	0.75
Acetic acid - ethanol - chloroform (1: 10: 89, v/v/v)	0.5
Ammonia - ethanol - chloroform (1: 10: 89, v/v/v)	0.81

Fig. 3. Ultraviolet spectrum of purified *M. coralloides* antibiotic.

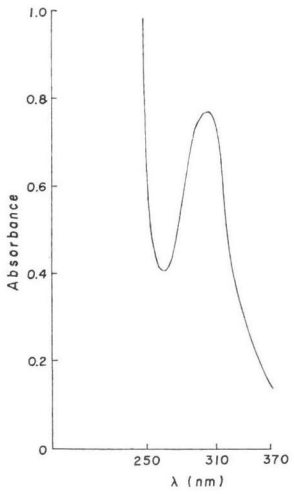
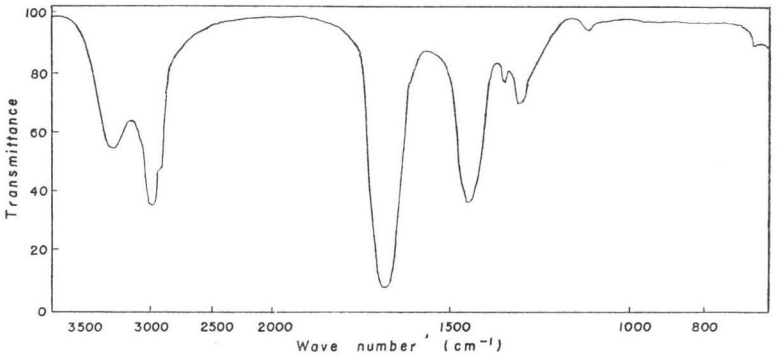


Fig. 4. Infrared spectrum of *M. coralloides* antibiotic.



position of antibiotic activity.

The active spot reacted positively with iodine vapours and negatively with sulphuric acid, ninhydrin, DRAGENDORFF reagent, hydroxylamine hydrochloride, dinitrophenylhydrazine, perchloric-hydrochloric-molybdate, and bismuth subnitrate and KI.

An absorption spectrum in methanol was carried out with the antibiotic yielding a single peak at 300 nm; no absorbance was detected in the visible spectrum (Fig. 3). Mass spectral analysis revealed a possible molecular ion of 260. The infrared spectrum of the neat antibiotic (performed on NaCl cells) is shown in Fig. 4.

Antimicrobial Spectrum

When the purification process was accomplished it was considered of interest to test whether or not the crude extract and the purified antibiotic showed the same activity against other bacteria. As it is shown in Table 3 no significant differences were found between both active fractions. No activity against yeasts was found.

Table 3. Antibacterial activity[a] of *M. coralloides* antibiotic.

Test organism	Crude extract[b]		Purified antibiotic[c]	
	2 Units	10 Units	2 Units	10 Units
Staphylococcus aureus	11	23	12	24
Bacillus subtilis	0	14	0	15
Escherichia coli	0	0	0	0
Klebsiella pneumoniae	0	0	0	0

[a] The results are expressed as inhibition zone diameter (mm).
[b] Organic phase (Fig. 2).
[c] Corresponds to the dry extract, dissolved in chloroform (Fig. 2).

Discussion

It is known that antibiotic production in bacilli occurs during sporulation[16,18] and also that conditions which favor sporulation in bacilli, fungi and actinomycetes stimulate antibiotic production[2]. According to ZÄHNER and MASS[22] antibiotics have an essential role in protection during the

resting cell stage. It is noteworthy to emphasize the fact that the *M. coralloides* antibiotic is produced during its vegetative growth and not in the resting cell stage.

The extraordinary resistance of the antibiotic to several physical and chemical agents has facilitated the extraction and purification process. The method of isolation has provided enough material to study some of its properties, but not sufficient material to attempt crystallization.

When assayed in TLC the antibiotic showed a polar and acid behavior. Its acid character was corroborated by electrophoretic experiments from which its low isoelectric point was determined. The chemical structure of this molecule is not known.

The antibiotic described here is different from that reported by PETERSON *et al.*[15] from *Sorangium*. Myxin shows a cherry red colour absorbing in the ultraviolet and visible spectra with several maxima. The antibiotic produced by *M. coralloides* differs in several of its properties from the *M. xanthus* TA antibiotic[17,20]. The *M. xanthus* TA antibiotic is produced during vegetative growth and myxospore formation; the *M. coralloides* antibiotic is produced during exponential growth only. Besides, the two antibiotics differ in their U.V. and mass spectra.

The demonstration of antibiotic production by a strain of *Myxococcus coralloides* is very interesting not only in relation with the knowledge of the biology of myxobacteria but also because of the possible use of this antibiotic in human beings, something which is practically unexplored in relation with myxobacteria.

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