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XANTHACIN, A BACTERIOCIN OF

MYXOCOCCUS XANTHUS

by

Thomas H. MacRae

A Thesis

Submitted to the Faculty of Graduate Studies Through the  
Department of Biology in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science at the  
University of Windsor

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1973

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## ABSTRACT

An extremely stable, particulate bacteriocin, xanthacin, has been found in Myxococcus xanthus fb after mitomycin C induction. Xanthacin is resistant to trypsin, protease type VI, RNase, DNase, sodium lauryl sulfate (SDS), acetone, ether, ultraviolet irradiation and autoclaving. A high titre preparation was obtained after partial purification by pervaporative concentration, differential centrifugation, molecular sieve chromatography, and density gradient centrifugation. Electron microscopy of platinum shadowed and uranyl acetate stained preparations revealed the presence of circular bodies of varied size which resembled membrane fragments.

#### ACKNOWLEDGEMENTS

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Thanks is also extended to all others who have helped with needed advice and assistance during this study, especially Dr. R. G. E. Murray of the University of Western Ontario for the use of the Phillips 300 Electron Microscope.

This investigation was supported in part by grant A1022 from the National Research Council of Canada.

Finally, I would like to dedicate this thesis to my parents for their understanding and assistance during my first years at university.

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## INTRODUCTION

The presence of rod-like structures, which were termed rhapsomes, has been reported in Saprospira grandis (Correll and Lewin 1968). Although it was claimed that they consisted of protein and ribonucleic acid (Correll and Lewin 1964), a recent report has confirmed only the presence of protein (Delk and Dekker 1972). No biological activity appears to be associated with rhapsosomes. Similar particles have been seen in the fruiting myxobacter, Archangium violaceum (Reichenbach 1965, 1967). Even though they also lacked demonstrable biological activity, Reichenbach was able to show that these particles were, in fact, bacteriophage tails.

Recent electron microscopic observation of a variety of myxobacteria in our laboratory revealed the presence of particles resembling bacteriophage or bacteriophage parts. It became of interest to determine whether any of these particles had biological activity.

Many bacteriophage-like particles produced by eubacteria, while not infectious, often have associated with them the ability to kill a narrow range of bacteria usually related to the producing strain. Such particles then represent one class of the substances known as bacteriocins. (Reeves 1965; Nomura 1967; Bradley 1967; Harting et al. 1972)

Defective bacteriophage-like bacteriocins consist of seemingly intact phages, phage heads, tails which may be extended or contracted,

sheaths and cores or combinations of these phage structures such as in: colicin 15 and colicin H (Mennigman 1965; Endo et al. 1965; Sandoval et al. 1965; Bradley and Dewar 1966), pyocins (Ishi et al. 1965; Higerd et al. 1967; Takeya et al. 1967) and particles from Proteus vulgaris (Coetzee et al. 1968).

Bacteriocin activity is almost always present in bacteriocinogenic cultures but only in small quantities. However, it is possible to increase these amounts by induction. Mitomycin C (Takeya et al. 1967; Foulds 1972; Timmis 1972;) and ultraviolet light (Foulds and Skerman 1969; Elgat and Ben-Gurion 1969) are the inducing agents most commonly used but hydrogen peroxide (Stickler et al. 1965) and azaserine (Sandoval et al. 1965) have produced satisfactory results. The dramatic increase in activity upon induction has facilitated the purification of bacteriocins by a variety of physical and chemical methods (Sandoval et al. 1965; Higerd et al. 1967; Reichle and Lewin 1968; Gagliano and Hinsdill 1970; Ellison and Kautter 1970; Timmis 1972; Foulds 1972). Purification, of course, is a prerequisite for identification of the bacteriocin particle and for morphological and chemical characterization.

This report is concerned with the induction, purification and characterization of a bacteriocin from Myxococcus xanthus fb. Of the strains examined and shown to produce bacteriophage-like particles, M. xanthus was of special interest since not only was it found to produce a wide variety of particles but it is the most thoroughly

studied of the myxobacteria and the only one for which a bacteriophage has been reported (Burchard and Dworkin 1966).

## MATERIALS AND METHODS

### Chemicals

The following chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri: Mitomycin C (crystalline - from Streptomyces caespitosus); Protease type VI (repurified from Streptomyces griseus); Ribonuclease type 1-A (5x crystallized from Bovine pancreas); and Deoxyribonuclease I (amorphous, from beef pancreas). Cesium chloride (CsCl) and sodium lauryl sulfate (SDS) were obtained from Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, New Jersey. Trypsin 1:250 (from pancreas) was obtained from Difco Laboratories, Detroit, Mich. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden. The remainder of the chemicals used were reagent grade.

### Microorganisms and Growth Conditions

The following Myxobacteria, from the University of Windsor culture collection, were used: Myxococcus xanthus fb ATCC - 25232, Cystobacter fuscus M205 and M430, Melittangium lichenicolum M154, Myxococcus fulvus M98, Myxococcus stipitatus M149, Myxococcus virescens M62, Stigmatella brunnea M162, and Stigmatella aurantiaca M341. Other bacteria used were: Escherichia coli ATCC - 11125 and Bacillus subtilis ATCC - 6051.

All working cultures were grown with shaking in the dark at 30°C in 125 ml screw cap flasks containing 30 ml SP medium (McCurdy 1963) and transferred every 4 - 5 days. Stock cultures were maintained on SP slants in the dark at 25°C and transferred every two weeks.

### Induction, Detection and Quantification of Bacteriocin Activity

For induction, 0.01 µgm per ml of mitomycin C was added to an 18 hour logarithmic culture of M. xanthus and incubated with shaking in the dark at 30°C for 24 hours.

Bacteriocin titres were determined by making either 2-fold or 10-fold serial dilutions of samples and spotting a loopful of each dilution on an overlay plate of Cystobacter fuscus M205, the routine indicator strain. The reciprocal of the highest dilution to show inhibition of growth was recorded as the activity.

The specificity of activity was determined with a variety of strains for the following three samples: unpurified 50,000 x g sediment, purified xanthacin and viable M. xanthus cells.

Overlay plates were prepared by making a 1:10 dilution of a stationary phase culture of the indicator strain in dilution medium (McCurdy 1963) and adding 1 ml of this dilution to 9 ml of 1% SP agar. This was then poured over a 2% agar base buffered with 0.01 M potassium phosphate buffer at pH 7.6 (phosphate buffer) and allowed to solidify. After spotting, overlays were incubated in the dark at 30°C. Results were read after 36-48 hours.

#### Purification of Bacteriocin Activity

To remove bacteria, induced cultures were centrifuged at 4,000 x g for 1 hour and 100 ml quantities of supernatant were decanted into sterile dialysis tubing. The culture supernatant was concentrated by pervaporation at room temperature in contact with phosphate buffer. The concentrated preparation was centrifuged at 20,000 x g for 30 minutes to remove cell debris, and the supernatant was centrifuged at 50,000 x g for 2 hours to sediment bacteriocin activity. The 50,000 x g sediment from a 600 ml culture was resuspended in 1.2 ml of phosphate buffer.

In preparation for molecular sieve chromatography, the 50,000 x g sediment was sonicated at full power with the Bronwill Biosonik sonicator for 60 seconds to disperse clumps. Any remaining clumps were removed by centrifugation at 20,000 x g for 30 minutes. The prepared sample was applied to a Sephadex G-200 column previously equilibrated at room temperature with phosphate buffer and was eluted from the column with the same buffer. Fractions, 2 ml in volume, were collected using the Isco Model 272 fraction collector and the presence of protein determined by measuring the OD<sub>280</sub> of each fraction with the Coleman Double Beam Spectrophotometer. The protein-containing tubes from each peak were pooled, centrifuged at 7,000 x g for 30 minutes and at 50,000 x g for 2 hours to sediment the bacteriocin. The sediment was resuspended in a volume of phosphate buffer equivalent to the original volume, and autoclaved at 121°C for 15 minutes.

After passage on Sephadex, 0.5 ml of the active sample was placed on a preformed cesium chloride gradient made by layering 4 ml of a 1.25 gm per ml solution of cesium chloride on 2 ml of a 1.30 gm per ml solution of cesium chloride in a 12 ml cellulose nitrate tube. The tube was filled with 5.8 ml of liquid paraffin and centrifuged at 105,000 x g for 1.5 hours (Beckman L2-65 B; Rotor no. Sw 41 Ti). Opalescent bands which formed in the gradient were removed with sterile 1.0 ml syringes fitted with 16 gauge hypodermic needles.

Protein was determined by the method of Lowry et al. (1951) with bovine serum protein as a standard and by absorption at

OD<sub>260-280</sub>. Carbohydrate was determined by the phenol method (Herbert et al. 1971).

#### Action of Chemical and Physical Agents on Xanthacin

Xanthacin was exposed to various enzyme treatments in the following reaction mixtures: One hundred µgm per ml of trypsin in 0.05 M Tris (pH 8.0) containing  $2 \times 10^{-3}$  M CaCl<sub>2</sub>; 50 µgm per ml of protease type VI in 0.05 M Tris (pH 8.0) containing  $2 \times 10^{-3}$  M CaCl<sub>2</sub>; 200 µgm per ml of RNase in 0.05 M Tris (pH 8.0); and 200 µgm per ml of DNase in 0.05 M Tris (pH 7.4) containing 0.008 M MgSO<sub>4</sub>. All incubations were at 37°C except for DNase which was at room temperature. At 0, 1, 4 and 8 hour intervals after mixing of xanthacin and enzyme, samples were removed, placed in a boiling water bath for 10 minutes to denature residual enzyme, cooled in an ice bath and bacteriocin activity determined.

The effect on xanthacin activity of 0.1% SDS and acetone after a 4 hour incubation and of absolute ether after 1 hour was determined.

To test for heat resistance, xanthacin samples were autoclaved for varying periods up to 1 hour at 121°C.

Resistance to ultraviolet (UV) irradiation was determined by exposure to UV irradiation from a General Electric 15 watt lamp at a distance of 23 cm for 1 hour.

#### Electron Microscopy

Samples were prepared by negative staining with 2% uranyl acetate and by shadowing with platinum in a Balzer's freeze etch



apparatus. Grids were examined with either a Phillips 300 or a Hitachi HU 12 electron microscope.

Phase Microscopy

Photomicrographs were taken using a Carl Zeiss (Jena) NfpK microscope with a Carl Zeiss (Jena) automatic exposure device.

## EXPERIMENTAL RESULTS

### Induction

Spotting 4,000 x g supernatants from various cultures of un-induced and ultraviolet induced strains of myxobacteria on indicator strains from the same order indicated little or no activity. However, induction of M. xanthus with mitomycin C resulted in increased activity against C. fuscus M205. Microscopic observation indicated lysis of the sensitive microorganism.

One hundred ml exponential (18 hour), early stationary (36 hour) and late stationary (48 hour) cultures of M. xanthus were exposed to various mitomycin C concentrations. Fifty thousand x g sediments were collected and assayed to determine the optimum concentration for induction; the results are presented in Table 1.

Bacteriocin production was accompanied by an increase in turbidity of the induced M. xanthus cultures as indicated by the Klett-Summerson Colorimeter. When induced cultures were examined by phase microscopy, some cells had lysed while others were elongated and had dense inclusions as demonstrated in Fig. 1.

### Purification

Following induction, the bacteriocin was partially purified as shown in Table 2. The 4,000 x g supernatant was concentrated by pervaporation which resulted in a clear yellow-brown solution with a specific activity of 0.0023 units per  $\mu\text{gm}$  of protein. The concentrate was centrifuged at 20,000 x g for 30 minutes prior to sedimentation of the bacteriocin at 50,000 x g for 2 hours. The specific activity at this stage was 0.061 units per  $\mu\text{gm}$  of protein.

Table 1. The effect of culture age and mitomycin C concentration on the induction of xanthacin.

Mitomycin C concentration ( $\mu\text{gm}$ per ml)	Activity*		
	18 hour	36 hour	48 hour
2.00	1	1	1
1.50	-	1	1
1.00	1	10	10
0.50	10	10	100
0.25	10	10	100
0.15	100	10	10
0.10	100	10	10
0.05	100	10	10
0.025	10	10	10
0.010	10	10	10
0.000	1	1	1

\*Reciprocal of the greatest dilution to show inhibition of growth.

Each determination is the average of two experiments.

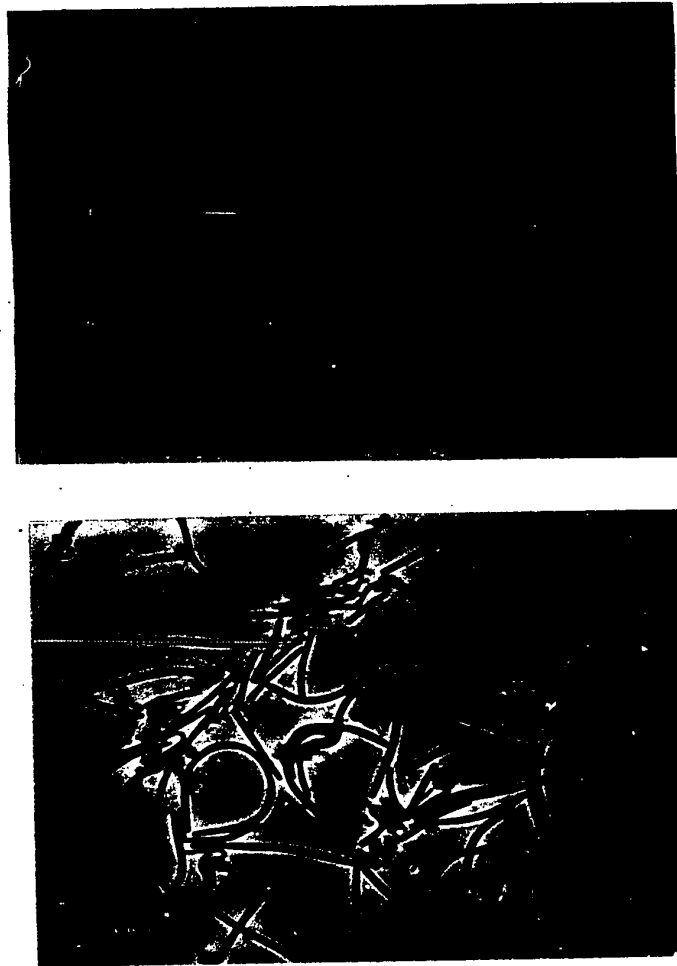


Fig. 1. Phase micrographs of Myxococcus xanthus; a, normal cells; b, mitomycin C induced cells after a 24 hour incubation. X 3,000. Marker represents 3  $\mu\text{m}$ .

Table 2. Purification of xanthacin

Step	Vol. ml.	Activity* units/ml	Protein µgm/ml	Specific Activity units/µgm protein	Yield %
4,000 x g supernatant	2320	2	852	0.0023	100
Concentrate	160	16	7975	0.0020	55
50,000 x g sediment untreated	5	256	4200	0.061	28
50,000 x g sediment sonicated, centrifuged	3.2	128	2650	0.048	8.6
Sephadex G-200	3.5	128	276	0.427	9.7
Cesium chloride density gradient centrifugation	0.63	8	-	-	-

\* Reciprocal of the greatest dilution to show inhibition of growth.

Molecular sieve chromatography on Sephadex G-200 resulted in two peaks as shown in Fig. 2. All activity was in the first peak and was sedimentable at 50,000 x g for 2 hours.

Three opalescent bands were visible following cesium chloride density gradient centrifugation. Xanthacin activity was associated with the top band which appeared near the top of the gradient.

Overall, up to and including molecular sieve chromatography, there was a 185 fold purification of the bacteriocin with a yield of 9.7%.

Chemical analysis of purified bacteriocin revealed the presence of protein. The high 260-280 absorption indicated nucleic acid and this was supported by the phenol test for carbohydrate which gave absorption at 480 nm, a result indicative of pentoses. Insufficient material prevented a precise quantitative chemical analysis of xanthacin.

#### Effect of Chemical and Physical Agents on Xanthacin

None of the agents tested, which included: trypsin, protease type VI, DNase, RNase, SDS, acetone, ether, UV irradiation and heat had any effect on xanthacin activity.

#### Specificity of Activity

The specificity of the three fractions tested is outlined in Table 3.

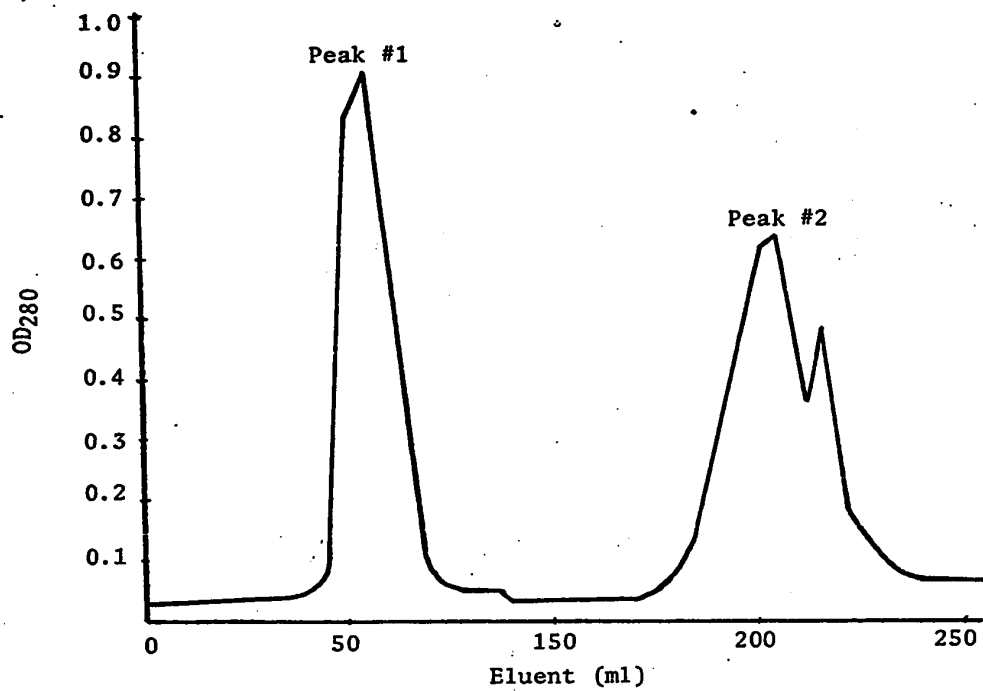


Fig. 2. Sephadex G-200 column chromatography of xanthacin. Elution buffer 0.01 M  $\text{PO}_4$  -  $\text{pH}7.6$ . Column volume = 150 ml.

Table 3. Specificity of xanthacin activity.

Indicator Organism	50,000 x g Sediment Unpurified	Purified Xanthacin	Viabile <u>M. xanthus</u> Cells
Cystobacter fuscus M205	+	+	-
Cystobacter fuscus M430	+	+	VS+
Melittangium lichenicolum	+	+	+
Myxococcus fulvus	S+	-	-
Myxococcus xanthus	-	-	-
Myxococcus stipitatus	-	-	-
Myxococcus virescens	-	-	-
Stigmatella aurantiaca	-	-	+
Stigmatella brunnea	-	-	VS+
Escherichia coli	-	-	-
Bacillus subtilis	-	-	-

+ denotes inhibition, - no inhibition, S+ slight inhibition and VS+ very slight inhibition.



### Electron Microscopy

Examination of unpurified preparations of xanthacin revealed membrane-like particles. Some of these particles were flat, some vesicular, and others were collapsed-vesicular (Fig. 3). Further electron microscopy at various stages of purification revealed similar objects (Figs. 4 and 5) with an increase in the number of flat and small vesicular particles in the final purified preparation (Fig. 5). It is probable that the variety of particles observed and the change in numerical ratio of one type of particle to another were due to purification techniques, with autoclaving causing the greatest changes (Figs. 4a and 4b). Shadowed samples exhibited structures similar to those observed in negatively stained preparations (Fig. 6). No particles were seen which resembled bacteriophage or parts of bacteriophage.

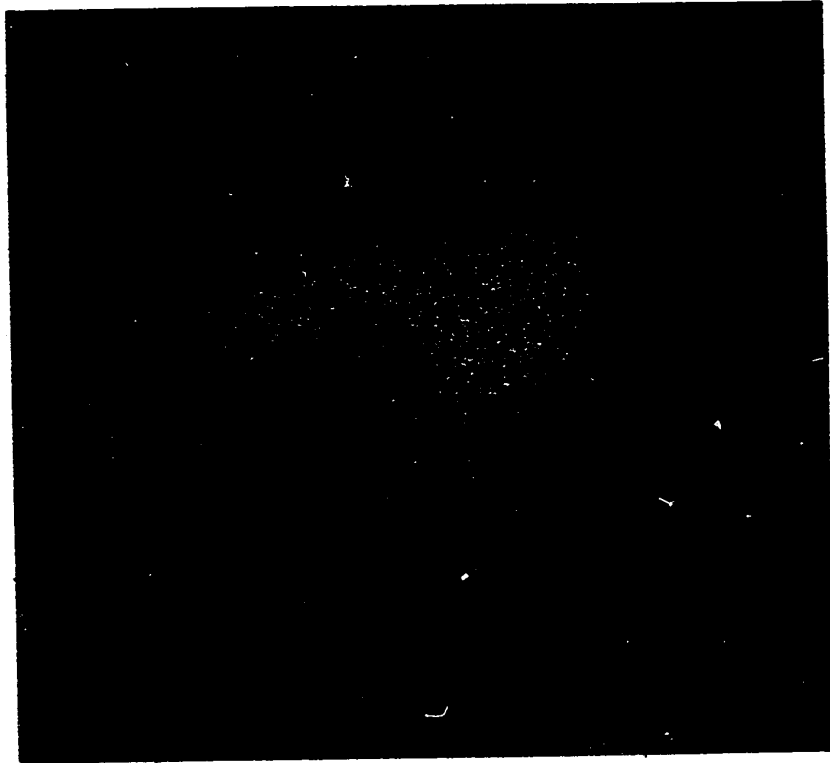


Fig. 3. Electron micrograph of negatively stained, unpurified xanthan. X 119,000. Marker represents 0.10  $\mu\text{m}$ . V, vesicular structure; C, collapsed vesicular particle; F, flat particle.



Fig. 3. Electron micrograph of negatively stained, unpurified xanthan. X 119,000. Marker represents 0.10  $\mu\text{m}$ . V, vesicular structure; C, collapsed vesicular particle; F, flat particle.

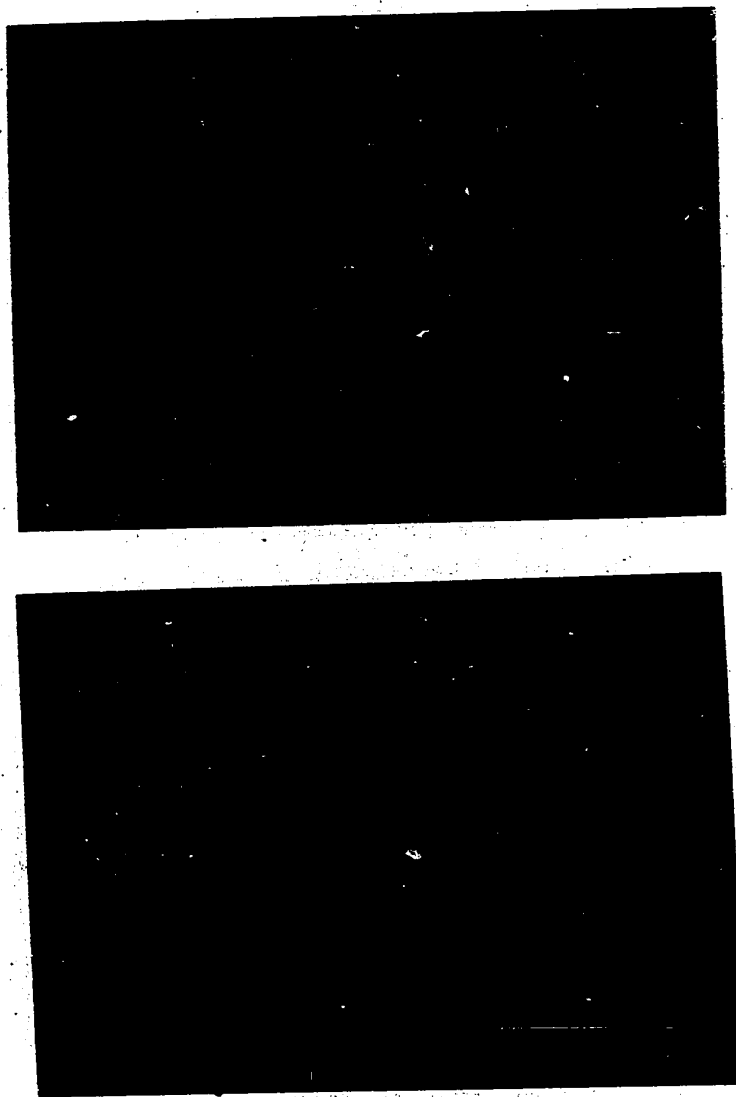


Fig. 4 Electron micrographs of negatively stained xanthacin after passage on Sephadex G-200; a, before autoclaving; b, after autoclaving. X 119,000. Marker represents 0.10  $\mu\text{m}$ .

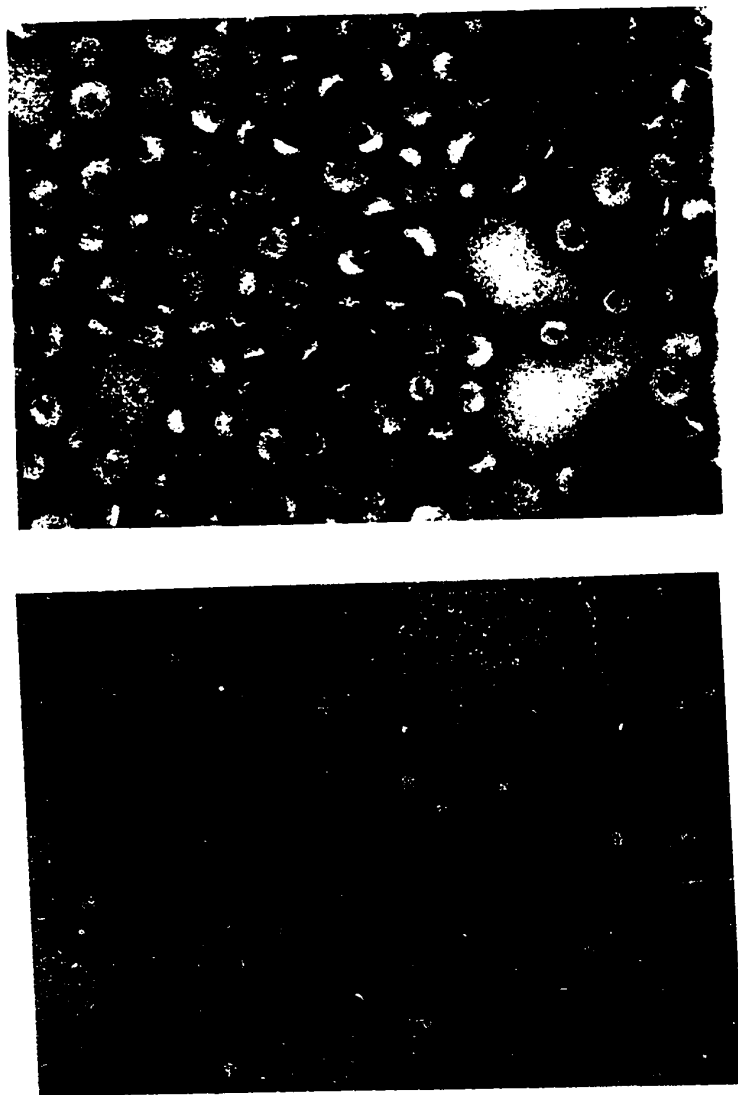


Fig. 4 Electron micrographs of negatively stained xanthacin after passage on Sephadex G-200; a, before autoclaving; b, after autoclaving. X 119,000. Marker represents 0.10  $\mu$ m.



Fig. 5. Electron micrograph of negatively stained xanthacin after CsCl density gradient centrifugation. X 119,000. Marker represents 0.10  $\mu\text{m}$ .

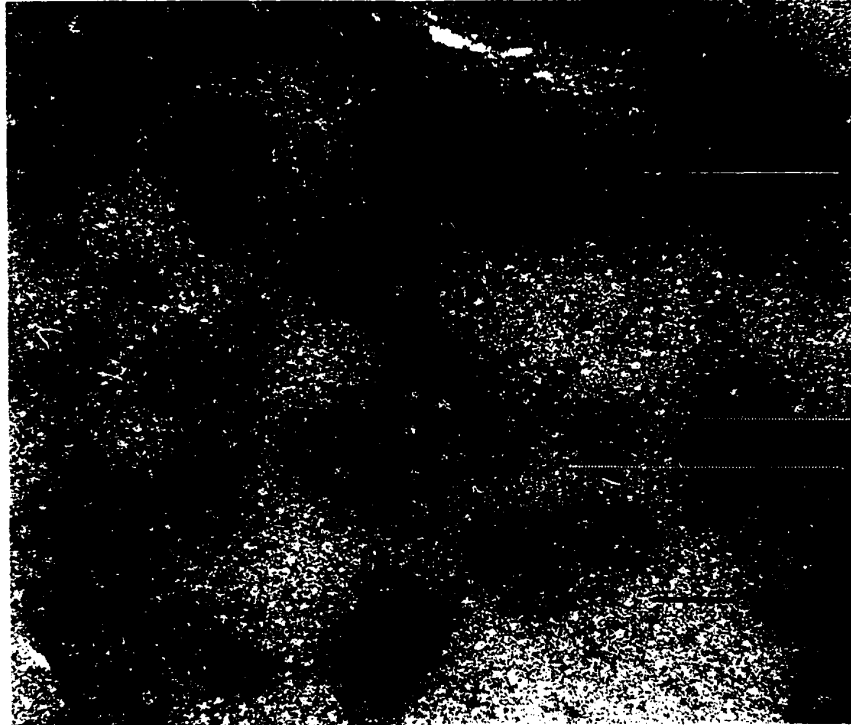


Fig. 5. Electron micrograph of negatively stained xanthacin after CsCl density gradient centrifugation. X 119,000. Marker represents 0.10  $\mu$ m.



Fig. 6. Electron micrograph of platinum shadowed, purified xanthacin.  
X 119,000. Marker represents 0.10  $\mu\text{m}$ . V, vesicular structure;  
C, collapsed vesicular particle; F, flat particle.



## DISCUSSION

The exposure of bacterial cells to mitomycin C often results in cell elongation (Suzuki and Kilgore, 1967; Suzuki et al. 1967) and bacteriocin production. Successful bacteriocin induction has generally involved logarithmic cultures but xanthacin induction, as compared to the induction of other bacteriocins, involved a longer exposure to mitomycin C as well as a slightly lower concentration of the chemical (Seaman et al. 1967; Takeya et al. 1967; Timmis 1972; Foulds 1972). The presence of bacteriocin was indicated by the formation of plaques on overlays of sensitive strains which became less transparent upon dilution, instead of breaking up into discrete plaques, as would be expected for bacteriophage.

Numerous phage-like bacteriocins have been purified (Sandoval et al. 1965; Takeya et al. 1967; Higerd et al. 1967; Jayawardene and Farkas-Himsley 1969) and chemical analysis has been reported for some of these particles. Pyocin consists only of protein (Kageyama 1964) as does a bacteriocin from Proteus vulgaris, even though contaminating carbohydrate and RNA were revealed in the analysis (Coetzee et al. 1968). A bacteriocin from Bacillus subtilis contains both protein and DNA (Seaman et al. 1964; Stickler et al. 1965). Preliminary investigation of the chemical composition of xanthacin revealed that protein, nucleic acid and carbohydrate are present but it is uncertain whether these are associated with the bacteriocin or with contaminating substances. Variation of particle

size and morphology as seen in the electron micrographs and the absence of indicators of chemical purity (Timmis 1972) cause the uncertainties.

Xanthacin resembles other bacteriocins in having a narrow range of activity (Bradley 1967; Coetzee et al. 1968). Results with M. fulvus (Table 3) are of particular interest as M. fulvus is inhibited by unpurified xanthacin but not purified xanthacin showing that some type of activity has been lost during purification. That other bactericidal activity was lost during purification is indicated by differences in the spectrum of activity of viable cells of M. xanthus as compared to unpurified and purified xanthacin.

No evidence was found for defective bacteriophage particles upon electron microscopic examination. Instead, the particles appeared to be membrane-vesicle structures varying in size and morphology. These various morphological types may indicate a lack of purity but it is also possible that harsh treatment during purification resulted in structural changes.

Thus, xanthacin, although resembling defective phage-like bacteriocins in sedimentability and resistance to enzymes, differs from them in extreme thermostability and ultrastructural characteristics. On the other hand, its particulate nature and trypsin resistance distinguish xanthacin from the second class of bacteriocins which are derived from the O-somatic antigen of other gram negative bacteria. There is some resemblance between xanthacin and staphylocin 414, a recently described bacteriocin produced by Staphylococcus

aureus (Gagliano and Hinsdill 1970). Staphylococcin appears to consist of membrane vesicles, is resistant to proteases and is also resistant, although less so than xanthacin, to heat. The chemical composition of staphylococcin is very similar to S. aureus membranes. There are differences between xanthacin and staphylococcin however, as staphylococcin cannot be induced by mitomycin C, is disassociated into subunits by SDS and has a wider range of activity as it is a product of a gram positive bacterium.

That xanthacin is neither a bacteriophage-like bacteriocin or an O-somatic antigen-type bacteriocin has been established. Further investigation is required before the composition of xanthacin can be specified, even though present evidence suggests xanthacin is composed of membrane vesicles. As such, it would represent a bacteriocin class intermediate between the two types now recognized for other gram negative bacteria.

#### SUMMARY

Even though the phage-like structures initially sought in this study were not found, a particulate bacteriocin, xanthacin, produced by Myxococcus xanthus fb has been observed after motomycin C induction, it being active against two strains of *Cystobacter* and one *Melittangium*. Xanthacin is the first bacteriocin reported for the fruiting Myxobacters and is among the most resistant of all bacteriocins. It is not clear what relationship or similarities xanthacin has with other bacteriocins. This question, and the question of its origin in the cell can only be answered when sufficient quantities are available to permit a definitive chemical analysis.

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