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Inhibition of *Paramecium caudatum* by an *Alteromonas luteoviolacea* Antibiotic

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An antibiotic factor obtained from *Alteromonas luteoviolacea* was shown to kill *Paramecium caudatum*. This substance was shown to be destroyed by boiling, sonication, or freezing. It passed through a 0.45-μm-pore-size filter and was not precipitated from a culture supernatant with ethyl alcohol. Its production varied with *A. luteoviolacea* strains and medium constituents. It was also shown to be distinct from the *A. luteoviolacea* antibiotic that affects bacterial cells.

In a study in which bacteria were used as food for protozoa, it became apparent that strains of the gram-negative, purple-pigmented marine bacterium *Alteromonas luteoviolacea* killed *Paramecium caudatum*. *Alteromonas* species are known to produce antibiotics effective against procaryotic cells (bacteria) (1, 3, 5). This study demonstrated that the antibiotic that killed the eucaryotic cells of *P. caudatum* was unique and distinguishable from that affecting bacteria.

MATERIALS AND METHODS

Synthetic media. Artificial seawater as formulated contains no phosphorus (2) and is normally used with peptones and yeast extract which provide variable amounts of phosphorus.

Additional single-carbon compounds were added to give a 0.5% final concentration in a phosphate buffer-artificial seawater solution. $(NH_4)_2SO_4$ was added to yield a 0.1% final concentration. Modified ZoBell broth contained 0.5% peptone and 0.1% yeast extract in artificial seawater to which 1.5% agar was added for a solid medium. A 0.5 M sodium potassium phosphate buffer (pH 7.8) was diluted to give various phosphate concentrations.

Cultures. The *A. luteoviolacea* strains were isolated from Kinko Bay in southern Japan and previously characterized (4, 5). The *Staphylococcus aureus* culture was beta-hemolytic and coagulase positive. It, along with *Escherichia coli* and *Proteus mirabilis*, was a departmental stock culture. *Vibrio damsela* ATCC 33539 was obtained from the Centers for Disease Control, Atlanta, Ga. *P. caudatum* was obtained from the Carolina Biological Supply Company, Burlington, N.C., and maintained on a sterile pondwater nonnutrient agar slant medium.

A 7-day culture of *A. luteoviolacea* grown in various synthetic media and modified ZoBell broth was used for the susceptibility tests of the antibiotics against *P. caudatum* and *S. aureus*. An aliquot (10 ml) of a 40-ml culture was placed in a boiling water bath for 5 min. Another was frozen at −20°C for 24 h and thawed. Aliquot 3 was maintained in an ice bath and then sonicated with a Branson sonic power source (Branson Sonic Power Co., Danbury, Conn.) at power 8 for 4 min. An untreated aliquot was the control. Each aliquot was subsequently centrifuged in the cold, and the supernatant was collected. The optical densities of the supernatants at 570 nm were determined. The supernatants were tested for viable cells by plating on ZoBell agar, and a 0.5-ml sample of each was added to a culture of *P. caudatum*. Each culture contained between 20 and 40 organisms in sterile pondwater over a nonnutrient agar slant. A sterile filter-paper disk dipped in each supernatant was placed on a Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plate previously swabbed with *S. aureus*. One loopful (0.01 ml) of the pellet was added to a *Paramecium* culture, and a sterile filter-paper disk dipped in the pellet was placed on a Trypticase soy agar plate previously swabbed with *S. aureus*. Inhibition zones were measured after 2 days of incubation at 25°C. The *Paramecium* cultures were incubated for 1 day at 25°C, and the antibiotic toxity was determined by observing the viability of the organisms with a dissecting microscope. Death was indicated by the disappearance of all *P. caudatum* in the liquid phase of the tubes and no recovery in 24 h or upon subculture.

Antibiotic extraction. Cells were grown in ZoBell broth for 4 days at 25°C and harvested by centrifugation in the cold. The supernatant was passed through filters (0.8- and 0.45-μm pore size), and 2 volumes of ethanol was then added in the cold to effect the precipitation of the antibiotic. The precipitate was collected by centrifugation in the cold and suspended in 10 ml of a 0.2 M sodium phosphate buffer (buffer B) at pH 8. After dialysis against distilled water in the cold for 24 h, the precipitate was collected and resuspended in 1 ml of the buffer.

A sample (100 ml) of the supernatant was dialyzed in the cold for 24 h against 80% ammonium sulfate, and the precipitate was collected in buffer B. After dialysis against distilled water, the precipitate was collected in 1 ml of buffer B.

The cell pellet was suspended in 400 ml of distilled water, and one aliquot was sonicated in an ice bath; another was frozen at −70°C for 24 h and thawed. Aliquot 3 was boiled for 10 min and cooled, and aliquot 4 was used as a control. The cell pellets were collected by centrifugation in the cold and suspended in buffer B. The supernatant from these centrifugations was treated with 2 volumes of 95% ethanol for 72 h in the cold; the precipitate was collected and suspended in buffer B.

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TABLE 1. Effect of phosphate concentration on growth of *A. luteoviolacea* in artificial seawater with single carbon sources[a]

Strain	Cell count (log_{10} CFU) in ZoBell broth control	Phosphate concn (M)	Cell count (log_{10} CFU) with single carbon source:[b]			
			Glucose	Glycerol	Acetate	Succinate
9K-V2	8.6	0.025	4.4	4.4	5.5	5.9
		0.0007	6.2		5.0	3.3
9K-V7	8.7	0.025			5.9	3.2
		0.0007	4.3	2.3		
9K-V8	8.6	0.025		3.0	4.0	4.7
		0.0007	5.8	1.9		
9K-V10	8.8	0.025	7.5	4.1	3.2	4.6
		0.0007	6.9	7.6		5.1

[a] Cultures grown with pyruvate or lactate as the carbon source were unaffected by the phosphate concentration.

[b] Counts were made after 7 days of incubation at 25°C.

RESULTS

All four *A. luteoviolacea* strains grew on glucose, glycerol, acetate, succinate, and pyruvate used as single carbon sources. Both growth and pigmentation varied with both the strain and the phosphate concentration in the medium, which varied from 0.025 to 0.0007 M. Typical results with the two extreme phosphate concentrations are shown in Table 1. The effects of both the medium and the phosphate concentration on antibiotic activity against both *S. aureus* and *P. caudatum* are shown in Table 2. With two strains (9K-V2 and 9K-V7), the antibiotic activity against *P. caudatum* which occurred with the cells grown in Zobell medium was lost when a synthetic medium and various phosphate concentrations were used. However, the addition of phosphate to a pyruvate or glucose synthetic medium retained the anti-*Paramecium* activity of strain 9K-V10, although the antibiotic inhibition by the supernatant against *S. aureus* was lost. Strain 9K-V8 never demonstrated antibiotic activity against *Paramecium* cultures. This was also true for strain 9K-V10, grown in a synthetic glycerol medium (Table 2). Boiling either the supernatant or the pellet from cultures of strain 9K-V10 that were grown in all media resulted in the loss of antibiotic activity against *S. aureus* but not against *P. caudatum*. Freezing and thawing had no effect on either antibiotic, whereas sonication destroyed the antibiotic activity against *S. aureus* in the supernatants but not in the pellets and had no effect on anti-*Paramecium* activity. Although the cells grew well in the low-phosphate glycerol medium (Table 1), they had no antibiotic activity against either microorganism.

The filtered culture supernatant inhibited both staphylococcal and *Paramecium* cultures, although the inhibitory activity against *P. caudatum* was routinely lost after ethanol treatment, and the inhibitory activity against *S. aureus* was lost after ammonium sulfate dialysis (Table 3). The filtrate and its ethanol precipitate were autotoxic to the antibiotic-producing strain, but the toxicity was always significantly less than that against *S. aureus*. This autotoxic factor, unlike the activity of the antibiotic against *S. aureus* and *P. caudatum*, appeared limited to the cells themselves, because it was absent from the supernatant that resulted from the treated cells. Boiling destroyed all antibacterial activity, whereas anti-*P. caudatum* activity was retained. Cultures of *E. coli*, *P. mirabilis*, and *V. damsela* grown and extracted by the same procedure had no antibiotic effect on *Paramecium* cultures, thus eliminating the classic gram-negative endotoxin effect.

TABLE 2. Effect of phosphate on antibiotic production by *A. luteoviolacea* strains[a]

Strain	Medium (phosphate molarity)	Inhibition of *P. caudatum* by:		Inhibition of *S. aureus* by:	
		Supernatant	Pellet	Supernatant	Pellet
9K-V2	ZoBell	+	+	−	−
9K-V7	ZoBell	+	−	−	−
9K-V8	ZoBell	−	−	−	+
9K-V10	ZoBell	+	+	+	+
	Pyruvate				
	0.025	+	+	−	+
	0.0007	+	+	−	+
	Glucose				
	0.025	+	+	−	+
	0.0007	+	+	−	+
	Glycerol (0.0007)	−	−	−	−

[a] +, Inhibition; −, no inhibition.

DISCUSSION

The data indicate that antibiotic production by *A. luteoviolacea* varies significantly with different strains as well as with the growth medium and the phosphate concentration in the medium. In the nonsynthetic media commonly used, the

TABLE 3. Inhibitory effects of extracted samples of *A. luteoviolacea*

Sample[a]	Inhibitory effect on[b]:		
	A. luteoviolacea 9K-V10	*S. aureus*	*P. caudatum*
Filtrate	12	24	+
Filtrate ethanol precipitate	36	56	−
Filtrate ammonium sulfate precipitate			+
Untreated cells	20	32	+
Supernatant			+
Ethanol precipitate	26	48	−
Sonicated cells	18	32	+
Supernatant		10	+
Ethanol precipitate	32	56	−
Frozen and thawed cells	16	40	+
Supernatant		24	+
Ethanol precipitate	32	52	−
Boiled cells			+
Supernatant			+
Ethanol precipitate			−

[a] Filtrate, culture supernatant passed through 0.45-μm-pore-size filter. Supernatants were obtained after centrifugation of untreated and treated cell suspensions. Ethanol precipitate obtained from supernatant.

[b] Numbers are zones of inhibition in millimeters. +, Death of cells; −, no cell death.

phosphate concentration is not controlled and may account for variable antibiotic production. At least three distinct antibiotic effects were demonstrated: (i) a primary antibiotic effect against *S. aureus*, associated with both the *A. luteoviolacea* cells and their culture supernatants, which was lost after dialysis with ammonium sulfate; (ii) an antibiotic factor which was autotoxic for *A. luteoviolacea* and was not released from the cells by either sonication or freezing and thawing; and (iii) a unique antibiotic affecting *P. caudatum*, which was notably heat stable and was lost after ethanol extraction. The latter is apparently not the classic gram-negative endotoxin, since several common gram-negative bacteria similarly extracted had no toxic effect and *E. coli* strains are commonly used as food in *Paramecium* cultures (6, 7).

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