Antibiotic Production by Anaerobic Bacteria[1]

NANCY O. STURGEN AND L. E. CASIDA, JR.

Pennsylvania State University, University Park, Pennsylvania

Received for publication July 31, 1961

ABSTRACT

STURGEN, NANCY O. (Pennsylvania State University, University Park), AND L. E. CASIDA, JR. Antibiotic production by anaerobic bacteria. Appl. Microbiol. **10**:55–59. 1962.—Soils from aerobic and anaerobic sources were investigated for the possible presence of bacteria which produce antibiotics under anaerobic conditions of growth. The screening techniques devised for this study yielded 157 soil bacteria which, during anaerobic growth, produced antibiotic activity against aerobic test bacteria.

Studies on choice of media, presence of oxygen, and changes in antibiotic activity during growth indicated that representative strains of these bacteria produced mixtures of antibiotics. The activity was heat labile.

In recent years there has been an intensive search for microorganisms which produce antibiotics during growth on artificial media. Many sources of microorganisms have been examined for the presence of these forms. However, with few exceptions, the microbiological techniques utilized for the examination of these sources have been those which would yield aerobic microorganisms. Thus, the possibility of anaerobic production of antibiotics by microorganisms seems to have been somewhat overlooked, although there are a few reports of such occurrences for specific microorganisms. Miller (1959) described the isolation of an anaerobic *Bacteroides* species from the intestine of a mouse which had received streptomycin by stomach tube. In vitro and under anaerobic growth conditions this organism produced an antibiotic which inhibited the growth of certain strains of *Salmonella*, *Proteus*, *Pseudomonas*, and *Staphylococcus*.

There have been several reports of antibiotic production by lactic acid producing species of *Streptococcus* and *Lactobacillus* (Whitehead, 1933; Mattick and Hirsch, 1944; Oxford, 1944; Hirsch and Grinsted, 1951; Hirsch and Wheater, 1951; Wheater, Hirsch, and Mattick, 1951; Vincent, Veomett, and Riley, 1955). In a few instances (Whitehead, 1933; Oxford, 1944; Berridge, 1949), it was determined that these antibiotics may have been polypeptides.

The present study was undertaken to determine whether anaerobic or facultative soil microorganisms produce antibiotics under anaerobic growth conditions, or at least conditions of low oxidation-reduction potential. In the course of this study, a screening procedure was devised which routinely yielded these microorganisms from soil.

MATERIALS AND METHODS

Source of microorganisms. All isolations of microorganisms were made from soil. Soil samples included 5 from fresh water swamps, 1 from a salt water swamp, 3 from garden or greenhouse soils, and 2 from forest soils. All soil samples were kept in glass screw-capped jars until used, and water was added occasionally to approximately maintain the original moisture content of the soils. Stock cultures of anaerobic soil isolates were maintained in screw-capped tubes of freshly steamed Bacto[2] cooked meat broth.

Media. The media, other than those commercially available, used in these studies were as follows: Medium I-7 contained (per liter): glucose, 1 g; beef extract, 5 g; peptone, 3 g; yeast extract, 2 g; K_2HPO_4, 3 g; L-cysteine, 1 g; agar, 15 g. Medium I-11 was composed of (per liter): peptone, 5 g; beef extract, 3 g; glucose, 1 g; yeast extract, 1 g; agar, 15 g. Medium T-1 was similar to medium I-11 except that agar was not included. Medium P-2 contained (per liter): peptone, 5 g; beef extract, 3 g; glucose, 5 g; yeast extract, 1 g. K_2HPO_4, 1 g; corn steep liquor, 10 ml; L-cysteine, 1 g. Medium P-3 was composed of (per liter): tryptone, 5 g; beef extract, 3 g; yeast extract, 1 g; glucose, 1 g; KH_2PO_4, 2 g; L-cysteine, 1 g. Medium P-1 contained (per liter): Bacto dehydrated liver infusion broth, 35 g; Bacto dehydrated veal infusion broth, 22 g; K_2HPO_4, 1 g; L-cysteine, 1 g. All media were adjusted initially to pH 7.0.

Preparation of spread plates. Sterile agar medium was steamed just prior to use and poured without cooling into plastic Petri plates (15 by 90 mm). The plates then were placed in a refrigerator to promote rapid cooling and reduce the tendency for oxygen absorption by the medium. As soon as the agar had hardened, aliquots of 0.1, 0.5, or 1.0 ml of soil diluted 1:10 in a solution containing 0.1% each of $NaHCO_3$ and L-cys-

[1] Authorized for publication on July 24, 1961, as paper no. 2580 in the journal series of the Pennsylvania Agricultural Experiment Station.

[2] Difco Laboratories, Inc., Detroit, Mich.

teine, pH 7.4, were added to the surfaces of the agar and spread with a glass rod. Each dilution of soil was plated in quadruplicate. The plates receiving 0.5 or 1.0 ml of soil dilution usually yielded on incubation a continuous film of bacterial growth on the surface of the medium. Plates spread with 0.1 ml of soil dilution produced separate, isolated colonies.

Conditions for anaerobic culture incubation. Anaerobic incubation of agar and broth cultures of soil isolates was carried out in alkaline pyrogallol vessels containing tubes of reduced methylene blue to indicate the absence of oxygen (SAB, 1957). The anaerobic chambers were large desiccators or cylindrical jars with plate glass covers sealed with plasticine. The methylene blue solution was prepared as recommended except that the primary stock solution of methylene blue was aged for at least 1 month at room temperature to increase its sensitivity to oxygen. Also, 6 ml of 0.1 N (instead of 10 N) NaOH were diluted to 100 ml.

In most instances, the methylene blue indicator solution remained reduced during the incubation period. All cultures were discarded if the methylene blue indicated an oxygen leak. At harvest of the cultures the methylene blue tubes were placed in the open at room temperature to be sure that the indicator would recolorize on exposure to oxygen. This procedure detected the few instances in which the indicator solution had been damaged at the time of preparation.

Assay of broth cultures for antibiotic activity. Cells of anaerobic soil isolates were removed from broth cultures by centrifugation in the culture tubes, and the supernatant solutions were adjusted, where necessary, to pH 6.8 to 7.2. However, in most instances, pH adjustment was not required. Sterile filter paper antibiotic assay discs (12.7 mm)[3] were saturated with 0.1-ml portions of culture supernatant solutions and immediately placed on the surfaces of seeded agar test plates. Five discs were added to each plate.

The seeded agar test plates were prepared by spreading 0.1-ml portions of diluted cultures of aerobic test organisms on the surfaces of medium I-11 in Petri plates. The test organisms were 24-hr medium T-1 broth cultures diluted in such a manner as to give confluent growth on the plates. These test plates were incubated aerobically for 18 hr, at which time the zones of antibiotic activity were determined as the total diameters in millimeters of the resulting inhibition zones.

RESULTS

Screening of soil samples. Preliminary isolation trials yielded no soil fungi or actinomycetes producing antibiotic activity under anaerobic growth conditions. Therefore, only bacteria were considered in the present studies.

[3] Schleicher and Schuell Company, Keene, N. H.

Soil samples were diluted and prepared as spread plates on various agar media. The dilutions were such that on anaerobic incubation for 1 week at room temperature a continuous film of bacterial growth resulted, and antagonistic bacterial colonies were evidenced by a halo of inhibited growth around the colonies. By this procedure, 157 isolations of antagonistic bacteria were made and transferred to tubes of cooked meat broth. The majority of these isolates were gram-positive rods resembling species of *Clostridium*, although 16 of the isolates were short, gram-negative rods. The soils from aerobic and anaerobic natural environments yielded approximately the same numbers of isolates. Of the isolates, 48 were obtained from plates of liver veal agar, 35 from medium I-7, 28 from Sabouraud dextrose agar (Bacto), and 27 from Littman oxgall agar.

Antibiotic production by soil isolates in broth cultures. The 157 soil isolates were grown in the presence and absence of oxygen in medium P-1, a medium of low oxidation-reduction potential. Inoculum for each isolate, as a cooked meat broth culture, was added at 10% to tubes of freshly steamed and cooled medium P-1. The cultures were incubated 1 week at 30 C both anaerobically and in the presence of oxygen, then assayed for antibiotic content against a series of aerobic

TABLE 1. *Antibiotic activity of soil isolates grown in medium P-1 in the absence of air*

Isolate no.	Test microorganisms and inhibition zone diameters										
	Bacillus cereus	*Escherichia coli*	*Pseudomonas fluorescens*	*Serratia marcescens*	*Staphylococcus aureus*	*Sarcina lutea*	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Rhizopus* species	*Penicillium chrysogenum*	*Streptomyces griseus*
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
27-6						35		16		22	
24-7	21	26	16	21	24	27			22		
24-13		16	15								
24-15				20	21	18					
24-17						20		22			
24-19						16		24			
24-22				18		19	15				
24-23						20	15				
24-24			21	27				23	26		
24-25						18		25	18		
24-27		16	18					17			
24-28		19							20		
24-32								23	22		
24-33			23			23		25	24		
24-34						20	23	28			
24-35							16		30		
24-37	18		15		26						
9-6	35		17	30	30		30	22	28		24
12-9						28	22				
13-6		15			15		16				
19-4	22			17							
23-4					16	22					
27-1										17	30
27-2			17							17	17

test organisms. Of the 157 isolates, 24 were selected for further study (Table 1). These isolates in the absence of oxygen exhibited zone diameters of at least 15 mm and antagonistic activity against at least 2 of the test organisms. Isolates 12-9 and 9-6 were gram-negative rods; the rest were gram-positive sporeforming rods.

TABLE 2. *Antibiotic activity of soil isolates grown in medium P-1 in the presence of air*

Isolate no.	Test microorganisms and inhibition zone diameters										
	Bacillus cereus	*Escherichia coli*	*Pseudomonas fluorescens*	*Serratia marcescens*	*Staphylococcus aureus*	*Sarcina lutea*	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Rhizopus* species	*Penicillium chrysogenum*	*Streptomyces griseus*
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
27-6								19	17	20	
24-7			22	35			24	18			
24-13					15			19			
24-15		20	15	18	17			23	21		
24-17											
24-19								19	17		
24-22		15									
24-23		19	15	15		21	20	21	17		
24-24	17	23		15				24	37		
24-25	15			16							
24-27											
24-28											
24-32			20				23	22			
24-33	37	15	23	18			30	19			
24-34				23							
24-35			16	18			21				
24-37				16							
9-6	38				28			20	28		30
12-9	15							19			
13-6										21	
19-4							22		20		
23-4						21					
27-1					15		18				
27-2					16						

TABLE 3. *Effect of media on anaerobic production of antibiotics by isolates 9-6, 12-9, and 24-37*

Isolate no.	Culture media	Test microorganisms and inhibition zone diameters						pH of cultures at harvest
		Escherichia coli	*Pseudomonas fluorescens*	*Serratia marcescens*	*Staphylococcus aureus*	*Sarcina lutea*	*Saccharomyces cerevisiae*	
		mm	*mm*	*mm*	*mm*	*mm*	*mm*	
9-6	P-1	14			14		18	6.9
	P-2	16		15			16	7.2
	P-3			18	14		16	7.2
12-9	P-1				13	15	16	6.6
	P-2			17	16		17	7.0
	P-3		16	15	14	15	16	7.0
24-37	P-1		18	17		14	18	6.6
	P-2		14	20			20	4.3
	P-3		16	15			17	7.0

Antibiotic production by the 24 isolates in medium P-1 in the presence of oxygen is presented in Table 2. Isolates 24-17, 24-27, and 24-28 grew under these incubation conditions, but did not produce antibiotic activity. The other isolates exhibited antibiotic activity in the presence of oxygen, but the spectrum of inhibited test organisms often differed from that in the absence of oxygen. As is shown later, these results may reflect a differing growth rate when oxygen is present above the medium.

Effect of media on anaerobic antibiotic production. Isolates 9-6, 12-9, and 24-37 were chosen for these studies as representing differing Gram morphologies, oxygen sensitivities, spectra of inhibited test organisms, and soil sources. Isolates 12-9 and 9-6 were small gram-negative rods isolated from a fresh water and a salt

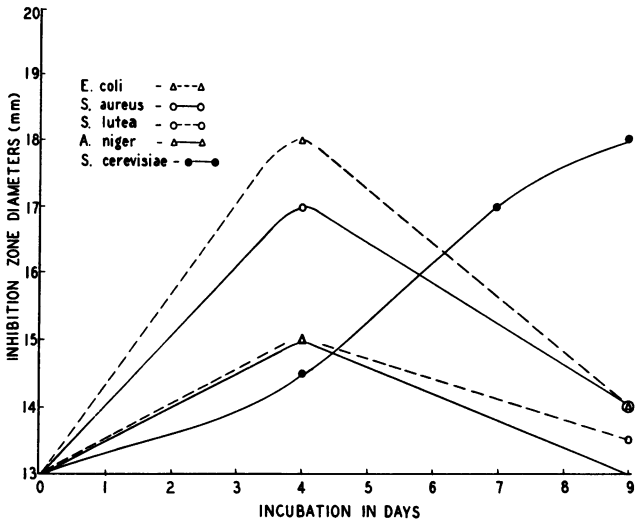

FIG. 1. *Antibiotic production by isolate 9-6 during anaerobic growth.*

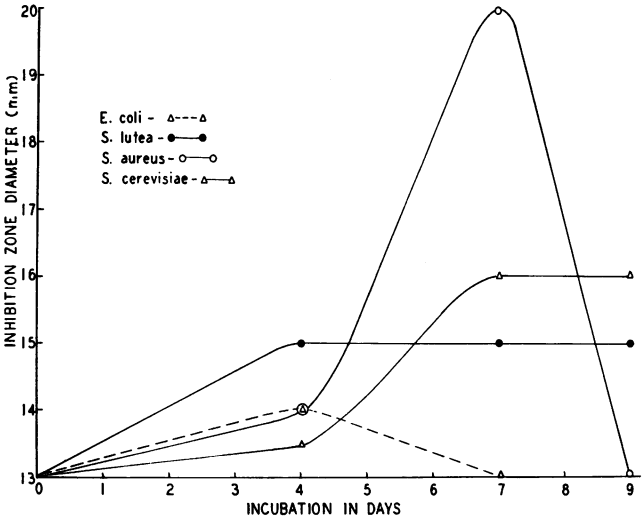

FIG. 2. *Antibiotic production by isolate 12-9 during anaerobic growth.*

water swamp, respectively. Isolate 24-37 was a gram-positive rod with a large terminal spherical spore and swollen sporangium, and was isolated from an aerobic greenhouse soil.

Various media were tested with these organisms to find what effect different nutrients might have on the amount of antibiotic produced and on the spectrum of inhibited test organisms. Tubes of media P-1, P-2, and P-3 were inoculated with 7-day-old medium P-1 broth cultures of the isolates and incubated anaerobically 1 week at 30 C. The antibiotic activity produced in these media (Table 3) indicated that, although there was little difference in yields between media for any one isolate, there were definite differences in the spectrum of inhibited test organisms. This may indicate that each isolate produces a mixture of antibiotics.

Spectrum of antibiotic activity during growth of isolates. If a microorganism has produced more than one antibiotic during growth in a given medium, then samples of cultures taken at various time intervals during growth should show changes in the antibiotic inhibition spectrum characteristic of the individual antibiotics. This was demonstrated by culturing isolates 9-6, 12-9, and 24-37 in medium P-1 anaerobically at 30 C for periods of 0, 4, 7 and 9 days. The spectrum of inhibited test microorganisms and relative antibiotic activity for each isolate are shown in Fig. 1, 2, and 3. Thus these isolates each apparently produced two or more antibiotics during growth.

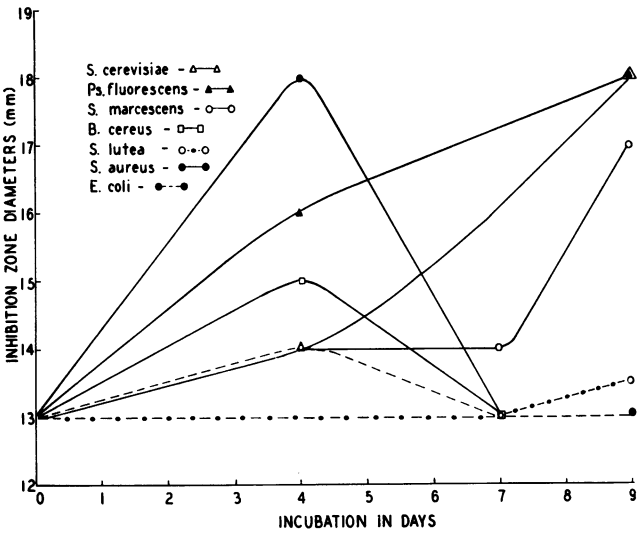

FIG. 3. *Antibiotic production by isolate 24-37 during anaerobic growth.*

TABLE 4. *Effect of heat on antibiotic activity in culture broth preparations of isolates 9-6, 12-9, and 24-37*

Isolate no.	Heat treatment	Test microorganisms and inhibition zone diameters					
		Pseudomonas fluorescens	*Staphylococcus aureus*	*Bacillus cereus*	*Sarcina lutea*	*Saccharomyces cerevisiae*	*Aspergillus niger*
		mm	mm	mm	mm	mm	mm
9-6	Control	15	24	30			16
	Heated	0	0	0			0
12-9	Control	14	14			17	
	Heated	0	0			0	
24-37	Control		16		17		
	Heated		0		0		

Heat stability of antibiotic activity. The stability toward heat of the antibiotics produced by these isolates was tested by heating 10-ml aliquots of medium P-1 culture supernatant solutions, adjusted to pH 7, for 5 min in an Arnold sterilizer. The preparations were immediately cooled in ice water after heating. As may be seen in Table 4, all antibiotic activity in these preparations was destroyed by the heat treatment.

DISCUSSION

The culture isolation procedure described in the present study yielded anaerobic or facultative soil bacteria capable of producing antibiotics under anaerobic conditions of growth. The isolates were obtained from anaerobic as well as aerobic soils, and thus these microorganisms may have a wide distribution in nature.

It is not known what anaerobic soil microorganisms actually were inhibited by the antagonistic bacterial colonies on the original soil isolation plates, but it would appear that they were not clostridia. In a series of experiments not reported, soil dilution plates with isolated colonies were sprayed with suspensions of sporulated cultures of various species of *Clostridium*. On further anaerobic incubation of the plates, there was no indication of antagonistic activity of the soil bacteria against the added clostridia. Also, giant colonies of the soil isolates described in the present study did not inhibit the growth of various clostridia streaked up to the giant colonies.

The anaerobic antagonistic bacteria isolated from soil, in a few instances at least, produced more than one antibiotic substance. This was particularly evident when the culture broths were tested for antibiotic activity at various time intervals during growth. Thus, the relative activity of a culture broth against various aerobic test organisms changed with the period of incubation. The presence at any one time of several antibiotics in a culture broth and the change in antibiotics present with time made it difficult to study the effects of conditions of incubation and media on antibiotic production. Slight changes in these conditions altered the growth rates and hence the relative amounts of the antibiotics present at any one sampling.

Although there was no attempt made at chemical characterization of the antagonistic materials produced anaerobically by the soil isolates, it is believed that

they are antibiotics and not merely inhibitory metabolic products such as organic acids or amines. When these organisms were cultured anaerobically in media in which antagonistic activity was produced, the pH values of the media rarely changed from the near neutral initial pH value. Also, when active neutral pH culture preparations were heated, the activity toward aerobic test microorganisms disappeared.

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