Studies on the Antibiotic Substances from Actinomyces

(5th Report)

"Secondary Screening" Test of 15 Strains of Actinomyces, Specially Antagonistic to Acid-fast Bacteria

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In the first step of the study on the antagonistic actinomyces, 360 active strains¹⁾ were selected by streak plate method (so-called "primary screening") and classified into 4 groups (Table I).

TABLE I
Classification of 360 Active Strains of Actinomyces

1st group	inhibitory to gram positive, negative and acid-fast organisms	201 (56 %)
2nd group	inhibitory to gram positive and acid-fast organisms	130 (36 %)
3rd group	inhibitory to gram positive bacteria only	14 (4 %)
4th group	inhibitory to acid-fast bacteria only	15 (4 %)

For last 2 years many efforts were taken to investigate the 1st group actinomyces (inhibitory to Gram positive, Gram negative and acid fast bacteria) i.e. to divide this group of actinomyces further in details by the specificities of culture filtrates (so-called "secondary screening")²⁾ and to isolate the representative antibiotics of this group in crystalline forms (streptomycin²⁾³⁾ and many streptothricin-like substances⁴⁾). Many antibiotics belonging to the 1st group inhibited acid-fast bacteria too and especially streptomycin is remarkably potent to *My. tuberculosis* (human type). Furthermore, Kelner and Morton⁵⁾ reported the isolation of an "anti-smegmatis factor" from the culture filtrate of a strain of actinomyces.

The 4th group actinomyces, however, were so characteristic in their specific antagonistic activity against acid-fast bacteria, that it was thought

most interesting to prove their potency in their culture filtrates using several kinds of culture media and to elucidate their specific properties by comparison of their antibacterial spectra with the others according to several methods (cup-, dilution- and superposing method) and of antibacterial activities in test media of several pH.

EXPERIMENTAL

Methods and Materials

1) Culture media: After many experiments the following media were used in order to prove the antibacterial activities of 15 actinomycetes in their culture filtrates:

For surface culture (semi-solid media containing 0.25% agar)

- (1) Modified Waksman media, containing 3% glucose.
- (2) Modified Waksman media, containing 2% glycerol.

For shaking culture

- (1) Modified Waksman media, containing 1% glycerol.
- (2) Modified Waksman media, containing 1-2% glycerol and 0.35% CaCO_3 .
- (3) Modified Waksman media, containing 1% starch.
- (4) Original Waksman media, containing 1% glucose.

2) Assay method: On the 5th, 10th and 15th day in the case of surface culture, and on the 2nd, 4th and 6th day, in the case of shaking culture, samples of each culture fluid were filtered, heated at 100°C for 5 minutes and tested for their activities

(1) Dilution method: The culture filtrates were diluted at first 5 or 10 fold and then 2 fold successively with the test medium (4% glycerol broth, pH 6.5). Total volume of culture media was 5 cc. each. Their activities were tested upon *Mycobacterium phlei*, *My. smegmatis* and *My. avium* starting from 10 fold. For *Mycobacterium tuberculosis* (human type, Aoyama B strain) modified Lockemann media (pH 6.5) were used starting from 5 fold.

(2) Cup assay: Unheated culture filtrates were filled in the cup at the center on glycerol agar plates and stored in the ice-box overnight. After 24 hours, 7 test bacteria (*My. phlei*, *My. smegmatis*, *My. avium*, *My. sp.* (Clegg type), *B. anthracis*, *Staph. aureus* and *E. coli*) were streaked radially around the cup and incubated at 37°C for 2 days. The length of inhibition zone was measured in mm.

(3) One-dimensional diffusion method⁶⁾ was also used to test the activity of filtrates on *Staphylococcus* and *E. coli*. Because this method was found to be most sensitive to prove the slightest activity on these microorganisms, it was expected to be able to prove by this method the specificity of filtrates to acid-fast bacteria only.

(4) Filtrates showing activity in dilution method, were assayed again by dilution method using 4% glycerol broth of pH 6.5 and 7.5 in order to find out a suitable pH of test medium.

All the results which referred to following 3 points are shown in Tables II, III and IV.

(1) The specificity of antibiotic spectrum of culture filtrates. (dilution method and cup assay)

(2) pH of test medium, at which culture filtrate was most active.

(3) Diffusibility of antibiotics in cup assay: These observations enabled us to presume the chemical character of antibiotic substance contained in the filtrate.

Results

1) It was able to prove antibiotic activities in culture filtrates of 11 out of 15 strains, using several kinds of culture media in surface or shaking culture. Though their activity was not so powerful in general, the activity of filtrates in any assay methods corresponded almost with that of "primary screening."

TABLE II
Method of Cultivation

Strain No.	Culture method	Culture media	Incubation period (days)	pH at maximum potency
0-130	shaking	1% glycerol (0.35% CaCO_3)	4	7.0
364	surface	1% glycerol	12	7.6
436	surface	3% glucose	15	6.4
497	surface	2% glycerol	15	7.8
0-30				
0-101	shaking	1% glucose	4	6.0
0-128	shaking	1% glycerol	4	9.4
388	shaking	1% glycerol (0.35% CaCO_3)	4	7.2
309	shaking	1% glycerol	4	6.0
287	surface	2% glycerol	10	5.8
395	surface	3% glucose	10	8.2
450				
57				
27	shaking	1% glycerol	4	5.4
456				

TABLE III
Classification by the "Primary Screening"

Strain No.	Character on asparagin-glucose agar			Chromogenic (+) or not (-), on nutrient both agar	Results of streak plates (length of inhibition zone, in mm)		
	Basic mycel.	Aerial mycel.	Soluble pigment		Sm.*	Av.*	Phlei*
0-130	faint yellow	gray green	—	—	1.0	2.0	1.0
364	white	gray white	—	+	3.5	8.0	7.0
436	faint brown	white	yellow	—	1.8	2.5	2.0
497	faint yellow	gray white	—	—	0	2.3	2.0
0-30	faint yellow	red brown	—	+	5.4	3.8	3.2
0-101		white	—	—	6.7	4.5	4.0
0-128	faint yellow	yellow brown	—	+	10.5	2.5	1.0
388	white	white	—	—	1.0	1.0	0
309	white	rose	—	+	0	6.0	6.8
287	white	white	—	—	0	1.8	4.1
395	white	gray brown	—	+	0	0	7.0
450	faint yellow	gray white	—	—	0	0	2.0
57	white	gray white	—	+	0.5	0	1.0
27	faint blue	faint blue	—	+	0.5	0	0
456	white	white	—	+	0	1.5	0

* Sm; indicates *My. smegmatis*, Av; *My. avium*, and Phlei; *My. phlei*.

TABLE IV
Results of "Secondary Screening"

	Cup assay							Dilution method				One-dimensional diffusion method		Active pH of filtrate
	Sm.	Av.	Phlei	Clegg	B. anth.	Staph. aureus	E. coli	Sm.	Av.	Phlei	Human type*	Staph. aureus	E. coli	
0-130	0	0	0	0	3.2	0	0	<5	10	10	<5	—	—	alkaline
364	0	2.2	3.6	0	0	0	0	<5	10	80	<10	—	—	alkaline
436	0	0	2.0	0	2.4	0	0	<5	<5	20	<5	±	—	acidic
497	9.0	0	0	0	0	9.7	6.0	<5	10	10	5	+	+	acidic
0-30	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	acidic alkaline acidic
0-101	0	0	0	0	0	0	0	10	<5	10	<5	+	—	
0-128	7.8	0	0	0	0	0	0	10	<5	<5	<5	—	—	
388	0	0	0	0	0	0	0	10	10	10	<5	—	—	

309	0	0	0	0	0	0	0	<5	10	40	<5	—	—	acidic
287	0	0	4.1	0	5.1	16.3	0	<5	10	20	<5	+	+	neutral
395	5.3	0	2.3	0	0	0	0	<5	<5	<5	<5	+	—	
450	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	
57	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	
27	0	0	0	0	0	0	0	<5	<5	5	5	—	—	acidic
456	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	

* Indicates *My. tuberculosis* (human type).

2) Although in some cases antibiotic spectra by cup assay method coincided good with those of "primary screening," they did not in the other. This fact has been already experienced in the screening of the 1st group actinomyces.

3) Antibiotic activity of filtrates was proved in some strains by dilution method only and could not be found out in cup assay, while some were detectable in cup assay only, but could not be proved in dilution method, suggesting different diffusibilities of each filtrate.

4) Filtrate from surface culture were sometimes higher active than that of shaking culture, so both culture methods must be tried in any case.

5) As it was expected, the antibiotic substances produced by 4th group actinomyces were almost not inhibitory to Gram-positive and negative organisms and specially active to acid-fast bacteria only. Some strains of this group, however, showed this specificity only in "primary screening", and in "secondary screening", they were proved to be somewhat inhibitory to non acid-fast organisms too. In latter case, *B. anthracis* was often rather sensible than the other, as already anticipated in "primary screening".

6) Properties of active filtrates are somewhat different from each other, showing that all of them may probably produce unknown specific antibiotic substances respectively. A new antibiotic substance specially antagonistic to *My. phlei* was now isolated and crystallized by Ouchi⁷⁾ from the culture filtrate of Act. No. 364. If each of these antibiotic is purified in the future, they would certainly contribute to reveal the classification and fermentative metabolism of acid-fast bacteria.

7) It was not able to obtain more highly active filtrates to *My. tuberculosis* (human type) from this group. The 4th group of actinomyces, however, were proved to be very characteristic in the aspect of specific activity to acid-fast organisms.

SUMMARY

Fifteen strains which showed a specific activity to acid-fast bacteria only in streak plate method, were tested using several kinds of culture media on their antibiotic potency in the culture filtrates, and studied further on the nature of antibiotic substances from the point of their own antibiotic spectra, active pH and diffusibility.

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