An Antibiotic Substance Produced by a Gram-positive, Sporeforming Bacillus Isolated from Soil.*

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Since the antagonistic action of sporeforming bacilli against many kinds of bacteria were found by Duclaux¹⁾ 1898, Kimmelstiel²⁾ Rosenthal³⁾ Much and Sartorius,⁴⁾ Franke and Ismet⁵⁾ etc. reported on the studies about these phenomenon. But René J. Dubos⁶⁻⁷⁾ was the first to study in detail the chemotherapeutic application of the substances extracted from a culture of the active bacteria.

He named the substances at first isolated by him "gramicidin" and "tyrocidine."

These substances, however, were quite toxic for living bodies and it was expected that their use would be limited to local application. A search to find, therefore, suitable antibiotics among the cultures of spore formers was performed very vigorously, and many kinds of these substances were isolated such as "bacillin" by Foster⁸⁾ et al, "subtilin" by Jansen⁹⁾ et al, "colistatin" by Gause¹⁰⁾ and "bacitracin" by Johnson¹¹⁻¹²⁾ et al. In our country also similar substances were isolated by Anzai¹³⁾, by Nishimura¹⁴⁾, by Suda¹⁵⁾, by Kochi¹⁶⁾ and by Katsunuma.¹⁷⁾

The author recently had the opportunity to read the literature about "polymyxin" by Stansley et al,¹⁸⁾ and "aerosporin" by Brownlee et al¹⁹⁾ al, concerning antibiotics produced by sporeformers.

All these substances except "bacitracin" and a few other antibiotics seem to be more or less toxic or inactivated in the living body or no report was made about its purification in detail.

Under the leadership of Prof. M. Kuroya, the author sought an antibiotic sporeformer among the collection of strains isolated from the soil in many places in Tohoku and Kanto district, and obtained one active strain.

* The outline of the article was reported at the 21st general meeting of Japanese bacteriologist on 2nd May, 1948.

After extraction and purification of the water-soluble antibiotic from this culture, the author ascertained that its toxicity was very slight. The present paper is concerned with this data, which are being compared with that of "bacitracin" by Johnson et al.

Isolation and Selection of Strains.

At first the suspension of 1 g of soil in 5 cc saline was heated at 75°C for 30 minutes, in order to kill non-sporeforming, vegetative cells. One loopful of this suspension was streaked on a nutrient agar plate and incubated at 37°C for 24 hours. From many different colonies of spore-forming bacilli produced on agar plate, 251 strains were isolated on the nutrient agar slants. The antibiotic activity of these strains was tested by the dilution method.

The culture medium was as follows:

Meat extract	10 g	} pH adjusted to 7.2
Peptone	10 g	
KH ₂ PO ₄	1 g	
Na ₂ HPO ₄ ·12H ₂ O	0.25 g	
NaCl	5 g	
Agar	2.5 g	
Aq. dest.	1000 cc	

The agar was added as the supporting material of bacterial pellicles and the phosphates was used for buffering the pH range of medium during cultivation. Vessels were 200 cc flat shaped flasks and 40 c.c. of culture fluid were bottled into each flask and one loopful of bacteria cultivated on agar slant was inoculated into each bottle. The assay was performed by the broth serial dilution method, the "Terashima" strain of *Staphylococcus aureus* being used as the test organism. The culture filtrate was tested after the heat sterilisation at 100°C for 10 minutes. Among 251 strains isolated from soils of different places one active strain (No. 164) was found by such a procedure.

Bacteriological Characteristics of Strain No. 164.

This strain could grow easily on the nutrient agar and in the nutrient broth very abundantly at 37°C, but in the peptone water its growth was restricted. On the nutrient agar small yellowish non-transparent S-form colonies were produced, while R-form colonies were found only infrequently.

The broth cultures were turbid homogeniously at first, but in a few days they became transparent and very fragile, thin pellicles were formed at the same time. Löffler's medium was not liquefied. Gelatin medium was liquefied only on the surface layer in a dish form. On the potato slant

grayish-white, viscous colonies were formed. Acid was produced in glucose, maltose and sucrose, but no gas production was observed. Lactose was not fermented.

Indol production was negative, catalase-reaction positive. The organisms survived often even after the heating at 100°C for 30 minutes.

The organisms were Gram positive, 1.0–3.5 μ long, their ends were blunt, long chained. Spore central, oval, actively motil.

Regarding these findings, the organism seems to be very similar to the *Bacillus subtilis* group described by Topley and Wilson,²⁰⁾ and Bergey.²¹⁾

Media for the Production of Antibiotic.

With reference to culture media for the production of antibiotic by the spore formers, many kinds has been developed, such as Dubos's casein hydrolysate media for "gramicidin," Foster's yeast extract agar media for "bacillin" production, and Johnson's synthetic or soybean extract media for "bacitracin" production and so forth.

With these media in mind, several experiments were conducted with media of different compositions and the following medium was selected as a most suitable one for the production of the antibiotic.

Meat extract	10 g
Peptone	10 g
KH ₂ PO ₄	1 g
Na ₂ HPO ₄ ·12H ₂ O	0.25 g
NaCl	5 g
Agar	2.5 g
"Dokudami" (<i>Houttuynia cordata</i>) concentrated extract	2 cc
Aq. dest.	1000 cc

The medium was adjusted to pH 7.2 with sodium hydroxide and sterilized by autoclaving for 20 minutes at 15 pounds pressure.

So-called "Dokudami extract" was prepared as follows: The dried pieces of stems and leaves of "Dokudami" (*Houttuynia cordata*) plant was extracted in amount of 200 g with 5000 cc water at 100°C for 5–6 hours and filtered.

The yellowish brown extract thus obtained was concentrated to 1/10 in vacuo and added at the rate of 2 cc per liter of basal media.

This extract promoted in a suitable amount the production of the antibiotic as shown in Table I. It was seen from the table that this media gave the highest titer (1:640) by the serial dilution method against "Tera-shima" strain after the incubation for 1 day only and that there was an optimal amount (2 cc per liter) of "Dokudami" extract in order to bring

TABLE I.

The Needed Amount of "Dokudami" Extract to Promote the Production of Antibiotic.

No.	Amount of extract (cc per l basal media)	Incubated for									
		1 day		2 days		3 days		4 days		5 days	
		pH	Potency	pH	Potency	pH	Potency	pH	Potency	pH	Potency
1	0	7.2	1:10	7.4	1:10	7.4	1:10	8.0	1:10	8.2	1:10(+)
2	0.0002	7.2	1:40	7.4	1:20	7.4	1:10				
3	0.002	7.2	1:80	7.4	1:40	7.4	1:10				
4	0.02	7.2	1:80	7.4	1:40	7.4	1:10				
5	0.2	7.2	1:160	7.2	1:80	7.4	1:80				
6	2	7.2	1:640	7.2	1:160	7.6	1:80	7.6	1:80	8.0	1:80
7	4	7.2	1:320	7.4	1:160	7.6	1:40	7.6	1:40	8.0	1:10
8	8	7.2	1:160	7.6	1:160	7.8	1:20	7.8	1:20	8.4	1:10
9	12	7.2	1:160	7.8	1:80	7.8	1:40	7.8	1:40	8.4	1:10
10	16	7.2	1:160	7.6	1:160	7.8	1:80	7.8	1:80	8.2	1:10
11	24	7.2	1:160	7.4	1:160	7.8	1:80	7.8	1:80	8.2	1:80
12	32	7.2	1:40	7.6	1:160	7.6	1:80	7.6	1:80	8.2	1:80
13	64	7.2	1:40	7.6	1:160	7.6	1:160	7.8	1:80	8.0	1:80

Test organism:—*staphylococcus aureus* (Terashima strain).

The potencies were expressed in dilution titer of culture filtrate.

The extracts were concentrated to 1/10 of their original volume.

about a highest potency of the culture filtrate.

The active principle contained in "Dokudami" extract was determined by further study to be a sort of phytosterol. In the latter part of this work the following media were used for the large scale production of the antibiotic.

Meat extract	10 g
Peptone	10 g
KH ₂ PO ₄	1 g
Na ₂ HPO ₄ ·12H ₂ O	0.25 g
NaCl	5 g
Agar	2.5 g
0.1% Cholesterol alcohol solution	10 cc
Aq. dest.	1000 cc

In this media Cholesterol promoted the production of the antibiotic

substances equally well as the active phytosterol isolated from "Dokudami" plant. Regarding the details a report will be made elsewhere (Okabe and Hosisima²²).

Addition of a trace of metal salts such as iron, copper, zinc, and manganese, and several kinds of sugars, such as glucose and lactose was not adequate for the antibiotic production.

Optimum temperature for antibiotic production was 30°C–32°C.

Stability of the Antibacterial Substance Contained in Supernatant.

a) Influence of the preservation.

The potency of the centrifuged supernatant of the culture fluid, which was sterilized at 100°C for 10 minutes and kept at pH 5.0 at room temperature (13–28°C), was found to be constant during 30 days and decreased slowly until its potency dropped to 50% after 120 days. Therefore, the antibiotic can be preserved at weakly acidic pH range for a long time.

b) Influence of the heating.

When the active fluid was heated at pH 2.0, pH 3.0, pH 5.0, pH 7.0, pH 9.0, and $\frac{N}{10}$ NaOH for 30 or 60 minutes, at 60°C or 100°C, its potency decreased to 1/2 at pH 9.0 for 30 minutes at 60°C, to zero at $\frac{N}{10}$ NaOH for 30 minutes at 60°C, but at pH 3.0–5.0 it only slightly decreases even after the heating for 60 minutes at 100°C.

c) Influence by oxidation and reduction.

The decrease of potency is not caused by aeration of the centrifuged supernatant for 3 hours at room temperature, and by addition of sodium hyposulfite to the concentration of 1% and by keeping it for 3 hours at room temperature.

d) Influence by filtration.

The potency decreased to 1/10 when filtered through Seitz filter and to zero when filtered through a Berkefeld filter (w). There was no decrease in potency when filtered through filter paper.

e) Bactericidal property.

The centrifuged supernatant (bacteriostatic dilution titre 1:320) diluted 320 times was not bacteriocidal, but that diluted 80 times or less was shown to be bactericidal.

Toxicity of the Centrifuged Supernatant.

The intraperitoneal injection of 2 cc centrifuged supernatant (dilu-

tion potency 1:320) did not cause the death of mice weighing 15 g.

Extraction and Purification of Active Substance.

Various methods of purification such as precipitation of protein from the filtrate at its isoelectric point, absorption by active carbon, extraction by butanol, ether, etc. have been reported.

The following procedures were carried out and their corresponding results recorded.

The isoelectric point of the centrifuged supernatant of the culture fluid was pH 1.0–2.0. The protein precipitated by this method absorbed almost all the active principle in the fluid. Further purification, however, was difficult, because the active substance could not be extracted with alcohol, methanol or other organic solvents as it has been made by Dubos and others in case of “gramicidin” or “tyrocidin.”

Absorption of active substance on 2% carbon from culture fluid was complete at pH 6.0, whereas the elution from the carbon was not possible in spite of using many kinds of organic solvents. When the centrifuged supernatant was shaken with the same volume of n-butanol, it was proved that the active agent was completely taken up by n-butanol. The further purification, therefore, was conducted according to the method of purification of bacitracin, because it was shown already by Johnson et al, that bacitracin dissolved easily in n-butanol in a similar way as this antibiotic.

The harvest was centrifuged at its maximum titre (after one day's culture at 30°C) at 3000 r.p.m. for 30 minutes and the supernatant after adjusting pH to 9.0 was shaken well with 2/3 volume n-butanol in the separatory funnel and was left at room temperature for 24 hours, until almost all the active substance was removed into n-butanol layer, which showed a slightly yellowish tinge. The butanol solution was evaporated under reduced pressure at 30°C–40°C, being diluted with distilled water, in order to facilitate the evaporation and to keep the potency constant during the evaporation. When the butanol solution was reduced to ca. 1/3 of its original volume, the addition of distilled water was discontinued and the solution was further evaporated to 5–10 cc, until the active substance separated out as a solid in the pure butanol solution. When the suspension was centrifuged, almost all active substance (90%) was present in the precipitate. This precipitate was washed with absolute alcohol and ether 2–3 times respectively and dried in vacuo. The substance obtained was dissolved in warm butanol-water mixture and the same treatment described above was repeated once more.

The dried substance was yellowish-white semicrystallized powder.

From 2000 cc of centrifuged supernatant, 0.145 g. of such a powder

was obtained, which showed a potency of 1:4,000,000 (dilution titre).

The total potency of the powder was corresponding to 82.3% of the total potency of original supernatant (Table II).

TABLE II.
Extraction and Purification of Active Substance.

Test organism:—*Staphylococcus aureus*, Terashima strain.

P Potency expressed in dilution titre.

Antibiotic Spectrum of the Active Substance.

As illustrated in Table III, it showed against *Staphylococcus aureus* (Terashima and F.D.A. 209-p strain) a dilution titre of 1:4,000,000, against *Staphylococcus albus* 1:8,000,000. For *Streptococcus hemolyticus* 1:3,200, for pneumococcus I, II, III types 1:16,000 using 1% serum broth as test medium and for *Corynebacterium diphtheriae* 1:32,000 dilution titre.

Among acid fast bacilli, against *My. phlei* 1:320,000, against *My. smegmatis* and *My. tuberculosis* (aviane type), 1:10,000 using 4% glycerol broth as test medium.

For Gram negative bacilli, it showed a lower titre than 1:2,000. So this substance was effective almost selectively against Gram positive bacteria.

TABLE III.
Antibiotic Spectrum.

	Strain	Titre		Strain	Titre
Gram positive bacteria	<i>Staphylococcus aureus</i> , Terashima	4,000,000	Gram negative bacteria	<i>Esch. coli</i>	2,000
	<i>Staph. aureus</i> , F.D.A. 209-P	4,000,000		<i>Eberth. typhi</i>	2,000
	<i>Staph. citreus</i>	4,000,000		<i>S. paratyphi</i>	2,000
	<i>Staph. albus</i>	8,000,000		<i>S. schotmülleri</i>	2,000
	<i>Pneumococcus</i> Type I.	16,000		<i>Bacterium paradysent.</i> <i>sonnei</i>	2,000
	"Type II.	16,000		<i>Vibrio cholerae</i>	4,000
	"Type III.	16,000		<i>Proteus vulgaris</i>	2,000
	<i>Cory. diphtheriae</i>	32,000		<i>Enterococcus</i>	2,000
	<i>Bac. anthracis</i>	126,000		<i>Neisseria meningitidis</i>	2,000
	<i>Myc. tbc, avium</i>	10,000		<i>Pseudomonas pyocyanea</i>	2,000
	<i>Myc. phlei</i>	320,000			
	<i>Myc. smegmatis</i>	10,000			
	<i>Strept. hemolyticus</i>	3200			
	<i>Micrococcus tetragenus</i>	10,000			

Toxicity of the Substance.

(1) Hemolytic toxin.

This substance does not show any hemolysis for cattle red blood cells at 1:1000 dilution.

(2) The intraperitoneal injection of 20 mg of this substance in a mouse weighing 20 g did not show any toxic effects during the observation for one week.

Chemical Property of the Substance

1 per cent solution of the substance showed following chemical reactions:

- 1) Biuret reaction (—)
- 2) Molisch's reaction (—)
- 3) Xanthoprotein reaction (—)
- 4) Millon's reaction (—)
- 5) Sakaguchi's reaction (—)

- 6) Chlor reaction (—)
- 7) Phosphor reaction (—)
- 8) Nitrogen reaction (+)
- 9) Vohl's reaction (Sulphur) (—)

The substance was soluble in water and water butanol mixture, not soluble in methanol, ethanol, acetone and other organic solvents.

SUMMARY.

1) A strain of Gram positive, spore forming bacilli was isolated selectively inhibitory for Gram positive bacteria.

2) This strain seemed to belong to the *Bacillus subtilis* group according to the morphological and biochemical properties.

3) The colony on the nutrient agar plate was S type, unlike the other antagonistic strain already reported.

4) The medium for the production of the antibiotic consisted of meat extract 10 g, peptone 10 g, KH_2PO_4 1 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.25 g, NaCl 5 g, conc. "Dokudami" extract 2 cc and tap water 1000 cc.

5) The 24 hours culture of this strain in the medium at 30°C showed 1:640 dilution titre for "Terashima" strain.

6) The active substance was found to be stable toward the heating at weakly acidic pH, toward the preservation for 6 months and toward the oxidation by aeration.

7) The concentrated centrifuged supernatant was bactericidal.

8) This substance was soluble in water and butanol containing water and insoluble in alcohol, ether, acetone, methanol and butyl acetate at pH 7.0.

9) This substance could be extracted by shaking with butanol at pH 8.0–9.0. By this property its purification was possible to a certain extent.

10) This antibiotic substance was active only for Gram positive bacteria. The dried powder showed 1:4,000,000 dilution titre for *Staphylococcus aureus* (Terashima strain.)

11) Hemolytic activities were negative in a dilution of 1:1000 of culture filtrate.

12) The M.L.D. of this substance was proved to be larger than 1000 mg per Kg of body weight of mice. The substance, therefore, seemed to be applicable for chemotherapeutic use.

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