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An Antibiotic against *Bacillus amyloliquefaciens* from *Streptomyces* sp. KM-48

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*Streptomyces*屬 KM-48 菌株가 生成하는 *Bacillus amyloliquefaciens*에 對한 抗生物質

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One of *Streptomyces* sp. was screened as an antibiotic producing strain against *Gram* positive bacteria specifically from about one hundred *Actinomycetes* collections. The isolated KM-48 strain, indexed in our collection, was secreted an anbitiotic in nutrient broth culture. The active principle was extracted with acetone from the lyophilized solid of cultured filtrate. The nonadsorbed elute in aluminium oxide was isolated as a purified homogeneity which revealed a single spot under ultra-violet beam on avicel thin layer chromatography. The isolated antibiotic was sensitive specifically against the Gram positive bacteria and fungi. About fifty percent inhibition of the control growth on *Bacillus amyloliquefaciens* was 18*ug/ml*. The antibiotic was lavile against thermotreatment and stabled in acidic condition remarkably. The KM-48 was identified as a similar strain to *Streptomyces tsusimaensis* on its morphological and cultural characteristics.

The synthesis and secretion of extracellular enzymes by *Bacillus amyloliquefaciens* are examining as a model system for investigating how proteins are secreted through cell layers. For study of the secretory mechanism of extracellular enzymes,[1-6] a new antibiotic which inhibits the peptidoglycan synthesis of *Gram* positive bacteria will be isolated. If the peptidoglycan synthesis were inhibited by an antibiotic, the normal secretion of protein through cell walls might be blocked. By comparison of the secretion in normal cells with abnormal ones, the regulatory factor related to transport in cell wall might be elucidated. That is the reason why we want to isolate an antibiotic which reacts to peptidoglycan of *Gram* positive bacteria specifically.

Among anbitiotics inhibiting the peptidoglycan synthesis, ß-lactam antibiotic which inhibit cell division and cell elongation by inactivating enzymes that are involved in the biosynthesis of peptidoglycan or murein, was one of the most famous ones after discovery by Flemming in 1929.[7] After that vancomycin,[8-9] cycloserine,[10] tunicamycin,[11-15] bacitracin,[16] enramycin[17] and ristocetin,[18-19] etc., were also isolated as a specific inhibitor against peptidoglycan synthesis. Moreover, the tunicamycin might be one of the most appropriate antibiotics for studing the secretory problems because of its accumulation activity[20] of extracellular enzymes in the periplasmic space in *Gram* positive bacteria, and also this antibiotic was refined on its target site in peptidoglycan synthesis pathway.[21-24] However, we tried to isolate another new antibiotic that might be useful for investigating this problems.

For these reasons, various soil samples from the outskirts of Daegu city was collected, and isolated *Actinomycetes* for screening the antibiotic producing strains. One of *Streptomyces* species indexes KM-48 in our collection was screened finally. And, identification of the strain and antibiotic spectrum were observed.

Materials and Methods

Strains

Bacillus amyloliquefaciens which producing liquefying

type of α-amylase was used. *Escherichia coli, Bacillus subtilis* NA64,[25] *Saccharomyces cerevisiae, Candida albicans, Aspergillus niger* and *Rhizopus japonicus* were also used in antibiotic spectrum test. These strains were stocked in this laboratory.

Isolation of antibiotic producing strains

About 100 kinds of soil samples from Palgong Mt. near Daegu city and the campus of this university were collected. An *Actinomycetes* from the saline suspensions of these soil samples was isolated with glucose-asparagine medium by dilution or streaking methods. Composition of the isolating medium was as follows: Glucose 1.0%, Asparagine 0.05%, NaCl 0.01%, K_2HPO_4 0.05% and Agar 1.5%.

Screening of the antibiotic producing strains

In the first screening test, 21 strains were isolated by diffusion method in nutrient agar plate, and 4 strains in the second screening test. Finally, the most powerful antibiotic producing bacteria was isolated and designated as a KM-48 in this collection. This screening test was needed that the antibiotic action should react to the *Gram* positive bacteria specifically, because of the cell layer differences.

Culture and purification

B. amyloliquefaciens was inoculated and incubated to 10 ml of NB (nutrient broth) medium in L-test tube for 24 hrs at 37°C by shaking. The antibiotic producing *Actinomycetes* were cultured in 10 ml of glucose-asparagine medium at *Erlenmeyer* flask for 5 days at 30°C by standing. The cultured filtrate was lyophilized and extracted with acetone three times using each time a volume of the solvent corresponding to about one-fourth of the volume of broth. The acetone extracts were combined and removed acetone by evaporation *in vaccuo*. The oily materials of soluble components in acetone was column chromatographed with aluminium oxide. None adsorbed elute in the column was isolated and evaporated as a purified substance (Fig. 1).

Antibiotic spectrum

Growth media was used 0.8% of NB broth for bacteria and 1.5% of malt extract broth in fungi under the addition of purified antibiotic substance by dilution method. The growth was determined by checking turbidity in OD 550 nm after culture for 15 hrs at 30°C.

Characterization of *Streptomyces* KM-48

The microorganism was incubated on various medium at 30°C for 20 days. The morphological properties and cultural characteristics of the strain were observed on synthetic and organic media with agar-cylinder culture method. Carbohydrate utilization was determined after culture at 30°C for 20 days in the minimal medium of *Pridham and Gottlied*[27], containing 1% of each carbohydrate. Sodium chrolide tolerance test was used with glucose-asparagine medium containing maximal concentration of 20% of sodium chloride.

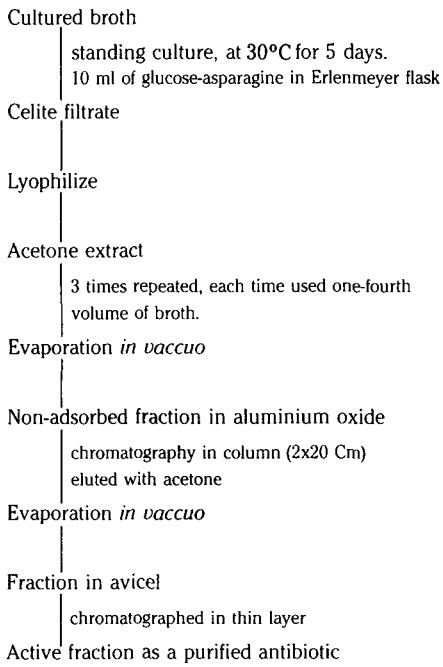

Fig. 1. Purification of Antibiotic

Results

Screening of the antibiotic producing strains;

The most powerful strain for producing growth inhibitory substance against *B. amyloliquefaciens* was screened finally KM-48 in our collection. The antibiotic spectrum of 1st screened strain against *Gram* positive and negative bacteria was listed in Table 1. KM-29, 30, 48 and 51 revealed relatively strong activity on growth inhibition of *B. amyloliquefaciens* specifically by disc method. In the final screening test, KM-48 was revealed the most powerful activity by dilution method. The minimal inhibition concentration was 2 ug/ml (Table 2).

Purification of the active substances

The non-adsorbed fraction in aluminium oxide was

Table 1. Antibiotic inhibition by streaking method.

strain		Distance of growth inhibition (Cm)							
		E. coli		*B. subtilis* NA_{64}		*B. subtilis* B_7		B. *amyloliquefaciens*	
KM	2	0	0	0.3	0.3	0.6	0.5	0.5	0.6
	6	0	0	0.5	0.7	0.5	0.3	0.3	0.6
	8	1.2	1.8	0.8	1.4	0	0	0.3	0.4
	10	0	0	0.3	0.5	0.5	0.4	0.5	0
	11	0	0	1.0	1.0	0.1	0.3	0.3	0.3
	12	0.3	0.1	0.2	0	0.2	0.1	0	0
	14	0	0	0.8	1.6	—	0.3	0.5	0.5
	15	—	0.2	0	0	0.7	0.4	0.6	—
	16	0	0	0.2	0.2	0.1	0.2	0.1	0
	27	0.2	0.2	—	0.9	—	0.2	—	2.2
	29	1.1	1.0	1.4	0.8	2.2	—	1.8	1.6
	30	1.0	1.0	0.7	0.9	3.5	—	1.8	2.0
	34	0	0	0.5	0.9	0.2	0.3	0.3	0.3
	35	—	1.0	1.7	1.4	0	0	2.0	1.4
	37	0.2	0.2	0.3	—	0.3	—	0.3	—
	41	0	0	0	0	1.4	1.6	0	0
	48	0	0	0	0	0.8	0.8	0.7	0.5
	51	0	0	0	0	—	1.3	1.9	1.4
	63	1.0	1.0	0.2	0.2	0	0	0	0
	65	0.7	0.5	1.5	1.5	1.6	1.6	1.3	1.3
	67	1.8	1.5	0.3	0.4	0.5	0.5	0.5	0.5

The strains which inhibit the growth of *gram* positive bacteria were secreened with streaking method. The *actinomycete* was inoculated in two plates of nutrient agar and cultured at 37°C for 5 days.
The strains were inoculated across the *actinomycete* and cultured at 30°C for 1 day and inhibition distance was determined. Underlined strains were isolated in 2nd screening test.

Table 2. Final screening by dilution method

strain		culture	Dilution per ml of medium (ml)							
			0.50	.25	.13	.06	.03	.02	.01	control
KM	29	22 hr	+	+	+	+	+	+	++	++
		47	+	+	+	++	++	++	++	++
	30	22	−	+	+	+	+	+	+	
		47	−	+	+	+	+	++	++	
	48	22	−	−	−	−	−	+	+	
		47	−	−	−	±	±	+	+	
	51	22	±	±	±	±	±	+	+	
		47	±	±	±	+	+	+	+	

Streptomyces strain KM-48 was screened with dilution method.
Bacillus amyloliquefaciens was inoculated in nutrient broth medium containing dilution series of cultured solution and cultured at 37°C for 47 hr.

growth: −; no growth, ±; poor, +; good, ++; excellent

chromatographed in avicel thin layer by developing with n-butanol:acetic acid:water = 4:1:2. The purified substance was shown a single spot in Rf value of 0.98. This spot emitted a blue-white fluorescence under ultraviolet irradiation Fig. 2.

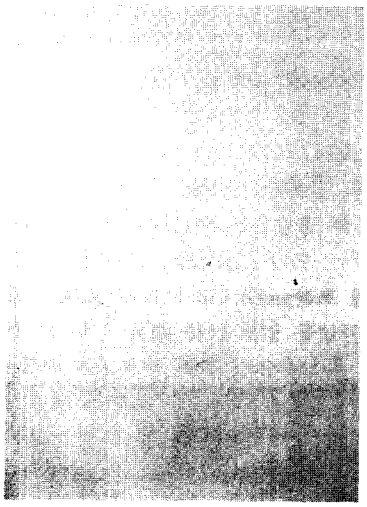

Fig. 2. Avicel thin layer chromatogram

The purified sample was developed in n-buthanol:acetic acid:water = 4:1:2 for 2 hr. Blue-green fluorescence was emitted under UV.

Antibiotic spectrum of the antibiotic

The inhibitory activity was tested by dilution method. This antibiotic was more sensitive to *B. amyloliquefaciens* than *E. coli*. The fifty percent inhibition occured in 13.5 ug/ml to *B. amyloliquefaciens* and 92.5 ug/ml to *E. coli*. Moreover, the fungi was more sensitive than bacteria. Fifty percent inhibitory concentration to *Candida albicans*, *Aspergillus niger* and *Rhizopus japonicus* were 6.5 ug/ml, 3.0 ug/ml and 9.0 ug/ml, respectively. So this antibiotic might react against the growth of *Bacillus* and fungi specifically (Fig. 3).

Temperature and pH stability

The KM-48 antibiotic was very stable at 40°C, because most of the activity remained after treating 4 hrs at above temperature. However, it was inactivated perfectly by 1 hr treatment at 70°C or 3 hrs at 60°C Fig. 4. The antibiotic was also treated in various pH from 3 to 10 for 9 hrs at 30°C with *McIlvaine*, *Clark* and *Lubs* and glycine-NaOH buffers. After treatment in each pH, the solution was nutralized with N-NaOH or N-HCl and the remaining activity was determined by checking the growth inhibition. Fig. 5 shows that this substance was stable in acid, while it was much lavile in neutral and alkaline pH.

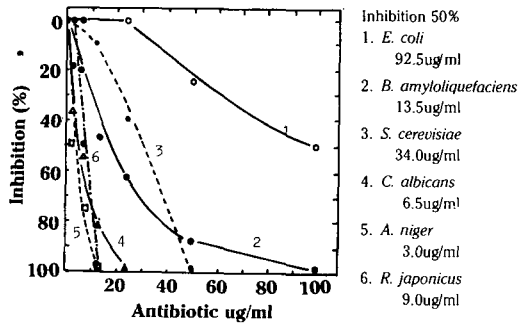

Fig. 3. Antibiotic inhibition to microorganism

The examined microorganisms were cultured in nutrient broth or yeast extract medium at 30°C for 15 hr. Each concentration of antibiotic from *Streptomyces* KM-48 was added to the medium in initial phase of the growth and cultured 24 hr more. The growth was determined in O.D. 550nm.

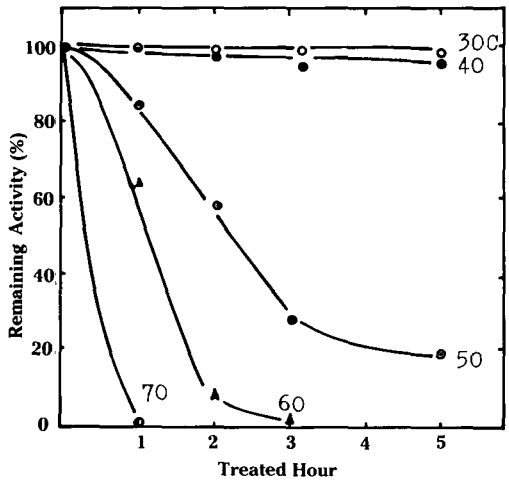

Fig. 4. Heat stability

The substance was treated at various temperature for a given time. After thermal treatment, *B. amyiloliquefaciens* was cultured in nutrient broth medium containing a treated substance at 30°C for 15 hr. Activity of the substance not treated was set at 100%.

Characterization of KM-48

The strain was incubated in various media at 30°C for 20 days, and the growth ability, aerial mass color and soluble pigment under various conditions were observed. This organism grew well in organic medium except Czapek's agar, glycerol agar and glucose agar. Sporephores was straight to fluxuous (Fig. 6). Surface of spores are smooth and spores are ellipsoidal. Acid production from glucose is negative. Growth type on glucose broth is pellicle type.

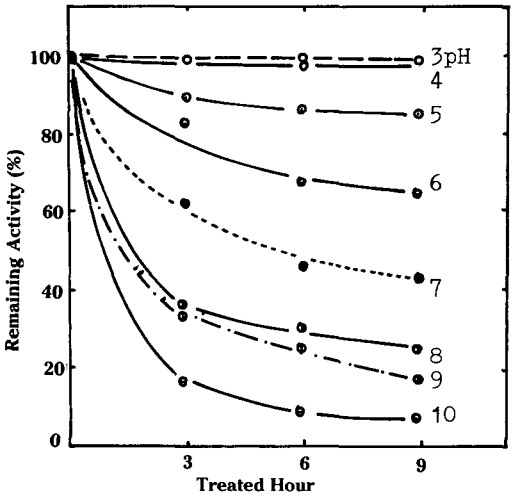

Fig. 5. pH stability

The substance was treated in the various pH range for 9 hr. at 37°C. After treatment, remaining activity was tested as the inhibition of *B. amyloliquefaciens*. Activity of the substance not treated was set at 100%.

Fig. 6. Aerial mycelia of *Streptomyces* sp. KM-48 (2,000X)

Aerial mycelium was usually colored with pale yellowish white to light yellowish brown, and soluble pigment was brown in liquid culture of organic medium (Table 3). Carbohydrate utilization was listed in Table 4. D-Glucose, D-fructose, D-galactose, D-mannitol and salicin was utilized somewhat well, whereas L-rhamnose and I-inisitol was not. The morphological characteristics were listed in Table 5. Aerial mass color was yellow and melanoid pigment in potato plug was produced. Gelatine was liquefied after 10 days culture at 30°C. Fifty ug/ml of streptomycin inhibited cell growth perfectly, and nitrate was reduced to nitrite. Sodium chloride tolerance was 5%, but 10%.

Among many species of *Streptomyces* described in *Bergey's manual of determinative bacteriology*, *Streptomyces tsusimaensis*[28] appears to be the most closely related one to this KM-48 strain in morphological property, color of aerial mycelium or synthetic agar and some kinds of biochemical characteristics. However, it differs from the growth in Czapeck's agar, glucose and glycerol agar, pigment formation in organic medium, aerial mass color, acid formation and carbohydrate utilization. Moreover, their antibiotic activity against *Escherichia coli* and fungi was different than that of *S. tsusimaensis*.[32]

Table 3. Cultured characteristics

media	growth	aerial mycelium	soluble pigment
Czapeck's agar	-		
potato dextrose agar	+ +	w y	d b
yeast-extract agar	+	w y	b
glucose asparagine agar	+	w y	d b
glucose peptone agar	+ +	w y	l b
peptone yeast-extract glucose agar	+ +	w y	d b
asparagine NB glucose agar	+ +	w	d b
glycerol potato agar	+ +	w y	d b
glycerol agar	-		
nutrient-broth potato agar	+ +		d b
glucose agar	-		
peptone agar	+	w y	l b

The KM-48 was cultured in various kinds of media at 30°C for 2 weeks.

growth: -; no growth ±; poor +; good + +; excellent
aerial mycelium: w; white y; yellow
soluble pigment: d; dark l; light b; brown

Discussion

From about 100 kinds of soil samples originating from the outskirts of Daegu city, one of *Streptomyces* species indexes KM-48 in our collection was isolated as an antibiotic producing strain against *Bacillus amyloliquefaciens*

Table 4. Comparison of two strains in carbohydrate utilization.

carbohydrate	S. tsusimaensis (32)	KM-48
D-glucose	+	+
D-mannose	+	+
D-fructose	+	+
D-mannitol	+	+
D-galactose	+	+
L-arabinose	+	+
sucrose	+	+
D-xylose	+	+
salicin	+	+
I-inositol	±	-
raffinose	±	+
L-rhamnose	±	-
inulin	-	-

Various kinds of carbohydrate was added to the basal medium of Pridham & Gottlieb, and cultured at 30°C for 15 days.

Abbreviations; +; good and fair growth, ±; faint growth -; no growth.

Table 5. Comparison of *Streptomyces* sp. KM-48 with *S. tsusimaensis* in morphological and biochemical characteristics.

character	*S. tsusimaensis* (32)	KM-48
Aerial mass color	yellowish grey	pale yellowish brown
Spore chain	straight flexuous	straight flexuous
Spore surface	ellipsoidal smooth	ellipsoidal smooth
Melanois pigment	positive	positive
Gelatine liquefaction*	positive (strong)	positive (weak)
Nitrate reduction	positive	positive
Acid formation from glucose-peptone**	positive	positive
Milk petonization	positive	positive
NaCl tolerance	—	5%, but, 10%
Streptomycin	—	sensitive
Starch hydrolysis	positive	positive

*: The strain was cultured in glucose-asparagine medium containing 20% gelatine at 23°C for 15 days.
**: cultured at 28°C for 10 days.

specifically which is a powerful strain of secreting extracellular hydrolases. The screened antibiotic did not inhibit the growth of *Escherichia coli* severely which did not secret extracellular enzymes like *B. amyloliquefaciens,* under the same concentration of antibiotic to *B. amyloliquefaciens.*

The inhibitory principle in the cultured broth of KM-48 was purified as a homogeneity with an avicel thin layer chromatography from non-adsorptive elute of aluminium oxide after acetone extraction from the lyophilized broth of NB culture. This antibiotic substance was emmited with blue-green fluorescence as a single spot under UV beam. The recovered ratio of antibiotic in the final step of purification was 36% from the activity of cultured filtrate of glucose-asparagine medium. This substance was labile to thermotreatment and alkaline pH, while it was stable in acid pH range.

While, in the course of testing for antimicrobial activities of KM-48, we have found that this antibiotic shows a specifically high activity against *Bacillus amyloliquefaciens:* Fifty percent inhibitory concentration was 13.5 ug/ml, and moreover, inhibits the hyphal growth of *Candida albicans* or *Aspergillus niger.*

The isolated strain, *Streptomyces* sp. KM-48, might be the similar strain to *Streptomyces tsusimaensis* on its cultural and morphological characteristics by the identification with *Berey's Manual of Determinative Bacteriology.* In the literature the *S. tsusimaensis* produces a valinomycin as an K^+ ionophore antibiotics.[29-31] But the antibiotic spectrum[32] had revealed different patterns from valinomycin on its specific inhibition against various bacterial strains and hyphal fungi. As a results, it might be a new antibiotic and might be useful as a specific inhibitor related with the extracellular enzyme secretion, because of its sensitivity to the cell layer of *Gram* positive bacteria.

要 約

細胞外 酵素를 分泌하는 ***Bacillus amyloliquefaciens***의 生育을 選澤的으로 阻害하는 抗生物質을 分離하고저 大邱 近郊의 土壤試料 100여점을 채취하여 ***Streptomyces*** sp. KM-48株를 最終 選別하였다. 이 菌株의 Nutrient Broth 培地에서의 培養沪液으로부터 活性成分을 acetone 抽出, Al_2O_3 chromatography, avical TLC를 하여 UV 照射下에서 靑綠色 형광을 나타내는 單

一物質로 精製하였다. 이 物質은 主로 그람양성균과 酵母, 곰팡이 등에 強한 抗菌効果를 나타내었으며 ***Bacillus amyloliquefaciens***에 對한 50% 生育阻害濃度가 18μg/ml이었다. 이 物質은 熱에 比較的 弱하였으며 酸에는 強하게 그 活性이 維持되었다. 菌株의 形態 및 培養性에서 ***Streptomyces tsusimaensis***의 類以菌으로 同定되었다.

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