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Production of Antibacterial Substances by *Pseudomonas glumae*

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Forty eight strains of ***Pseudomonas glumae*** were tested for the antibiosis against 7 species of phytopathogenic bacteria, *Agrobacterium* ***tumefaciens,*** *Clavibacter* ***michiganensis*** subsp. ***michiganensis,*** ***Xanthomonas*** *campestris* pv. *citri,* ***X.*** *campestris* pv. *oryzae, Erwinia* ***carotovora*** subsp. ***carotovora, P.*** *solanacearum,* ***P. syn'ngae*** pv. ***syn'ngae.*** The productivity was tested using the plate chloroform method. All strains of ***P. glumae*** used in this study produced antibacterial substances against, at least, one indicator. The exudate from the culture plate obtained through freezing and thawing showed antibiotics against phytopathogenic bacteria used as indicators. While, no production of the antibacterial substances was observed in the various liquid media. However in the agar-extract amended liquid medium, antibacterial substances were produced. These results indicated that water-soluble nutrients from agar will be necessary for the production of antibacterial substances. The antibacterial substances produced by *P. glumae* were dialyzable, heat labile and stable to trypsin, pronase, DNase, RNase treatments and UV irradiation.

INTRODUCTION

Antibiotics such as Blasticidin S (Yonehara et al., 1958), Kasugamycin (Umezawa et ***al.,*** 1966), Polyoxin (Isono et al., 1955), Validamycin (Iwasa et al., 1970) and Mildiomycin (Harada and Kishi, 1978) were originally obtained from Actinomycetes and has been practically used as agricultural chemicals. While, it has been well-documented that some plant pathogenic bacteria and saprophytic bacteria isolated from phylloplane or rhizosphere are known to produce antibiotics (Newhook, 1954; Leben, 1964; Chakravarti et al., 1972; Swinburne, 1973). Among these antibiotics, pyoluteorin, pyrrolnitrin and tropolone produced by some strains of pseudomonads (Howell et *al.*, 1979 and 1980) and agrocin 84 produced by *Agrobacterium radiobacter* ***84*** (Roberts et ***al.,*** 1977), were purified and characterized.

Hirayae and Wakimoto (1987) reported that the phytopathogenic bacteria ***Pseudomonas gladioli*** pv. ***gladioli E-14*** isolated from rice plants produced antibacterial substances effective against some plant pathogenic bacteria. Since ***P.*** *glumae* resembled to ***P. gladioli*** pv. ***gladioli*** in bacteriological properties and pathogenicity to rice seedlings and grains, the antibiosis of ***P. glumae*** was expected. In this experiment, the production of antibiotics by ***P. glumae*** was tested using indicators of several plant pathogenic bacteria which are widely distributed, causing serious plant diseases throughout the world.

MATERIALS AND METHODS

Bacterial strains

Forty eight strains of ***Pseudomonas ghmae*** Kurita et Tabei shown in Table 1 were used in this experiment. These strains were collected from various localities in Japan and preserved in the Laboratory of Plant Pathology, Kyushu University.

To detect the productivity of antibacterial substances of ***P. glumae,*** seven species or pathovars of plant pathogenic bacteria, which are distantly related to each other, were used as indicators (Table 2). These bacterial indicators were known to be distributed widely causing serious diseases in the world.

In order to preserve these bacteria, they were stabbed into PSA medium (potato semi-synthetic agar medium: potato (300 g) decoction 1 liter, 2 g $Na_2HPO_4 \cdot 12H_2O$, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 5 g peptone, 15 g sucrose, 15 g agar, pH 7.0) in test tubes and cultured at an optimum growth temperature for 2 to 3 days. The cultures were submerged with liquid paraffine sterilized previously by heating at 150°C for 1 h and were then kept at 27°C. The cultures were renewed once a year.

For long term preservation, the bacteria were lyophilized with PS broth and kept at 4°C.

Detection of productivity of antibacterial substances

To detect the productivity of antibacterial substances of *P. **ghmae,*** a fresh 1-2 days culture of each bacterial strain of ***P. ghmae*** on the PSA slant medium was transferred to the center (or to 3 spots on each plate separate from each other) of the plate of YPDA medium (yeast peptone dextrose agar medium: 3 g yeast extract, 0.6 g peptone, 3 g dextrose, 15 g agar, 1 liter of distilled water, pH 7.2) in a 9 cm Petri dish. The plate was then incubated at 30°C for 2 to 3 days. When the bacteria matured to form colonies of several mm in diameter, the plate was turned upside down. A chloroform (0.5 ml) was dropped on the filter paper placed in the lid, and the Petri dish was kept at room temperature for 2 h. After complete exclusion of chloroform vapor, the suspension of indicator bacteria (conc. ca. 10^8 cfu/ml, 5 ml) mixed with 0.5% plain agar medium (melted and kept at 50°C) was overlaid. The plate thus prepared was incubated at 30°C for 2 days. If an inhibition zone appeared, its width was measured to estimate the activity or productivity of antibacterial substances.

The productivity of antibacterial substances in various liquid media such as TTC medium (10 g peptone, 1 g casein hydrolysate, 5 g glucose, 0.05 mg triphenyl tetrazolium chloride, 1 liter of distilled water), YPD and PS was examined under shaking and still culture conditions. The culture fluids were collected periodically and sterilized by filtration through a Millipore filter (0.2 μm) and the activity was measured by paper disk method.

Preparation of the agar-extract amended liquid medium and the productivity of the antibacterial substances in this medium

To examine the effect of agar extract on antibiotic production, agar extract was prepared.

Thirty grams of agar powder (Katayama Chemical Co. Ltd) was suspended in 1,000 ml of distilled water, stirred on a magnetic stirrer overnight at 4°C and filtered

Table 1. Strains of ***Pseudomonas glumae*** used in this experiment.

Strain	Isolated from	Variety	Origin	Strain	Isolated from	Variety	Origin
N7401	Rice seedling	Unknown	NIAES"'	8028	Rice grain	Unknown	KNAES
N7501		〃	〃	1	〃	〃	〃
N7502	〃	〃	〃	111	〃	〃	〃
N7503	〃	〃	〃	2	〃	〃	〃
N7504	〃	〃	〃	Ku8101	〃	Koshihikari	KU[d]
N7505		〃	〃	Ku8102	〃	〃	〃
YN7805	Unknown	〃	〃	Ku8103	〃	〃	〃
YN7810	〃	〃	〃	Ku8104	〃	Hayahikari	〃
YN7825	〃	〃	〃	Ku8105	〃	〃	〃
742	〃	〃	〃	Ku8106		〃	〃
750	〃	〃	〃	Ku8111	〃	Unknown	〃
752	〃	〃	〃	Ku8112	〃	〃	〃
805		〃	〃	Ku8113	〃	〃	〃
806		〃	〃	Ku8114	〃	〃	〃
Pl-22-1	〃	〃	〃	Ku8115	〃	〃	〃
Pl-22-2	〃	〃	〃	Ku8116	〃	〃	〃
Pl-22-3		〃	〃	Ku8117	〃	〃	〃
Pl-22-4	〃	〃	〃	Ku8119	〃	〃	〃
So-l	Rice grain	〃	FARC[b]	Ku8120	〃	Reihou	〃
8001	〃	Asominori	KNAES[c]	Ku8121		〃	〃
8012	〃	〃	〃	Ku8122	〃	〃	〃
8015	〃	〃	〃	Ku8123	〃	Nishihomare	〃
8017	〃	Nagomasari	〃	Ku8124	〃	〃	〃
8020		〃	〃				

a) National Institute of Agro-Environmental Sciences.
b) Fukuoka Agricultural Research Center.
c) Kyushu National Agricultural Experiment Station,
d) Kyushu University.

Table 2. Phytopathogenic bacteria used as an indicator for testing productivity of antibacterial substances.

Indicator bacteria	Abbreviation	Origin")
Agrobacterium tumefaciens Ku7411	A.t	KU
Clavibacter michiganensis subsp. ***michiganensis*** N6601SR(3)[b]	C.m	NIAES
Erwinia cartovora subsp. ***carotovora*** N7129	E.c	〃
Pseudomonas *solanacearum* C319	P.sol	KTES
l? *syringae* pv. *syringae* I	P.syr	NIAES
Xanthomonas *campestris* pv. *citri* N6113-1	X.c	〃
X. *campestris* pv. *oryzae* T7174SR(12)[b]	x.0	HAES

a) KU: Laboratory of Plant Pathology, Kyushu University.
NIAES: National Institute of Agro-Environmental Sciences.
KTES: Kagoshima Tobacco Experimental Station.
HAES: Hokuriku Agiricultural Experiment Station.
b) Streptomycin resistant mutant induced at KU.

through filter paper. Agar-extract amended liquid medium was prepared by adding 10 g of peptone, 5 g of D-glucose, 1 g of casein hydrolysate in 1 liter of the agar-extract fluid and autoclaved. Bacterial strain 750 of *P. glumae* was inoculated (conc. ca. 10^7cfu/ml) into the agar-extract amended liquid medium and incubated at 30°C under still conditions. A time-course observation on the bacterial growth, antibacterial production and alteration of pH value was conducted.

Siderophore production

To examine if the growth inhibition of the indicators are due to the competition for Fe^{3+}, test for antagonistic activity were carried out on KB plates (20.0 g proteose peptone # 3 (Difco), 2.5 g $K_2HPO_4 \cdot 3H_2O$, 6.0 g $MgSO_4 \cdot 7H_2O$, 15.0 g agar, 15 ml glycerol, 1 liter of distilled water) supplemented with $FeCl_3$ so as to be 50 or 100 μmol Fe^{3+}/l (18 μmol : 1 mg/l). Growth inhibition of indicator bacteria was estimated after incubation for 2 days at 30°C, based on the width of clear inhibition zones.

Tolerance of antibacterial substances to various treatments

For heat treatment, 2 ml sample of antibacterial substances was put into a test tube and incubated at 60, 70 and 100°C for 10 min in a water bath, respectively. Autoclave-treatment (120°C for 10 min) was also conducted. For ultraviolet (UV) irradiation, 5 ml sample of antibacterial substances was placed in a Petri dish (9 cm in diameter) and irradiated by UV lamp (Toshiba GL-15) from a distance of 30 cm for 60 min stirring on a magnetic stirrer. For enzyme treatment, a sample of antibacterial substances was mixed with one of the following enzymes: Pronase (100 μg/ml + 0.01 M $CaCl_2$), Trypsin (100 μg/ml), RNase (100 μg/ml), and DNase (20 μg/ml + 10 mM $MgSO_4$) and incubated at 30°C for 3 h with shaking. After chilling, antibacterial activity was measured by paper disk method.

RESULTS

Production of antibacterial substances by *P. glumae* on YPDA medium

The majority of the strains of ***P. glumae*** formed inhibition zones on some indicator bacteria as shown in Table 3, suggesting the productivity of an antibacterial substance(s). The activity spectra, however, varied greatly depending on strains of ***P. glumae.*** Out of **47** strains of ***P. glumae,*** **7** formed inhibition zones on the lawn of *Agrobacterium* ***tumefaciens,*** **22** on *Clavibacter* ***michiganensis*** subsp. ***michiganensis,*** 19 on *Erwinia* ***carotovora*** subsp. ***carotovora,*** and 26 on *P. solanacearum,* ***28*** on *Xanthomonas campestris* pv. *citri,* and 27 on X. *campestris* pv. *oryzae.*

Antibiosis on various media against *P. solanacearum*

The antibacterial activity differed depending on the kind of media used (Table 4). All strains of *P.* ***glumae*** used in this study, however, showed antibiotic activity toward ***P. solanacearum*** C319 on TTC medium.

Production of antibacterial substances by *P. glumae* in liquid media

The culture filtrates of YPD, PS and TTC liquid media did not show any antibacterial activity (Table 5). However, in the case of agar-extract amended broth,

Table 3. Production of antibacterial substances by ***Pseudomonas glumae.***

Strain	Indicator[a]						
	A.t	C.m	E.c	P.sol	P.syr	x.c	x.0
N7401	−[b]			−	++		
N7501	−		+++	+++	+	+++	+++
N7502		+		−	++	−	−
N7503	−	+		++	+	(+)	(+)
N7504	−		−		+	−	
N7505		+	−		++		+
YN7805	−			−	++	−	
YN7810	−		−		++		−
YN7825	−			−	+		
742		−	+++	+++	+	+++	+++
750	−	+++	+++	+++	(+)	+++	+++
752		++	+++	+++	(+)	+++	+++
805		+++	+++	+++	+	+++	+++
806		+		+	+	++	++
Pl-22-1		+++	+++	+++	+	+++	+++
P1-22-2	−		−		(+)		(+)
Pl-22-3		+	(+)	+++	(+)	+++	+++
P1-22-4		−	(+)	(+)	+		+
So-l			+++	+++	+	+++	+++
8001	−	+++	+++	+++	+	+++	+++
8012		−			+		(+)
8015		+		−	+		+
8017	++	+	++	+++	+	+++	+++
8020	++	+	++	+++	+	+++	+++
8028	−	+	−		+		−
I	++		+	+++	+	+++	++
III	++		++	+++	+	+++	+++
2	(+)		++	+++	+	+++	+++
Ku8101	−	−		−	+	−	−
Ku8102	−	+		−	+	−	−
Ku8103			−	−	++	−	
Ku8104		+		+++	++	++	++
Ku8105		+	−		++	++	++
Ku8111		−	−		+		−
Ku8112		++	+++	−	+	+++	(+)
Ku8113		+++	+++	−	(+)	+++	+++
Ku8114			−	++	(+)	+	+
Ku8115	−		++	+++	+	+++	++
Ku8116	−			++	+		−
Ku8117		−		+	+	+	+++
Ku8119		+	−	+++	++	+++	+++
Ku8120		+		+++	++	+++	+++
Ku8121		+++	+++	+++	++	+	+++
Ku8122	+	−		++	+	+	+++
Ku8123	++		++	−	+	++	−
Ku8124	++	−	++	++	+	++	

a) See Table 2.
b) Strain of P. glumae was spot-inoculated on YPDA plate and cultured for 72 h at 30°C. The plate was treated with chloroform vapor, overlaid with indicator bacteria and incubated at 30°C for 24-48 h.

Activity index : − not detected, (+) doubtful, + below 5 mm, ++ 5-10 mm, +++ above 10 mm of the width of inhibition zone.

Table 4. The formation of anti-P. *solanacearum* substance(s) on various media.

Strain	Media			Strain	Media		
	Y PDA"'	PSA[b]	TTC[c]		YPDA	PSA	TTC
N7401	−[d]		+	8028	−	−	+
N7501	+++	−			+++	++	+
N7502		+	+++	111	+++	++	+++
N7503	++		++	2	+++	+	++
N7504	−		++	Ku8101	−	+	++
N7505	−	++	+++	Ku8102		−	+
YN7805			++	Ku8103		−	++
YN7810	−	++	++	Ku8104	++		
YN7825		+	++	Ku8105		++	++
742	+++		+++	Ku8106	+++	+	++
750	+++		+++	Ku8111	−	+	+++
752	+++	++	+++	Ku8112		+++	+
805	+++	+++	+++	Ku8113	−	+	+++
806	+	++	++	Ku8114	++	++	++
Pl-22-1	+++		+++	Ku8115	+++		+
Pl-22-2	−		++	Ku8116	++	++	+
P1-22-3	+++		+	Ku8117	+	−	
P1-22-4	+		+	Ku8119	+++	−	
So-l	+++	−	++	Ku8120	+++		+++
8001	+++		+	Ku8121	+++	+++	+++
8012	−	+	+	Ku8122	++	++	++
8015	−		+	Ku8123	−	+	++
8017	+++	−	+	Ku8124	++	+	++
8020	+++	−	++				

a) YPDA: yeast peptone dextrose agar medium.
b) PSA: potato semi-synthetic agar medium.
c) TTC: tetrazolium chloride medium.
d) Width of inhibition zone : − not detected, + below 5 mm, + + 5-10 mm, + + + above 10 mm. ***P.*** *solanacearum* strain C319 was used as an indicator.

Table 5. Antibacterial spectra of the substances produced by ***Pseudomonas*** *glumae* strain 805.

Indicator code[b]	Activity"'		
	shown by the plate-chloroform method[c]	of the exudate from agar medium[d]	of the culture filtrate"'
A.t	−	−	−
C.m	++	+	
E.c	++	+	−
P.sol	++	+	
P.syr	+	±	−
x.c	++	+	−
x.0	++	+	−

a) - not detected, ± faint activity, + below 5 mm, + + above 20 mm of the width of inhibition zone.
b) See the Table 2.
c) The bacterium was spot inoculated on YPDA plate and cultured for 48 h at 30°C, treated with chloroform vapor, and overlaid with indicator bacteria.
d) YPDA medium on which the producer was grown for 48 h at 30°C was frozen at −20°C, and thawed at room_ temperature to obtain exudate.
e) Cultured in various liquid media (YPD, PS, TTC) at 30°C.

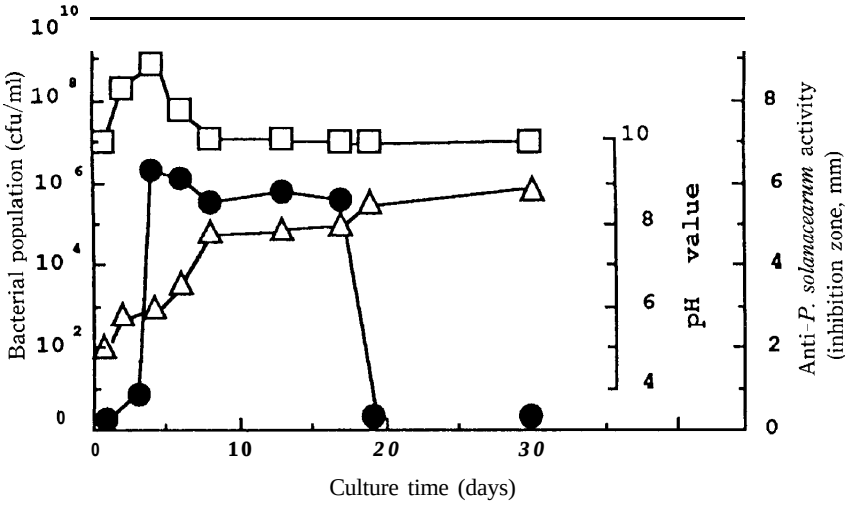

Fig. 1. Production of anti-bacterial substances by ***Pseudomonas glumae 750*** in agar-extract amended liquid medium.
□: Growth ●: Activity △: pH value

Table 6. Extraction of antibacterial substances with organic solvents.

Solvent	pH	Activity[a] of	
		solvent layer	water layer
n-Butanol	3.0	−	+
	7.0	−	+
	12.0		+
Chloroform	3.0		+
	7.0		+
	12.0	−	+
Diethyl ether	3.0	−	+
	7.0	−	+
	12.0		+
Ethyl acetate	3.0	−	
	7.0		
	12.0	−	+
Petroleum ether	3.0		+
	7.0	−	+
	12.0	−	

a) Discs dipped in each solvent were dried completely and placed on the lawn of ***P. solanacearum***. Activity index; -not detected, +inhibition zone was formed.

the culture filtrate at 2 to 19 days of incubation under still culture conditions showed antibacterial activity (Fig. 1). When the bacterial population reached 7 $\times 10^8$ cfu/ml in the liquid medium after 4 days incubation, the antibiotic activity of the culture filtrate peaked and was maintained for 16 days.

Physical and chemical characteristics of anti-P. *solanacearum* substances

The antibiotic substances were dialyzable and not transferrable to non-polar organic solvents such as chloroform, petroleum ether, diethyl ether, ethyl acetate and *n*-butanol at any pH value (Table 6). These substances were tolerant to UV-irradiation, pronase, protease, trypsin, DNase and RNase, and antibiotic activity declined after heat treatment at 120°C for 20 min or at 100°C for 10 min (Table 7).

Siderophore production

Regardless of the addition of Fe, antibacterial activity of *P. glumae* was not varied (Table 8).

Table 7. Effect of various treatments on the activity of antibacterial substances produced by ***Pseudomonas glumae.***

Treatment			Activity"'
Enzyme[b)]	DNaae	(50 μg/ml)	+[e)]
	RNase	(50 μg/ml)	+
	Pronase	(100μg/ml)	+
	Trypsin	(100μg/ml)	+
UV irradiation"		60 min	+
Dialysis[d)]			+
Heat	60°C	10 min	+
	70°C	10 min	+
	100°C	10 min	−
	120°C	10 min	
Untreated control			+

a) ***P.*** *solanacearum* C319 was used as an indicator.
b) Treated for 60 min at the optimum temperature and pH.
c) Irradiated by Toshiba GL-15 lamp from 30 cm height.
d) Treated overnight.
e) −: not detected, +: activity.

Table 8. Effect of Fe^{3+} addition to King'B medium on the antibacterial activity.

Pseudomonas glumae	Amount of Fe^{3+} added (μmol/l)		
	0	***50***	***150***
750	+[a)]	+	+
805	+	+	+
N7504	+	+	+
Ku8112	+	+	+
Ku8121	+	+	+

a) +: Inhibition zone was formed.

DISCUSSION

Plant pathogenic ***Pseudomonas*** *glumae* strains used in this experiment formed growth inhibition zones on the lawn of various plant pathogenic bacteria used as indicators, suggesting the productivity of some antibacterial substances.

As causal agents for the formation of growth inhibition zones, antibiotics (Fravel, 1988), bacteriocins (Vidaver, 1976), bacteriophages (Okabe and Goto 1963) and bdellovibrios (Stolp, 1963) are well-known. In addition to these agents, siderophores (Leong, 1986) were also reported as an agent forming inhibition zones on some specific bacteria grown on the Fe^{3+} unsupplemented medium.

However, the antibacterial activity shown by *P. glumae* are considered to be neither bacteriocins nor bacteriophages, because the antibacterial substances did not form inhibition zone on the lawn of ***P.*** *glumae* strains and formed it on the indicator bacteria distantly related to *P.* glumae. Since the inhibition zones appeared regardless the Fe^{3+} concentration in the medium, siderophores could not concern with the growth inhibition.

In general, *P. glumae* used in this experiment did not produce the antibacterial substances in liquid media. While, the production was observed when water-extract from agar was amended. Some nutrients from agar will participate in the productivity of antibacterial substances by *P.* ***glumae.***

The antibacterial spectrum of *P.* ***glumae*** and productivity differed greatly depending upon strains, media used, and culture conditions, suggesting that strains of *P.* ***glumae*** could produce several kinds of antibacterial substance.

The isolation and identification of these substances will be the subjects of the future study.

The ability of the antibiosis of ***P. glumae*** had no correlation with the pathogenicity to rice plants and with the number and kinds of plasmids found in each strain (Kamiunten et ***al.*** 1985).

The non-pathogenic strains of ***P. glumae*** which have the productivity of the antibacterial substances will be useful for the biocontrol measure.

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