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Effect of Specific Ions in Agar on Antibiotic Production by *Burkholderia glumae*

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Burkholderia glumae produced antibiotic substance(s) on agar plate media, but none in various liquid media. Some minor elements in agar were considered to be involved in the antibiotic production. K^+, Ca^{2+}, Mg^{2+} and NH_4^+ were shown to play significant roles in the production by culturing the bacterium in liquid media supplemented with various inorganic chemicals. Highest production was observed when *B. glumae* was cultured in the liquid medium containing a mixture of $MgCl_2$, KCl and $CaCl_2$. No antibiotic production was observed in PGC broth containing $MgSO_4$which was substituted for $MgCl_2$ or $Mg(NO_3)_2$. These results suggest that specific cations and anions are involved in antibiotic(s) production by *B. glumae*.

INTRODUCTION

In previous papers (Wakimoto *et al.*, 1986; Furuya *et al.*, 1992a), it was shown that *Burkholderia glumae* has abilities to produce antibiotic substance(s) against various phytopathogenic bacteria including *Ralstonia solanacearum* and to suppress bacterial wilt of tomato under controlled conditions (Furuya *et al.*, 1991; 1992b). Attempts to obtain a large amount of the antibiotic substance failed because it was not produced in liquid media. Consequently, elements in agar were considered to be important factors in production of the antibiotic substance(s) (Yamasaki *et al.*, 1993; 1994). In this paper, qualitative and quantitative effects of some elements (cations and anions) on the antibiotic production were described.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study were derived from the culture collection of the Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University. Seven strains of *B. glumae* were used as the producers of the antibiotics. *R. solanacearum* C319 was used as an indicator bacterium for the antibiotic substance(s).

Detection of antibiotic production

B. glumae was cultured on YPDA (10 g of yeast extract, 10 g of peptone, 20 g of D-glucose, 15 g of agar, 1 liter of water, pH 7.0), PSA (2 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of $Ca(NO_3)_2 \cdot 4H_2O$, 5 g of sucrose, 15 g of agar, 1 liter of potato (300 g) decoction, pH 7.0)

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and TTC (10g of peptone, 5g of D-glucose, 1g of casein, 18g of agar, 50 ppm of 2,3,5-triphenyltetrazorium chloride, 1 liter of water) media at 30°C for 72 hr. After the bacteria were cultured on the media, the agar plates were frozen at -20°C for longer than 2 hr and thawed at room temperature for 2hr. By these treatments, exudate was obtained from the culture plates. The exudate was sterilized by filtration through a Millipore filter (0.2μm) and antibiotic activity was estimated by the penicillin-cup method.

To investigate the production of antibiotic substance in liquid media, *B. glumae* was cultured in YPD, PS and TTC liquid media at 30°C for 6 days under still conditions. The cultured fluid was sterilized by filtration as described above and the presence of antibiotic was examined by the penicillin-cup method.

Production of antibiotic substance(s) in the agar-extract amended (AePGC) broth

AePGC broth (10g of peptone, 5g of D-glucose, 1g of casein, 1 liter of agar-extract solution) was used to investigate the effect of agar-extract on the antibiotic production by *B. glumae*. Agar-extract solution was prepared by suspending a certain grams of agar powder (Katayama Chemical Co. Ltd.) in 1 liter of distilled water. Agar suspension was stirred overnight at 5°C, and filtered through a filter paper. Concentration of agar-extract solution was expressed as the amount (gram) of agar powder which was added to 1 liter of distilled water. *B. glumae* was cultured in AePGC broth at 30°C for 6 days under still conditions. Antibiotic production in the broth was examined by the penicillin-cup method.

Effects of minor elements in agar on antibiotic production

Since 30g of agar powder was calculated to contain NaCl (3.2×10^{0}mM), KCl (6.6×10^{-2}mM), $CaCl_2$ (2.8×10^{-1}mM), $MgCl_2$ (4.4×10^{-1}), NH_4Cl (6.2×10^{-2}mM) and Na_3PO_4 (1.1×10^{-1}mM) according to Uzuka (1992), a liquid medium, PGC broth (10g of peptone, 5g of D-glucose, 1g of casein, 1 liter of distilled water), was amended with each or mixtures of the inorganic chemicals so that the medium contained the same concentration of the respective elements. *B. glumae* was cultured in the amended PGC broth at 30°C for 6 days under still conditions. The effect of elements on the antibiotic production was estimated by the penicillin-cup method.

RESULTS

Antibiotic production by *B. glumae* in various liquid media

The exudate from the culture plates showed anti-*R. solanacearum* activity, but the culture filtrates of YPD, PS and TTC liquid media did not show any anti-*R. solanacearum* activity (Table 1). As shown in Table 2, the antibiotic production was observed in AePGC liquid medium which contained the agar-extract from 5 g agar powder.

Effect of various elements in agar on antibiotic production by *B. glumae*

As can be seen in Table 3, the antibiotic production was observed in PGC broth supplemented with KCl, $CaCl_2$, $MgCl_2$ or NH_4Cl. Highest antibiotic production was recorded in PGC broth containing $MgCl_2$. However, the degree of production was lower than that in AePGC medium. A test was performed to examine cumulative effect of two or more kinds of elements on the production. The cumulative effect of cation on the

Table 1. Production of antibiotic substance(s) by *Burkholderia glumae*

B. glumae strain	Anti-*Ralstonia solanacearum* activity[a]		
	shown by plate-chloroform method[b]	of the exudate[c] from agar medium	of the culture filtrate[d]
N7401	–	–	–
N7503	+	±	–
805	+++	+	–
N750	+++	+	–
Ku8113	+	±	–
Ku8117	–	–	–
Ku8121	+++	+	–

a) Activity index (semidiameter of inhibition zone): –, not detected; ±, faint activity; +, less than 5 mm; +++, greater than 10 mm.
b) *B. glumae* was spot-inoculated on YPDA, PSA and TTC plates and cultured at 30 °C for 3 days. Aftert treatment with chloroform vapor, an indicator bacterium, *R. solanacearum*, was overlaid.
c) YPDA, PSA and TTC media on which *B. glumae* was cultured at 30 °C for 3 days were frozen at −20 °C for above 2 hr and thawed at room temperature to obtain the exudate.
d) YPD, PS and TTC liquid media in which *B. glumae* was cultured at 30 °C for 6 days were filtered through Millipore filter (0.2 μm).

Table 2. Effect of agar-extract on the antibiotic production by *Burkholderia glumae*

B. glumae strain	Width of inhibition zone[a]					
	Concentration of agar-extract[b] (g of agar/l of water)					
	0.0	1	5	15	30	50
N7401	–	NT	NT	NT	NT	–
N7503	–	NT	NT	NT	NT	+
805	–	–	+++	+++	NT	NT
N750	–	–	+++	+++	+++	+++
Ku8113	–	NT	NT	NT	NT	+
Ku8117	–	NT	NT	NT	NT	–
Ku8121	–	–	+++	+++	NT	NT

a) Activity index (semidameter of inhibition zone): –, not detected; +, less than 5 mm; +++, greater than 10 mm; NT, not tested. *Ralstonia solanacearum* C319 was used as an indicator.
b) Concentration of agar-extract was expressed as the amount (gram) of agar powder which was added in 1 liter of distilled water.

production was remarkable when the mixture of $MgCl_2$, KCl and $CaCl_2$ was added to PGC broth (Table 4). As shown in Table 5, the production was observed in PGC broth containing $MgCl_2$ or $Mg(NO_3)_2$, but not $MgSO_4$.

Table 3. Effect of various cations on the antibiotic production by *Burkholderia glumae* N750[a]

Cation				
R^{+b}	R^{2+c}	R^{3+d}	Activity[e]	pH
Na			0.0 h[f]	7.22 m
K			1.3 j	6.69 k
NH_4			0.9 hj	6.87 j
	Mg		3.7 k	6.27 m
	Ca		0.3 h	6.81 j
		P	0.0 h	7.29 h
Na, K, NH_4	Mg, Ca	P	5.4 m	6.33 m
	Cont. (AePGC)		5.6 m	6.24 m

a) Cultured at 30 °C for 6 days. b) Monovalent cation. c) Divalent cation. d) Trivalent cation. e) Semidiameter of inhibition zone (mm). *Ralstonia solanacearum* C319 was used as an indicator. f) Values within each column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 4. Cumulative effects of various cations on the antibiotic production by *Burkholderia glumae* N750[a]

Cation				
R^{+b}	R^{2+c}	R^{3+d}	Activity[e]	pH
K, NH_4	Mg, Ca		6.5 h[f]	6.08 h
K, NH_4	Mg		5.4 hj	6.25 jk
K	Mg, Ca		7.0 h	6.10 h
	Mg, Ca		4.4 j	6.21 j
K	Mg		5.5 hj	6.27 jk
NH_4	Mg		1.6 k	6.31 k
K, NH_4			0.0 m	6.65 m
K	Ca		0.0 m	6.62 m
Na, K, NH_4	Mg, Ca	P	5.4 j	6.33 k
	Cont. (AePGC)		5.6 j	6.24 jk

a) Cultured at 30 °C for 6 days. b) Monovalent cation. c) Divalent cation. d) Trivalent cation. e) Semidiameter of inhibition zone (mm). *Ralstonia solanacearum* C319 was used as an indicator. f) Values within each column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 5. Effcet of various anions on the antibiotic production by *Burkholderia glumae* N750[a]

Anion			
R^{-}[b]	R^{2-}[c]	Activity[d]	pH
$MgCl_2$		3.7 h[e]	6.27 h
$Mg(NO_3)_2$		3.9 h	6.21 h
	$MgSO_4$	0.0 j	6.35 h

a) Cultured at 30 °C for 6 days. b) Monovalent anion. c) Divalent anion. d) Semidiameter of inhibition zone (mm). *Ralstonia solanacearum* C319 was used as an indicator. e) Values within each column followed by the same letter are not significantly different (P =0.05) according to Duncan's multiple range test.

DISCUSSION

Antibiotic production by *B. glumae* was observed in solid agar media, but not in liquid media. This phenomenon indicated an important role of element(s) in agar for the production (Furuya *et al*, 1992a). Since the existence of six ions (Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+} and P^{3+}) in agar was formerly documented (Uzuka, 1992), the effect of these minor elements on the production was examined. Among these cations tested, K^+, NH_4^+, Mg^{2+} and Ca^{2+} were revealed to participate in the antibiotic production. The effect of Mg^{2+} was particularly prominent. Moreover, highest production was recorded when the bacterium was cultured in PGC liquid medium containing the mixture of $MgCl_2$, KCl and $CaCl_2$. Thus, in liquid culture conditions, the presence of specific cations was necessary for the production of the antibacterial substance(s). The antibiotic production was observed in PGC broth containing $MgCl_2$ or $Mg(NO_3)_2$, but not in the broth supplemented with $MgSO_4$, suggesting that anions also play an important role(s) for the production.

Like the case of *B. glumae*, Mg^{2+} was shown to affect magnesidin production by *P. magnesiorubra* nov. sp. ATCC21856 (Gandhi *et al.*, 1973) and *Vibrio gazogenes* ATCC29988 (Imamura *et al.*, 1994). The effect of minor element, ion, has also been reported in pyoveridine production by *P. fluorescens* (Loper and Lindow, 1994).

The mechanisms of the promotive effects on antibiotic production by amendment of the minor nutrients in agar have not been elucidated in the present study. This will be an important subject for future study.

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