Antibacterial Activities of Metabolites Produced by *Erwinia* spp. against Various Phytopathogenic Bacteria

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The production of antibiotics by *Actinomycetes* species^{6,17,19} and the antagonistic activity of bacteria against plant pathogens^{4,5,10,18} have both been well documented. Previously, we also reported the production of anti-bacterial and -fungal substances by phytopathogenic pseudomonads and xanthomonads^{2,3,11-16,20}. In addition, the authors recently found *Erwinia* spp. which were active against various species of phytopathogenic bacteria.

Eighty strains of *Erwinia* spp. isolated in Japan and Thailand were screened for production of substances effective against seven species plant pathogenic bacteria. All strains were maintained on PSA medium (potato semi-synthetic agar medium; 5 g peptone, 15 g sucrose, 2 g Na₂HPO₄·12H₂O, 0.5 g Ca(NO₃)₂·4H₂O and 15 g agar/l of potato (300 g) decoction, pH 7.0).

The production of antibacterial substances was detected by the plate-chloroform method¹⁾. Each strain of *Erwinia* spp. was spotted on the surface of YPDA medium (yeast peptone dextrose agar medium; 0.6 g peptone, 3 g glucose, 3 g yeast extract and 15 g agar/l, pH 7.2) in a petri dish, incubated at 30°C for 48 hr and exposed to chloroform vapor for 2 hr. The plate was overlaid with 5 ml of melted YPDA medium containing 0.5% (w/v) agar and ca. 10⁷ cfu/ml of the indicator bacterium. Antibacterial activity was estimated by measuring the diameter of the inhibition zone around the colony of the producer after incubation at 30°C for 24-48 hr.

As shown in Table 1, thirty eight strains of *Erwinia* spp. which included *E. carotovora* subsp. *carotovora*, *E. chrysanthemi* pv. *chrysanthemi*, *E. chrysanthemi* pv. *zeae*, *E. chrysanthemi* (pathovar unidentified) showed antibacterial activity against at least one phytopathogenic bacterium used as an indicator. The activity spectra differed greatly among Thai and Japanese strains of *Erwinia* spp. Several strains of *E. carotovora* subsp. *carotovora* obtained in Thailand strongly inhibited the growth of *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas solanacearum*. None of the Thai and Japanese strains of *Erwinia* spp. showed any antibacterial activity against *P. cepacia* or *X. campestris* pv. *citri*.

The production of anti-*P. solanacearum* substance(s) by Thai strains of *E. carotovora* subsp. *carotovora* strains was tested by the plate-chloroform method on Kado's 523, MKA, KBA, MMA, PSA and PA in addition to YPDA. The ingredients of the media stated above are as follows: Kado's 523 medium (10 g sucrose, 8 g casein enzymatic hydrolysate, 4 g yeast extract, 2 g K₂HPO₄, 0.3 g MgSO₄·7H₂O and 15 g agar/l)⁸⁾; modified Kelman's agar (MKA) medium (10 g peptone, 5 g glucose, 1 g casein enzymatic hydrolysate and 15 g agar/l)⁹⁾; King's B agar (KBA) medium (20 g proteose peptone No. 3 (Difco), 1.5 g K₂HPO₄, 1.5 g MgSO₄·7H₂O, 10 ml glycerol and 15 g agar/l); Minimal agar medium (MMA) (0.1 g MgSO₄·7H₂O, 1 g (NH₄)₂SO₄, 3 g K₂HPO₄, 3 g KH₂PO₄, 5 g glucose and 15 g agar/l) and PA (15 g agar per liter of potato (300 g) decoction, pH 7.0).

The production of anti-*P. solanacearum* substance(s) by Thai strains of *E. carotovora* subsp. *carotovora* varied with the strains and media used. As can be seen in Table 2, all Thai strains strongly inhibited *P. solanacearum* on PSA and PA medium, whereas none did so on MMA medium. The majority of Thai strains of *E. carotovora* subsp. *carotovora* were active against *P. solanacearum* but all Japanese strains of the subspecies were negative. To elucidate the reason for the difference in antibacterial substance(s) production between Thai and Japanese strains of *E. carotovora* subsp. *carotovora*, data for other strains from different geographic regions must be accumulated. The activity of Japanese strains of *Erwinia* spp. against Thai indicator bacteria will be the subject for future study.

The production of anti-*P. solanacearum* substance(s) by Thai strains of *E. carotovora* subsp. *carotovora* in liquid media was also tested. In Kado's 523, MK, KB, MM, PS, P and YPD liquid media, the bacteria were

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Table 1. Antibacterial activity of *Erwinia* spp. from Thailand and Japan against phytopathogenic indicator bacteria on yeast potato dextrose agar

Producer	Width of inhibition zone ^{a)}							Source	
	Indicator ^{b)}							Geographic	Donor ^{c)}
	A.t.	C.m.	P.c.	P.sol.	P.syr.	X.c.	X.o.		
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>									
014-2	++	-	-	-	-	-	-	Thailand	LSPPM
131-1	-	+	-	+	+	-	-	"	"
131-2	-	-	-	-	-	-	+	"	"
168-7	-	+	-	+	-	-	-	"	"
435-2	-	+++	-	+++	-	-	++	"	"
435-6	-	+++	-	+++	-	-	+++	"	"
463-1	-	++	-	-	-	-	-	"	"
473-1	++	-	-	++	-	-	-	"	"
486-5	-	+	-	+	-	-	-	"	"
489-5	-	+++	-	+++	-	-	+++	"	"
493-1	-	+++	-	+++	-	-	+++	"	"
493-5	-	-	-	+	-	-	-	"	"
N7101	-	+	-	-	-	-	-	Japan	NIAES
N7128	-	+	-	-	-	-	-	"	"
N7129	-	+	-	-	-	-	-	"	"
N7157	-	+	-	-	-	-	-	"	"
Ku7514	-	+	-	-	-	-	-	"	KU
Sr79-33-3	-	-	-	-	-	-	+	"	HPAES
S8488	-	-	-	-	-	-	+	"	"
S84812	-	-	-	-	-	-	+	"	"
645ar	-	-	-	-	-	-	++	"	IU
ar13	-	-	-	-	+	-	-	"	"
K1	-	+	-	-	-	-	-	"	KU
B1	-	+	-	-	-	-	-	"	"
1B	-	+	-	-	-	-	-	"	YU
T14	-	-	-	-	-	-	+	"	IU
T21-1	-	-	-	-	-	-	++	"	"
2	-	-	-	-	-	-	++	"	"
<i>E. chrysanthemi</i> pv. <i>chrysanthemi</i>									
E8301	-	+	-	+	-	-	-	"	SZU
Ku7601	-	+	-	+	-	-	++	"	KU
<i>E. chrysanthemi</i> pv. <i>zeae</i>									
R7	-	+	-	-	-	-	+	"	SZU
R8	-	+	-	-	-	-	+	"	"
ALE829p	-	+	-	+	-	-	-	"	"
511-3	-	++	-	-	-	-	-	Thailand	LSPPM
(pathovar unidentified)									
342-15	-	+	-	+	-	-	++	"	"
342-16S	-	+	-	+	-	-	+	"	"
Ech33	-	+	-	-	-	-	+	Japan	KU
Ech44	-	+	-	-	-	-	+	"	"

a) Activity index (diameter of inhibition zone): -, not detected; +, less than 5 mm; ++, 5~10 mm; +++, greater than 10 mm.

b) Abbreviations for indicator bacteria: A.t., *Agrobacterium tumefaciens* Ku7411; C.m., *Clavibacter michiganensis* subsp. *michiganensis* N6203; P.c., *Pseudomonas cepacia* 356-2; P.sol., *P. solanacearum* C319; P. syr., *P. syringae* pv. *syringae* 1; X.c., *Xanthomonas campestris* pv. *citri* Ku7517; X.o., *X. oryzae* pv. *oryzae* Q7463.

c) Abbreviations for culture collections: LSPPM, Laboratory of Seed and Post-Harvest Disease, Plant Pathology and Microbiology Division, Department of Agriculture, Thailand; NIAES, National Institute of Agro-Environmental Sciences; KU, Kyushu University; HPAES, Hokkaido Prefectural Agricultural Experiment Station; IU, Iwate University; YU, Yamagata University; SZU, Shizuoka University.

Fig. 1. Growth of *Erwinia carotovora* subsp. *carotovora* 493-1 under shake culture conditions in PS liquid medium, pH of its culture filtrate and activity against *Pseudomonas solanacearum* C319 over time. ▲, Bacterial population; ■, pH of culture filtrate; ●, Anti-*Pseudomonas solanacearum* C319 activity as measured by the paper disk method.

Table 2. Anti-*P. solanacearum* activity^{a)} detected on various media by nine strains of *Erwinia carotovora* subsp. *carotovora* isolated in Thailand

Strain	Medium ^{b)}						
	PSA	PA	523	YPDA	KBA	MKA	MM
435-2	++ ^{c)}	++++	+++	++	++	-	-
435-6	++	++++	+++	+++	+	++	-
473-1	++	++	+++	+	-	-	-
475-1	++	+++	+++	-	-	-	-
489-4	++	+++	-	-	-	-	-
489-5	++	+++	+++	+++	+	++	-
493-1	++	+++	+++	+++	++	++	-
493-3	++	+++	+++	-	-	-	-
493-5	++	+++	+++	+	+	-	-

a) Anti-*P. solanacearum* activity was estimated by the plate-chloroform method using strain C319 as an indicator.

b) Ingredients of each medium were shown in the text.

c) Activity index (diameter of inhibition zone): -, not detected; +, less than 40 mm; ++, 40~60 mm; +++, greater than 60 mm.

cultured at 30°C for 12-120 hr under shake or still conditions. The bacterial population, antibacterial activity and pH in each culture fluid were sampled periodically during incubation. Sample activity was evaluated by the paper disk method¹²⁾ after the samples were sterilized by filtration through a Millipore filter (0.2 µm). Under shake-culture conditions in PS liquid medium, all Thai strains produced antibacterial substance(s), similar to the case of strain 493-1 indicated in Fig. 1. None of the strains inhibited the indicator bacteria in still culture. Our results suggest that the Thai strains of *E. carotovora* subsp. *carotovora* produced several kinds of antibacterial substances depending upon the strain/medium combination. Furthermore, an unidentified substance(s) in the

potato broth culture is favorable for the antibacterial substance(s) production. Ishimaru *et al.*⁷⁾ showed that strain C9-1 of non-pathogenic *Erwinia herbicola* produced at least two antibiotics, herbicolin O and I, which inhibited the growth of a wide variety of enterobacteria including *Erwinia* spp. Purification and characterization of the anti-*P. solanacearum* substance(s) produced by *E. carotovora* subsp. *carotovora* strain 493-1 are in progress. The utilization of these *Erwinia* spp. as a biological control agent after the deletion of pathogenicity is a subject for future study.

Literature cited

1. Furuya, N., Kushima, Y. and Matsuyama, N. (1992). Production of antibacterial substances by *Pseudomonas glumae*. J. Fac. Agric. Kyushu Univ. 37: 149-158.
2. Furuya, N., Kushima, Y., Tsuchiya, K., Matsuyama, N. and Wakimoto, S. (1991). Protection of tomato seedlings by pre-treatment with *Pseudomonas glumae* from infection with *Pseudomonas solanacearum* and its mechanisms. Ann. Phytopathol. Soc. Jpn. 57: 363-370.
3. Furuya, N., Okamoto, T., Kori, Y., Matsuyama, N. and Wakimoto, S. (1991). Control of bacterial seedling rot of rice by avirulent strains of *Pseudomonas glumae*. Ann. Phytopathol. Soc. Jpn. 57: 371-376.
4. Howell, C.R. and Stipanovic, R.D. (1979). Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69: 480-482.
5. Howell, C.R. and Stipanovic, R.D. (1980). Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70: 712-715.
6. Isono, K., Nagatsu, J., Kawashima, Y. and Suzuki, S. (1965). Studies on polyoxin, antifungal antibiotics. Part I. Isolation and characterization of polyoxin A and B. Agric. Biol. Chem. 29: 848-854.

7. Ishimaru, C.A., Klos, E.J. and Brubaker, R.R. (1988). Multiple antibiotic production by *Erwinia herbicola*. *Phytopathology* 78 : 746-750.
8. Kado, C.I. and Heskett, M.G. (1970). Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. *Phytopathology* 60 : 969-976.
9. Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology* 44 : 963-965.
10. Kerr, A. (1980). Biological control of crown gall through production of agrocin 84. *Plant Dis.* 64 : 25-30.
11. Kikutake, K., Matsuyama, N. and Wakimoto, S. (1991). Antagonistic activity of xanthomonads against phytopathogenic fungi and isolation of antifungal substance. *Ann. Phytopathol. Soc. Jpn.* 57 : 61-64.
12. Kunitake, S., Matsuyama, N. and Wakimoto, S. (1988). Production of proteinous anti-fungal substance(s) by *Pseudomonas avenae* Manns. *Ann. Phytopathol. Soc. Jpn.* 54 : 640-642.
13. Kunitake, S. and Matsuyama, N. (1989). Purification of anti-fungal substance produced by *Pseudomonas avenae* Manns. *Ann. Phytopathol. Soc. Jpn.* 55 : 366-368.
14. Matsuo, N., Matsuyama, N. and Wakimoto, S. (1981). Purification and some properties of a heat-stable antibacterial substance released from *Xanthomonas campestris* pv. *citri* Ku7101. *Ann. Phytopathol. Soc. Jpn.* 47 : 472-479.
15. Matsuo, N., Matsuyama, N. and Wakimoto, S. (1981). Purification and some properties of the bacteriocin-like substance produced by *Xanthomonas campestris* pv. *citri*. *Ann. Phytopathol. Soc. Jpn.* 47 : 480-487.
16. Matsuo, N., Matsuyama, N. and Wakimoto, S. (1981). Production of a specific bacteriocin by *Xanthomonas campestris* pv. *citri*. *Ann. Phytopathol. Soc. Jpn.* 47 : 571-574.
17. Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H. and Yonehara, H. (1958). Blastidicin S, a new antibiotic. *J. Antibiot.* 11 : 1-5.
18. Tsuyumu, S., Tsuchida, S., Makino, T. and Takikawa, Y. (1989). Antifungal activity in cell-free culture fluid of *Pseudomonas solanacearum*. *Ann. Phytopathol. Soc. Jpn.* 55 : 9-15.
19. Umezawa, H., Okami, Y., Hashimoto, T., Suhara, Y., Hamada, M. and Takeuchi, T. (1965). A new antibiotic, Kasugamycin. *J. Antibiot.* 18 : 101-106.
20. Wakimoto, S., Hirayae, K., Tsuchiya, K., Kushima, Y., Furuya, N. and Matsuyama, N. (1986). Production of antibiotics by plant pathogenic pseudomonads. *Ann. Phytopathol. Soc. Jpn.* 52 : 835-842.

和 文 摘 要

西岡正憲・古屋成人・中島信彦・松山宣明： *Erwinia* 属細菌代謝産物の各種植物病原細菌に対する抗菌活性

日本およびタイ国各地で分離された *Erwinia* 属細菌 80 菌株の *Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas cepacia*, *P. solanacearum*, *P. syringae* pv. *syringae*, *Xanthomonas campestris* pv. *citri*, *X. oryzae* pv. *oryzae* に対する抗菌物質産生性を調べた。その結果 *E. carotovora* subsp. *carotovora*, *E. chrysanthemi* pv. *chrysanthemi*, *E. chrysanthemi* pv. *zeae*, *E. chrysanthemi* (pathovar 未同定) を含む 39 菌株が 7 種指示菌の少なくとも 1 菌種に対して抗細菌活性を示す物質を産生した。一方, *P. cepacia* および *X. campestris* pv. *citri* に対し抗細菌活性を示す物質産生性菌株は認められなかった。タイ国産の *E. carotovora* subsp. *carotovora* 4 菌株は平板培地上において *C. michiganensis* subsp. *michiganensis*, *X. oryzae* pv. *oryzae* および *P. solanacearum* に対して顕著な増殖阻止円を形成した。またタイ国産の *E. carotovora* subsp. *carotovora* 493-1 株は、ジャガイモシヨ糖半合成液体培地あるいはジャガイモ煮汁液体培地で振とう培養した場合, *P. solanacearum* に対し抗菌活性を有する物質を産生した。

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