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Isolation of Endophytic Bacteria from *Solanum* sp. and Their Antibacterial Activity against Plant Pathogenic Bacteria

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Endophytic bacteria reside within plant hosts without causing disease symptoms. In this study, seventy three endophytic bacterial isolates were isolated from the roots of *Solanum* sp. plants. The isolates were further subdivided into 15 groups based on the phenotypic characteristics. Some of these isolates were preliminarily classified as members of the genus *Bacillus* and fluorescent pseudomonads. Of 73 isolates, 40 were found to have *in vitro* antagonistic activity against various plant pathogenic bacteria. The isolate designated Kutox1201 exhibited a wide range of inhibition against tested plant pathogenic bacteria. The Gram positive phytopathogenic bacterium, *Clavibacter michiganensis* subsp. *michiganensis* was inhibited to the most by the isolates tested. The ability of these isolates to suppress the growth of various phytopathogenic bacteria makes them potential biocontrol agents.

INTRODUCTION

Interest in the use of the microorganisms for biological control of plant diseases has increased owing to the growing environmental and health concerns created by the use of pesticides. The importance of environment–friendly plant protection methods has been greatly emphasized in the sustainable agriculture. The recent increase in publications on bacterial endophytes reflects an interest in their potential benefits in agriculture (Kobayshi and Palumbo, 2000).

Endophytic bacteria are bacteria that live in plant tissues without doing substantive harm to the plant or gaining any benefit other than residency (Kado, 1992). They have been found associated with numerous plant species, with most being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus* and *Azospirillium* (Chanway, 1996). As cited extensively by Kobayashi and Palumbo (2000), endophytic bacteria exist in a variety of tissue types within numerous plant species, suggesting ubiquitous existence in most if not all higher plant species. Mundt and Hinkle (1976) isolated bacteria from seeds and ovules of 27 different plant species. Moreover, endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants ranging from woody tree species such as oak (Brooks *et al.*, 1994) and pear (Whitesides and Spotts, 1991), to herbaceous crop plants such as sugar beets (Jacobs *et al.*, 1985) and maize

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(Fischer *et al.*, 1992; Lalande *et al.*, 1989; McInroy and Kloepper, 1995b).

Although the interaction between endophytic bacteria and their host plants is not fully understood, many strains promote plant growth (Hallmann *et al.*, 1997) by atmospheric nitrogen fixation (Boddey and Dobereiner, 1995) and increased resistance to pathogens and parasites (Hallmann *et al.*, 1997). Some species such as *Pseudomonas fluorescens*, *Curtobacterium luteum, Bacillus amyloliquefaciens* were reported to control plant pathogenic bacteria like *Clavibacter michiganensis* and *Erwinia carotovora* (van Buren *et al.*, 1993 and Sturz and Matheson, 1996).

Wild relatives of eggplants (*Solanum* sp.) are well–known to be resistant to some major soil–borne phytopathogens such as bacterial wilt caused by *Ralstonia solanacearum* and verticillium wilt (Kondou *et al.*, 2001). *Solanum aethiopicum* was reported to carry resistance to bacterial wilt, which is one of the most important diseases of eggplant (*Solanum melongena*). Moreover, traits of resistance against bacterial wilt have been identified in different wild relatives of eggplant such as *S. torvum, S. sisymbrifolium* and *S. aethiopicum* (Collonier *et al.*, 2001). However, the resistant mechanism associated with *Solanum* sp. has not been elucidated and little attention was paid to *Solanum* sp. endophytic bacterial population.

The present study was designed (i) to isolate endophytic bacteria from *Solanum* sp. plant root tissues, (ii) to characterize phenotypes of these isolates, and (iii) to screen these isolates for *in vitro* antagonism against phytopathogenic bacteria.

MATERIALS AND METHODS

Isolation of endophytic bacteria from *Solanum* sp.

Sixteen *Solanum* sp. plants were collected in 2002 in Hanoi, Vietnam. A 3 cm segment of *Solanum* sp. root was cut out. The segment was washed with tap water to remove the attached soil and dipped in 70% of ethanol and then in 3% sodium hypochlorite for 3 min. The section was rinsed 2 times in sterile distilled water. Each sample was macerated with a sterile blade in a droplet of sterile distilled water in a Petri plate. The suspension was diluted tenfold series and each dilution was streaked onto YPDA medium (yeast peptone dextrose agar medium: yeast extract 3 g, peptone 0.6 g, dextrose 3 g, agar 15 g, distilled water 1 liter, pH 7.2) and incubated at 30 °C for 24–96 h. Colony morphology of the bacterial isolates was recorded at 24, 48 and 96 h post–inoculation for the following characters: size, color, shape and growth rate. For preliminary phenotypic characterization, the distinct colonies were sub–cultured on YPDA slants at 30 °C. All isolates were stored as culture stocks either in sterile distilled water or in the mixture of 10% skimmed milk containing 0.05% L–glutamic acid and 20% glycerol kept at -20 °C until use.

Phenotypic characterization of bacterial isolates.

Bacterial isolates were characterized for the following traits: Gram reaction, heat tolerance at 100 °C for 20 min, fluorescence pigment production on King's medium B (KB), hypersensitive reaction (HR) on tobacco leaf, anaerobic growth, oxidase and catalase activity. The procedures were carried out as described by Schaad, N. W. (1988).

Table 1. Phenotypic characterization of the endophytic bacteria isolated from *Solanum* sp.

Isolate	Phenotypic groups	Characteristics [a]							
		Gram reaction	Heat tolerance	Fluorescent on KB	Anaerobic growth	Oxidase test	Catalase test	HR [b]	Frequency [c] %
KuTox101, 506, 510	1	+	−	−	±	−	+	−	4.1
KuTox102, 202, 514	2	+	+	−	+	−	+	−	4.1
KuTox201, 203, 801, 802, 901, 903, 904, 905, 1002, 1102, 1302, 1502, 1503, 1601	3	−	−	−	+	−	+	−	19.2
KuTox204, 502, 505, 507, 508, 512, 513, 708, 710	4	+	−	−	+	−	+	−	12.3
KuTox301, 308, 503, 706	5	−	−	−	−	±	+	−	5.5
KuTox302, 304, 401, 403, 501, 601, 602, 701, 702, 1101	6	−	−	−	−	+	+	−	13.7
KuTox303, 305, 404, 504, 704, 1402, 1501	7	+	−	−	+	+	+	−	9.6
KuTox306, 511	8	+	−	−	−	+	±	−	2.7
KuTox307	9	+	−	−	+	+	−	−	1.4
KuTox402	10	−	−	−	−	−	−	−	1.4
KuTox509, 711, 712, 713	11	+	−	−	−	−	+	−	5.5
KuTox703, 707, 709	12	−	−	−	−	−	+	−	4.1
KuTox705	13	+	−	−	±	−	−	−	1.4
KuTox902, 1001, 1003, 1201, 1203, 1301, 1303, 1401, 1403	14	−	−	+	−	+	+	−	12.3
KuTox1103, 1202	15	−	−	−	+	±	+	−	2.7

[a] +, positive; ± not clear; −, negative

[b] HR: Hypersensitive Reaction

[c] Frequency is based upon 73 isolates in total.

***In vitro* antagonism assay.**

Nine species of phytopathogenic bacteria were used as indicators for screening the *in vitro* antibacterial activity of the endophytic bacterial isolates (Table 2). These indicator bacteria were known to have worldwide distributions and cause serious plant diseases.

To detect the antibacterial activity of the endophytic bacterial isolates, the plate chloroform method (Chen *et al.*, 1981; Wakimoto *et al.*, 1986) was used. A fresh culture (1–2 days) of the producer from YPDA slant was transferred to the centre of YPDA plate. The plates were then incubated at 30 °C for 2–3 days. After the bacteria formed colonies several mm in diameter, the plate was turned upside down. A sheet of filter paper was placed in the Petri plate lid and 2 ml of chloroform was added to it and kept at room temperature for 3 hrs. After complete evaporation of chloroform, the indicator bacterial suspension (conc. *ca.* 10^8cfu/ml, 0.5 ml) was mixed with 5 ml of water agar (melted and kept at 50 °C) and overlaid on the plate and incubated at 30 °C for 2 days. If an inhibition zone appeared, its semi–diameter was measured. The direct assay was applied only for *Bacillus* sp. isolates. The pathogen was inoculated by streaking evenly on the plate and the endophytic bacterial isolate was spotted on the center of the plate and incubated at 30 °C for 2 days. The experiment was conducted in duplicate.

Table 2. Phytopathogenic bacteria used as indicators for testing the antibacterial activity of the endophytic bacterial isolates

Indicator bacteria	Source [a]	Abbreviations [b]
Ralstonia solanacearum ATCC 1169^T	ATCC	Rs
Agrobacterium tumefaciens ATCC23308^T	ATCC	At
Xanthomonas oryzae pv. *oryzae* Q7527 (V)	KNAES	Xo
Erwinia carotovora subsp. *carotovora* ATCC175713^T	ATCC	Ec
Xanthomonas campestris pv. *campestris* ATCC33913^T	ATCC	Xc
Clavibacter michiganensis subsp. *michiganensis* N 6601	NIAES	Cm
Burkholderia cepacia ATCC25416^T	ATCC	Bc
Pseudomonas syringae pv. *syringae* ATCC19310^T	ATCC	Ps
Acidovorax avenae subsp. *avenae* ATCC19860^T	ATCC	Aa

[a] Abbreviations for culture collections: ATCC, American Type Culture Collection;NIAES, National Institute of Agro–Environmental Sciences, Japan; KNAES, Kyushu National Agricultural Experiment Station, Japan

[b] Abbreviations for indicator bacteria in the Table 3

RESULTS

Endophytic bacteria in *Solanum* sp.

Based on the distinct colony morphology of shape, size and color, seventy three endophytic bacterial isolates were isolated from the roots of *Solanum* sp. It was observed that different *Solanum* sp. plants contained different population of endophytic bacteria (data not shown). Gram negative bacteria were dominant, accounting for 59% of the total isolates.

Table 3. Antibacterial activity of the endophytic bacteria isolated from *Solanum* sp.

Isolate	Width of inhibition zone [a]								
	Indicator [b]								
	Rs	Bc	Aa	Ps	At	Xo	Ec	Xc	Cm
KuTox102	−	−	−	+	−	+	−	−	−
KuTox514	−	−	−	+	−	(+)	−	−	−
KuTox203	−	−	−	+	−	−	−	+	−
KuTox204	−	−	−	+	−	−	+	(+)	++
KuTox404	−	−	−	−	+	−	−	−	−
KuTox502	−	−	−	−	−	−	+	−	+
KuTox505	−	−	−	−	−	−	−	−	+
KuTox506	−	−	−	−	−	−	++	−	++
KuTox507	−	−	−	−	−	−	−	+	++
KuTox508	+	−	−	+	−	(+)	−	+	−
KuTox509	−	−	−	−	−	−	−	−	+
KuTox511	−	−	−	+	−	−	−	−	+
KuTox512	−	−	−	−	−	−	−	−	+
KuTox705	−	−	−	−	−	−	−	−	+++
KuTox711	−	−	−	−	−	−	−	−	+
KuTox713	−	−	−	−	−	(+)	−	−	++
KuTox1402	−	−	+	(+)	+	−	−	−	−
KuTox902	−	−	−	−	−	−	−	−	+
KuTox1001	+	−	−	−	−	+	−	−	+
KuTox1003	(+)	−	+	+	+++	+	−	−	++
KuTox1201	+	−	−	(+)	+	+++	++	−	+
KuTox1203	−	−	+	(+)	+++	+	−	−	++
KuTox1301	+	−	−	−	−	+++	++	(+)	+
KuTox1303	−	−	+	+	+++	+	−	−	++
KuTox1401	−	−	−	−	−	+	+	−	+
KuTox1403	+	−	−	−	−	−	−	−	+
KuTox501	−	−	−	−	−	−	−	−	+
KuTox702	−	−	−	−	−	−	−	(+)	+
KuTox801	+	−	−	+	−	−	−	−	−
KuTox802	(+)	−	−	++	−	−	(+)	−	++
KuTox904	+	−	−	(+)	−	−	−	+	+++
KuTox905	+	−	+	(+)	−	−	−	−	+
KuTox1101	−	−	−	(+)	−	+	+	(+)	+
KuTox1102	−	−	−	−	−	−	−	(+)	+
KuTox1103	−	−	−	(+)	−	−	−	−	+
KuTox1202	−	−	−	++	−	−	−	−	−
KuTox1302	−	−	−	(+)	(+)	−	−	−	+
KuTox1502	+	−	−	++	−	−	−	−	+
KuTox1503	−	−	−	−	−	−	−	−	+
KuTox1601	−	−	−	−	−	−	−	−	+

[a] Inhibition zone diameter index: +++>10mm, ++5–10mm, +<5mm, (+) doubtful, − no zone of inhibition.

[b] See Table 2

Phenotypic characterization of endophytic bacterial isolates

For preliminary characterization, 73 endophytic bacterial isolates were divided into 15 phenotypic groups (Table 1). Thirty isolates were Gram positive bacteria, 3 of which (phenotypic group 2) with the typical characteristics of heat tolerance and colony morphology were preliminarily characterized as members of the genus *Bacillus*. Forty–three isolates were Gram negative bacteria, 10 of which (phenotypic group 14) were classified as members of *Pseudomonas* sp. on the basis of the following general characteristics: fluorescencé pigment production on KB, oxidase positive, and catalase positive. None of endophytic bacterial isolates induced hypersensitive reaction (HR) on tobacco leaf after 3–4 days post–inoculation. However, change in tobacco leaf color at inoculation sites from green to light yellow was observed. The highest percentage (19.2%) was found among the isolates belonging to the phenotypic group 3.

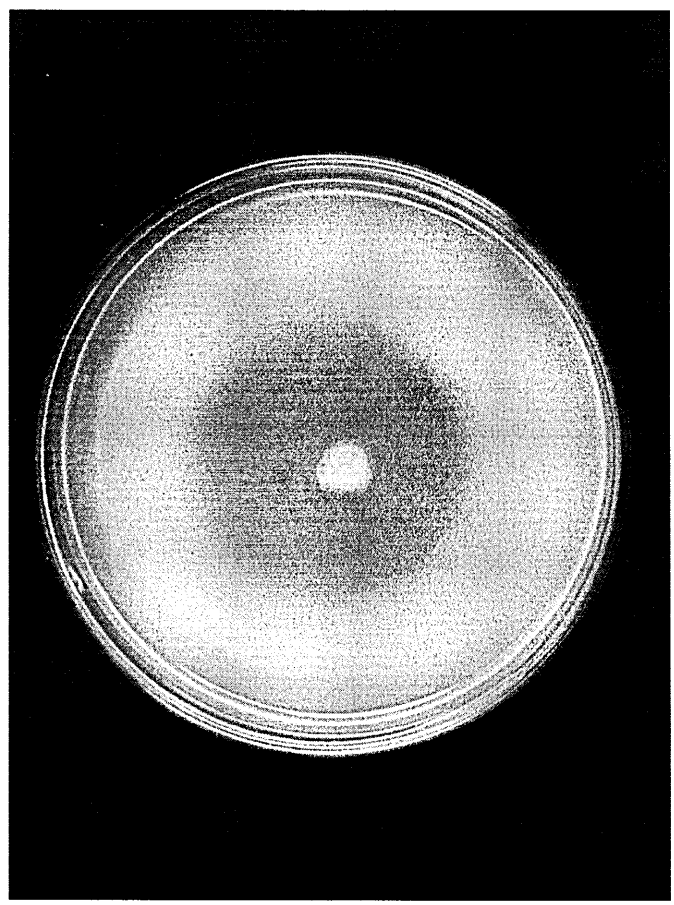

Fig. 1. Growth inhibition zone formed by endopytic bacterial isolate KuTox1201 on the lawn of *Xanthomonas oryzae* pv. *oryzae*.

***In vitro* antagonism of endophytic bacterial isolates**

Of 73 endophytic bacterial isolates screened for antagonism, 40 formed inhibition zones against 9 phytopathogenic bacteria tested. The semi-diameter of the inhibition zones around endophytic bacterial colonies ranged from 2 mm to 30 mm, indicating the production of antibacterial substances. The inhibitory activity of KuTox1201 against *Xanthomonas oryzae* pv. *oryzae* is shown in Fig. 1. Thirty-two endophytic bacterial isolates were active to *C. michiganensis* subsp. *michiganensis*. Meanwhile, their activity against *R. solanacearum*, *Acidovorax avenae* subsp. *avenae* and *X. campestris* pv. *campestris* was weak. Although some isolates showed the inhibitory activity against *R. solanacearum*, it was ambiguous. *Burkholderia cepacia* was not affected by the inhibitory activity of the endophytic bacterial isolates.

DISCUSSION

In this study, the roots of *Solanum* sp. were chosen as a source of endophytic bacteria. It was investigated that population densities of endophytic bacteria seem to be highest in the root (Quadt Hallmann and Kloepper, 1996). As a result, 73 isolates were isolated according to their colony morphology and subdivided into 15 phenotypic groups based on the phenotypic characteristics. It was evident that the number of Gram negative isolates was higher than that of Gram positive. Other workers reported the same result but working with other plants (reviewed in Hallmann *et al.*, 1997). The diversity of endophytic bacteria isolated from *Solanum* sp. was clarified. Several factors may explain the diversity including geographical distribution, plant age, and tissue type (Kobayashi *et al.*, 2000). This is the first report on the isolation and description of endophytic bacterial population from *Solanum* sp.

In vitro experiments on antibacterial activities of the isolates against phytopathogenic bactetria showed that many of the isolates, especially fluorescent pseudomonad isolates, possess the ability to inhibit the growth of several plant pathogenic bacteria. Fluorescent pseudomonads were known to suppress plant pathogenic microorganisms by the production of diverse microbial metabolites including antibiotics (Weller, 1988). The Gram positive phytopathogenic bacterium, *C. michiganensis* subsp. *michiganensis* was inhibited to the most by many of the isolates. This finding agrees with that of Foldes *et al.*, (2000) who observed that Gram positive bacteria were more sensitive to antimicrobial compounds produced by bacterial antagonists than Gram negative tested. Thus, the activity spectra of endophytic bacterial isolates were greatly different depending upon isolates, suggesting that several antibacterial substances participated in their activity.

This study revealed the diversity of culturable endophytic bacteria isolated from *Solanum* sp. and some of the isolates possess *in vitro* antagonistic activity against various plant pathogenic bacteria. Further detailed studies on endophytic bacterial population in *Solanum* sp. to establish their suitability as biocontrol agents are suggested. Furthermore, several points such as identification of effective biocontrol isolates and their protection mechanisms *in planta* need clarification.

REFERENCES

Boddey, R. M., O. C. de Oliveira, S. Urquiaga, V. M. Reis, F. L. de Olivares, V. L. D. Baldani and J.

Dobereiner 1995 Biological nitrogen fixation associated with sugar cane and rice: Contribution and prospects for improvement. *Plant Soil*, **174**: 195–209

Brooks, D. S., C. F. Gonzalez, D. N. Appel and T. H. Filer 1994 Evaluation of endophytic bacteria as potential biological control agents for oak wilt. *Biol. Con.*, **4**: 373–381

Chanway, C. P. 1996 Endophytes: they're not just fungi. *Can. J. Bot.*, **74**: 321–322

Chen, W. Y., E. Echandi and H. W. Spurr 1981 Protection of tobacco plants from bacterial wilt with avirulent bacteriocin–producing strains of *Pseudomonas solanacearum*. *Proc. Fifth Int. Cont. Plant Path. Bact., Cali.*, 482–492

Collonnier, C., K. Mulya, I. Fock, I. Mariska, A. Servaes, F. Vedel, S. Siljak–Yakovlev, V. Souvannavong, G. Ducreux and D. Shihachaki 2001 Source of resistance against *Ralstonia solanacearum* in fertile somatic hybrids of eggplant (*Solanum melongena* L.) with *Solanum aethiopicum* L. *Plant Sci.*, **160**: 301–313

Fischer, P. J., O. Petrini and H. M. Lappin Scott 1992 The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytologist*, **122**: 299–305

Hallmann, J., A. Quadt–Hallmann, W. F. Mahaffee and J. W. Kloepper 1997 Bacterial endophytes in agricultural crops. *Can. J. Microbiol.*, **43**: 895–914

Jacobs, M. J., W. M. Bugbee and D. A. Gabrielson 1985 Enumeration, location and characterization of endophytic bacteria within sugar beet roots. *Can. J. Bot.*, **63**: 1262–1265

Kado, C. I. 1992 Plant pathogenic bacteria. *In* "The prokaryotes", vol. 1. A, Balows, H.G. Truper, M. Dworkin, W. Harder and K. H. Schleifer (ed.), Springer–Verlag, New York, N. Y., pp. 659–674

Kobayashi, D. Y. and J. D. Palumbo 2000 Bacterial endophytes and their effects on plants and uses in agriculture. *In* "Microbial endophytes" C. W. Bacon and J. F. White (ed.), Marcel Dekker, Inc., New York, N. Y., pp. 199–233

Kondou, E. 2001 Resistance to soil–borne disease and crossing incompatibility of related wild *Solanum* species. *In* "Research report summary of Japan Agriculture and Forestry Research Center."

Lalande, R., N. Bissonnette, D. Coutlee and H. Antoun 1989 Identification of rhizo–bacteria from maize and determination of their plant–growth promoting potential. *Plant Soil*, **115**: 7–11

McInroy, J. A. and J. W. Kloepper 1995b Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil*, **173**: 337–342

Mundt, J. O. and J. O. Hinkle 1976 Bacterial within ovules and seeds. *Appl. Environ. Microbiol.*, **32**: 694–698

Quadt–Hallmann, A. and J. W. Kloepper 1996 Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. *Can. J. Microbiol.*, **42**: 1144–1154

Schaad, N. W. 1988 Initial identification of common genera. *In* "A Laboratory Guide for the Identification of Plant Pathogenic Bacteria", 2nd edition. Ed. N W Schaad. APS Press, St. Paul, MN, USA., pp 1–14

Sturz, A. V. and B. G. Matheson 1996 Population of endophytic bacteria which influence host–resistance to *Erwinia*–induced bacterial soft rot in potato tubers. *Plant Soil*, **184**: 265–271

Sturz, A. V., B. R. Christie and J. Nowak 2000 Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci.*, **19**: 1–30

Van Buren, A. M., C. Andre and C. A. Ishimaru 1993 Biological control of the bacterial ring rot pathogen by endophytic bacteria isolated from potato. *Phytopathology*, **83**: 1406

Wakimoto, S., K. Hirayae, K. Tsuchiya, Y. Kushima, N. Furuya and N. Matsuyama 1986 Production of antibiotics by plant pathogenic Pseudomonads. *Ann. Phytopath. Soc. Jpn.*, **52**: 835–842

Weller, D. M. 1988 Biological control of soil–borne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.*, **26**: 379–407

Whitesides, S. K. and R. A. Spotts 1991 Frequency, distribution, and characteristics of endophytic *Pseudomonas syringae* in pear trees. *Phytopathology*, **81**: 453–457