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Diversity and potential application of endophytic bacteria in ginger

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ABSTRACT. Here, 248 endophytic bacterial strains were isolated to assess the distribution and population diversity of endophytic bacteria in ginger plants. A total of 10.4 x 10^4 to 20.2 x 10^4 CFU/g fresh weight endophytic bacteria of different growth stages were isolated. Maximum bacterium numbers were obtained at the seedling stage. A total of 107 functional strains were screened, including 17 antibacterial strains and 90 indole acetic acid-producing strains. Based on 16S rDNA sequence restriction fragment length polymorphism and 16S rDNA sequences, these 107 strains were mapped and grouped into 16 genera. *Bacillus* and *Pseudomonas* were the dominant genera; however, the bacteria belonged to a tremendous range of genera, with the highest species richness being observed at the seedling stage. Sixteen strains exhibited antimicrobial activity against *Pythium myriotylum* Drechsler, while 7 strains exhibited antimicrobial activity against *Phyllosticta zingiberi* Hori. *Bacillus* was the dominant antibacterial strain. *Pseudomonas fluorescens*, *B. megaterium*, and *Enterobacter ludwigii* produced remarkably high levels of IAA. Only a few endophytic bacterial

strains were inhibited in fresh ginger juice. Most of these strains were present during seedling stage, including *Roseateles depolymerans*, *Chryseobacterium taiwanense*, *E. ludwigii*, *Agrobacterium larrymoorei*, *P. fluorescens*, and *Bacillus amyloliquefaciens*. This study indicates that the community of endophytic bacteria in ginger changes with the synthesis of antibacterial substances.

Key words: Endophytic bacteria; IAA; Ginger; Antifungal activity

INTRODUCTION

Ginger is the rhizomes of *Zingiber officinale* Roscoe (Zingiberaceae), and has been widely used as a spice and condiment in a range of forms. In addition to its food additive function, ginger has a long history of medicinal use in the treatment of a variety of human diseases, including common colds, fever, rheumatic disorders, gastrointestinal complications, motion sickness, diabetes, and cancer, among others (Kundu et al., 2009). Because of the high yields and economic benefits of ginger, Shandong Province has been developed into the largest ginger planting base in China. Ginger contains many nutrients, including saccharides, fats, proteins, carotenes, vitamin C, and a large number of microelements. Ginger also secretes various substances, such as gingerol, shogaol, zingerone, and ginger essential oil (Singh et al., 2003). Gingerol and ginger essential oil have antibacterial effects against *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces*, *Staphylococcus aureus*, *Aspergillus niger*, and *Rhizopus* (Zhao, 2008).

Endophytic bacteria play an important role in plant growth. Endophytes colonize plant tissues, and are able to interact among themselves and with invaders (e.g., pathogens); thus, influencing plant development. Evolutionarily, endophytes also appear to form an intermediate group between saprophytic bacteria and plant pathogens. Endophytic bacteria have been isolated from a variety of plants, because they ubiquitously inhabit most plant species, including sugar beet, several potato varieties, and poplar trees (Sessitsch et al., 2002; Dent et al., 2004; Taghavi et al., 2009). Many studies have shown that endophytes are widely present in plant tissues, such as the roots, stems, leaves, and flowers (Kobayashi and Palumbo, 2000). For instance, several genera have been isolated from legume tissues, including *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas*, *Pseudomonas*, and *Sphingomonas* (Sturz et al., 1997; Elvira-Recuenco and van Vuurde, 2000; Oehrle et al., 2000).

Many researchers have studied the breeding and cultivation characteristics of ginger. However, investigations about the endophytic bacteria that this plant harbors remain limited. Some endophytic bacteria have been screened in ginger, and were found to promote plant growth (Xie et al., 2009). Rajan et al. (2000) studied the effects of 4 isolates of endophytic bacteria on the growth of a ginger cultivar (cv.). They also assessed the suppressive activity of endophytic bacteria against *Phythium* sp, *Fusarium oxysporum*, and *Pratylenchus coffeae* in a pot experiment. Chu et al. (2011) analyzed the diversity of endophytes isolated from the ginger tuber.

Endophytes influence plant growth via several processes, including the production of plant hormones. One such hormone is indole-3-acetic acid (IAA), which is an essential phytohormone that is involved in different plant developmental processes (Liu et al., 2010). The

production of IAA is widely distributed among plant-associated bacteria. While the combined actions of several rhizobacteria seem to result in the promotion of plant growth, bacterial phytohormone production, particularly IAA, is still considered the primary mechanism that enhances the growth and yield of plants (Arkhipova et al., 2005). Another important function of plant growth-promoting bacteria is their antagonistic action against pathogenic microorganisms. Endophytic bacteria interact collaboratively with plant hosts, and are easy to cultivate *in vivo*. Thus, the investigation of endophytic bacterial functions and subsequent development of their use in plants is of importance to microbiologists and plant protection experts.

Because ginger secretes gingerol and other antibacterial substances during growth, changes in the endogenous bacterial population of ginger are expected to differ compared to bacterial populations in other crops. So the bacterial strains isolating from ginger were used in ginger, which are not only biocontrol or promoting-growth function, but also should be adaptation to different ginger growth stages (Policegoudra et al., 2007). This paper studies the ginger endophytic bacterial population change rule at ginger different growth stages, and provides strain resources and a theoretical basis for bacterial agents that promote the growth and bio-control of ginger. The current study is the first report about the evolutionary changes of endophytic bacteria throughout the different growth stages of ginger.

MATERIAL AND METHODS

Sampling

Healthy ginger plants were sampled at 3 different growth stages; specifically, the seedling stage, the stem and leaf vigorous growth stage, and the rhizome enlargement stage. Samples were collected from the same field plot in Qiujiadian, Taian, Shandong Province, China.

Surface sterilization of plants and isolation of endophytic bacteria

Total endophytic bacteria were isolated from the roots, stems, tubers, and leaves of ginger. Whole plants were first washed with tap water to remove attached clay. Ten grams of tissue from each of the specified plant parts was cut with a sterile surgical knife for surface sterilization. The collected plant materials were immersed in 75% ethanol for 2.5 min, rinsed with 3% sodium hypochlorite (NaClO) for 2 min, dipped in 75% ethanol for 30 s, and finally washed 5 times with sterile distilled water. To determine whether the sterilization process was successful, 100 µL water from the third rinsing was plated on R_2A medium (0.05% proteose peptone, 0.05% starch, 0.05% glucose, 0.05% yeast extract, 0.05% casein hydrolysate, 0.03% dipotassium phosphate, 0.03% sodium pyruvate, 0.0024% magnesium sulfate anhydrous, 2% w/v agar, pH 7.2 ± 0.2), which was also the medium used for the isolation and purification of endophytic bacteria (Kawai et al., 2002). The plates were incubated at 28°C for 1-3 days to determine surface sterilization efficacy. If bacterial colonies were not observed on the plates, the sterilization process was considered successful. All surface-sterilized samples were placed in a sterilized mortar, and thoroughly ground after the addition of 10 mL sterile distilled water. The resulting suspension was diluted 10-fold with sterile distilled water, and about 100 µL of each dilution was spread onto R_2A medium. Bacterial isolates were obtained after incubation at 28°C for 2-3 days. Glycerol was then added, and the purified isolates were kept frozen at -80°C until use.

Total DNA extraction and 16S rDNA PCR amplification

Total bacterial DNA isolation was completed according to the procedure of Murray and Thompson (1980). 16S rDNA PCR amplification was carried out with the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGCTACCTTGTTA CGACTTCACCCC-3') by using a (Biometra TGRADIENT) thermocycler. The PCR conditions were as follows: initial denaturation at 95°C for 4 min, followed by 32 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. Products of about 1500 bp long were monitored by electrophoresis on 1% (w/v) agarose gel at 110 V for 30 min in 1X Tris-boric acid-EDTA (TBE) buffer. PCR products were viewed by ethidium bromide (EB) staining and UV transillumination.

ARDRA analysis of 16S rRNA gene sequences and evolutionary analysis

A 3S Spin PCR Product Purification Kit (Shenergy Biocolor Bioscience and Technology Company, China) was used for PCR product purification following manufacturer protocols. Purified PCR products were digested using 3 restriction enzymes, *Alu*I, *Hae*I, and *Msp*I (TaKaRa Biotechnology Co., Ltd., Dalian, China), in separate reactions. The selection of these restriction enzymes was based on the study of Laguerre et al. (1994). Digestion reactions were performed for 4 h at 37°C in a reaction volume of 10 µL containing 5 µL purified PCR products, 1 µL commercially supplied 10X incubation buffer, 3.5 µL water, and 0.5 µL (10 U/µL) restriction enzyme. Reaction products (10 µL) were run on 2.5% (w/v) agarose gel in 1X TBE buffer for 2.5 h at 120 V under refrigeration. Agarose gels were stained, visualized, and digitalized as previously described.

Visible bands greater than 100 bp were used for dendrogram construction. From the banding patterns generated by each of the restriction enzymes, a binary data matrix was constructed based on the presence or absence of each band (coded as 1 or 0, respectively). To obtain a single pattern for each isolate, the banding patterns obtained from each of the enzymes were combined. The patterns were then used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) by a clustering algorithm that makes use of the Sorensen's coefficient along with fine optimization options, which was incorporated into the MultiVariate Statistical Package (MVSP) version 3.13h (GeoMem, Blairgowrice, UK).

16S rRNA gene sequencing and sequence analysis

Purified PCR products obtained from the isolates of each ARDRA group were sequenced. The NCBI (National Center for Biotechnology Information database) nucleotide-nucleotide BLAST (BLASTn) tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to locate closely identical sequences for the 16S rRNA gene sequences that were determined. High-similarity sequences, as well as 16S rRNA gene sequences of the type strains, were retrieved from the Ribosomal Database Project. The sequences were aligned using the CLUSTAL X software version 1.8. Evolutionary distances were calculated using the package TREECON software version 1.3b. Construction of a neighbor-joining tree and bootstrap analysis (1000 replicates) was also performed using TREECON (Tamura et al., 2007).

In vitro antagonistic spectrum bioassay

An antagonistic spectrum bioassay of the endophytic bacteria was performed with *Pythium myriotylum* Drechsler and *Phyllosticta zingiberi* Hori. Bacterial isolates were inoculated on the margin of the fungal colony with sterile toothpicks, and incubated at 28°C for 2-4 days. Growth inhibition was calculated using the formula R1/R2, where R1 is the maximum radius of the fungal colony away from the bacterial colony and R2 is the average radius of the bacterial colony.

Screening of IAA-producing bacterial isolates and quantitative analysis

IAA-producing bacteria were screened by growing the bacterial isolates on R_2A medium modified with L-tryptophan (200 mg/L) at 28°C and 180 rpm for 3 days. Fifty microliters each of the bacterial liquid cultures and Salkowski reagent were mixed and viewed on a white board background. After being kept in the dark for 30 min, IAA-producing bacteria were identified by a change in color from pink to red. The concentration of IAA-positive strain cultures was measured at 600 nm before centrifugation at 10,000 rpm for 10 min. The procedures of Glickmann and Dessaux (1995) were used to evaluate IAA production in the supernatant, and a standard curve was generated using pure IAA.

Detection of the bacteriostatic effect of fresh ginger juice

Peeled ginger was immersed in 75% ethanol for 2.5 min, and then rinsed with 3% NaClO for 2 min. The samples were then dipped in 75% ethanol for 30 s, and washed 5 times with sterile distilled water. The fresh ginger juice, obtained using a disinfected juicer, was used to produce 4 concentrations of ginger solution: 100, 50, 25, and 10%. Endophytic bacterial strains were suspended in physiological saline at concentrations ranging from 10^5 to 10^6. Sterilized filter papers (6 mm) were immersed in various concentrations of ginger juice for 12 h, and then dried at 28°C. The endophytic bacterial suspensions were then inoculated on the R_2A culture plates at a volume of 500 mL. The filter papers that had been immersed in ginger juice were placed on the culture plates containing endophytic bacteria. Each plate contained two of the filter papers for each concentration level, and experiments were performed in triplicate. The plates were incubated at 28°C for 1 day. The diameter of the observed inhibition zone was measured for each setup, and the data were recorded. Filter papers immersed in 0.5% $CuSO_4$ were used as controls.

RESULTS

Endophytic isolates of ginger plants

Based on phenotypic characterization, a total of 248 isolates were obtained (Table 1). Bacterial densities from different ginger growth stages were counted, with densities ranging from 10.4×10^4 to 20.2×10^4 CFU/g fresh weight. These data produced significant period differences. The highest density was observed at the seedling stage, whereas the lowest density was observed at the rhizome enlargement stage. We also found that ginger leaves have the largest number of endogenous bacteria with the lowest bacterial numbers being observed in stems and tubers (Table 1).

Table 1. Number of endophytic bacteria determined at different growth stages.

Growth stage	No. of isolates	Leaf density	Stem density	Tuber density	Root density	Population density
Seedling stage	95	12.1 x 10^4	1.0 x 10^4	0.8 x 10^4	6.3 x 10^4	20.2 x 10^4
Stem and leaf vigorous growth stage	88	8.3 x 10^4	0.6 x 10^4	0.6 x 10^4	4.7 x 10^4	14.2 x 10^4
Rhizome enlargement stage	55	5.4 x 10^4	0.5 x 10^4	0.4 x 10^4	4.1 x 10^4	10.4 x 10^4

Data are reported as CFU/g fresh weight.

Bacteria isolated from the seedling stage included *Bacillus*, *Chryseobacterium*, *Pseudomonas*, *Flavobacterium*, *Agrobacterium*, *Serratia*, *Roseateles*, *Sphingomonas*, *Leclercia*, and *Enterobacter*. Bacteria isolated from the stem and leaf vigorous growth stage included *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Agrobacterium*, *Aeromonas*, *Acetobacter*, *Roseateles*, *Sphingomonas*, *Enterobacter*, and *Pantoea*. Bacteria isolated in the rhizome enlargement stage included *Bacillus*, *Pseudomonas*, *Herbaspirillum*, *Agrobacterium*, *Ensifer*, and *Stenotrophomonas*.

Antimicrobial activity

All of the isolates were subjected to antagonism experiments. Among the endophytic isolates, 17 were found to be active against at least 1 of the 2 pathogenic fungi used in this experiment (Table 2). Sixteen strains with antagonistic effects against *P. myriotylum* Drechsler and 6 strains with pathogenic effects against *P. zingiberi* Hori were observed. Furthermore, 5 strains were observed to have antagonistic effects against both types of pathogenic bacteria.

Table 2. Antifungal activities of the endophytic isolates.

Isolate	*Pythium myriotylum* Drechsler	*Phyllosticta zingiberi* Hori
EG-14	+++	-
EG-42	-	+++
EG-47	+++	+
RS-163	+++	+++
RS-164	+++	++
RS-169	+++	-
RS-171	+++	-
RS-106	++	+++
RS-174	++	-
RS-178	+++	-
RS-179	++	-
RS-181	++	-
RS-183	++	-
RS-184	+++	-
RS-185	+++	-
RS-188	++	+
RS-195	++	+

Inhibition: (+) = 0 < R1/R2 < 1; (++) = 1 ≤ R1/R2 < 2; (+++) = 2 ≤ R1/R2 < 2.5; (-) = R1/R2 < 0. R1 is the maximum radius of the fungal colony away from the bacterial colony. R2 is the average radius of the bacterial colony.

ARDRA analysis, sequencing, and phylogeny

From the digestion patterns of the restriction enzymes, a dendrogram (Figure 1) was constructed based on the banding patterns obtained by the NTSYS 2.1 program.

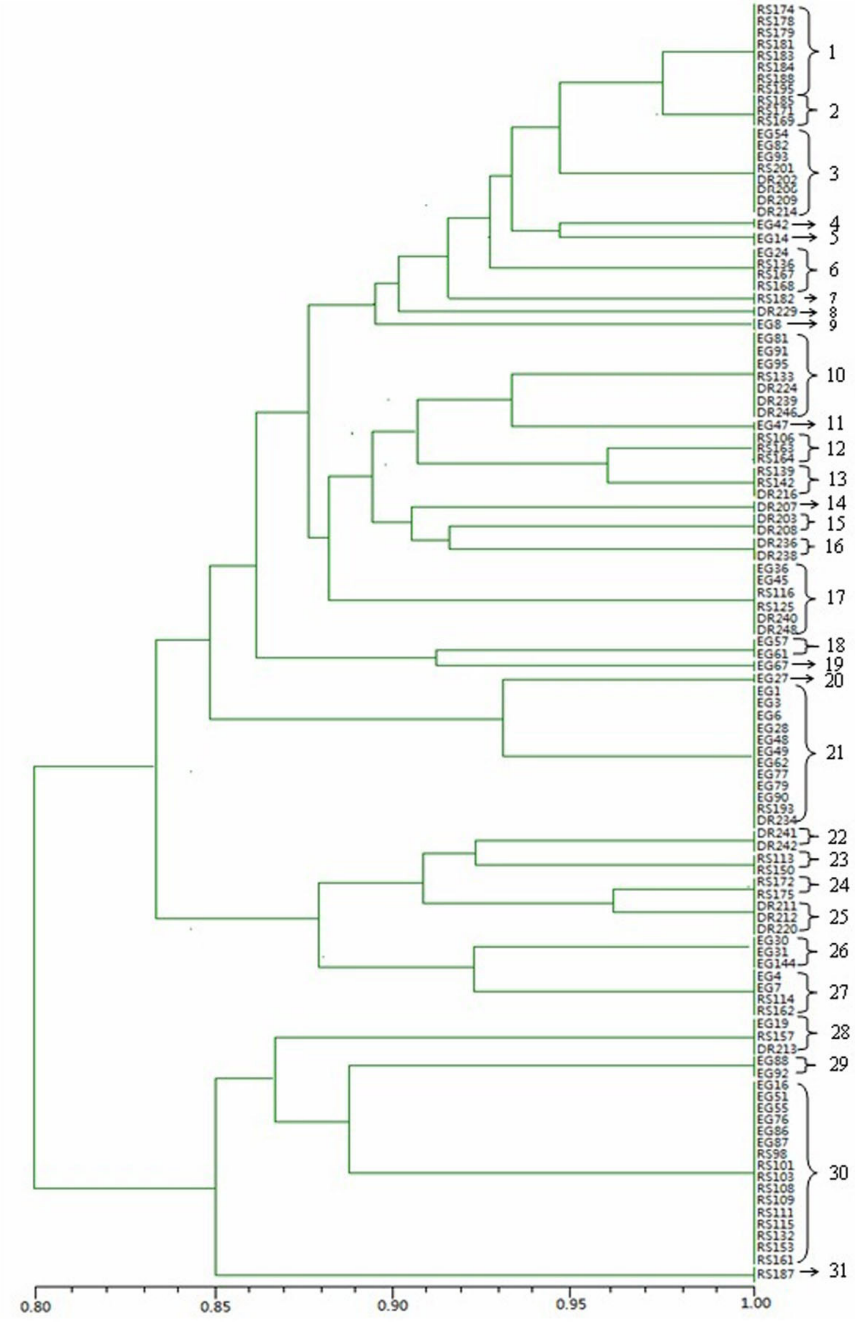

Figure 1. Dendrogram showing the relationships of 107 endophytic bacteria based on 16S-RFLP fingerprints obtained using cluster analysis.

The obtained isolates, 90 of which were IAA-producing and 17 of which featured activity against other strains, were divided into 31 clusters (or genotypic groups) with 100% similarity and 1 group with 80% similarity. Six large groups were formed (group 1, group 3, group 10, group 17, group 21, and group 30), with a further 15 small groups and 10 groups of just one isolate. The other groups contained various numbers of isolates. Thirty-one representative isolates from each group were sequenced and subsequently analyzed using the NCBI BLASTn program to retrieve annotated sequences (Table 3).

Table 3. Endophytic bacteria representing various 16S-RFLP groups and their closest affiliation according to 16S rRNA gene sequencing.

16S-RFLP groups	No.	Isolate	Accession No.	Closest NCBI strain and accession No.	Similarity (%)
1	8	RS178	KC122683	*Bacillus amyloliquefaciens* AB006920.1	99
2	3	RS169	KC122684	*Bacillus methylotrophicus* EU194897.1	99
3	8	EG54	KC122685	*Bacillus thuringiensis* D16281.1	99
4	1	EG42	KC122686	*Bacillus altitudinis* AJ831842	99
5	1	EG14	KC122687	*Bacillus cereus* AE016877.1	99
6	4	EG24	KC122688	*Bacillus megaterium* D16273.1	99
7	1	RS182	KC122689	*Paenibacillus hunanensis* EU741036.2	99
8	1	DR229	KC122690	*Herbaspirillum aquaticum* FJ267649.1	99
9	1	EG8	KC122691	*Chryseobacterium taiwanense* DQ318789.1	99
10	7	DR239	KC122692	*Pseudomonas fluorescens* D84013.1	99
11	1	EG47	KC122693	*Pseudomonas aeruginosa* X06684.1	99
12	1	RS163	KC122694	*Pseudomonas monteilii* AF064458.1	99
13	3	RS139	KC122695	*Pseudomonas taiwanensis* EU103629.2	99
14	1	DR207	KC122696	*Pseudomonas mendocina* AM088473.1	99
15	2	DR203	KC122697	*Pseudomonas putida* D84020.1	99
16	2	DR236	KC122698	*Pseudomonas pseudoalcaligenes* Z76666.1	99
17	6	RS116	KC122699	*Pseudomonas huttiensis* AB021366.1	99
18	2	EG57	KC122700	*Flavobacterium johnsoniae* AM230489.1	99
19	1	EG67	KC122701	*Flavobacterium reichenbachii* AM177616.1	99
20	1	EG27	KC122702	*Agrobacterium larrymoorei* Z30542.1	99
21	12	EG77	KC122703	*Agrobacterium tumefaciens* D14500.1	99
22	2	DR242	KC122704	*Ensifer adhaerens* AM181733.1	99
23	2	RS113	KC122705	*Aeromonas trotsa* X60415.2	99
24	2	RS172	KC122706	*Acetobacter pasteurianus* X71863.1	99
25	3	DR220	KC122707	*Stenotrophomonas maltophilia* AB294553.1	99
26	3	EG30	KC122708	*Serratia nematodiphila* EU036987.1	99
27	4	EG4	KC122709	*Roseateles depolymerans* AB003623.1	99
28	3	EG19	KC122710	*Sphingomonas yabuuchiae* AB071955.2	99
29	2	EG88	KC122711	*Leclercia adecarboxylata* JN175338.1	99
30	16	EG16	KC122712	*Enterobacter ludwigii* AJ853891.1	99
31	1	RS187	KC122713	*Pantoea ananatis* U80196.1	99
Total	107				

The phylogenetic tree (Figure 2) constructed from the 16S rDNA sequences showed that 31 isolates were clustered into 17 different genera. *Pseudomonas*, *Bacillus*, *Agrobacterium*, and *Enterobacter*, consisting of 23, 25, 13, and 16 isolates, respectively, were the dominant genera identified.

IAA-producing species

A total of 90 IAA-producing endophytic bacteria were obtained from the 3 growth stages. IAA production ranged between 7.45 and 162.90 mg/L^{-1} (OD_{600})$^{-1}$ (Table 4). Quantitative analysis revealed that samples with high levels of IAA production included *Pseudomonas*, *Pantoea agglomerans*, *Aeromonas*, *Serratia*, *Enterobacter asburiae*, and *Rhizobium*.

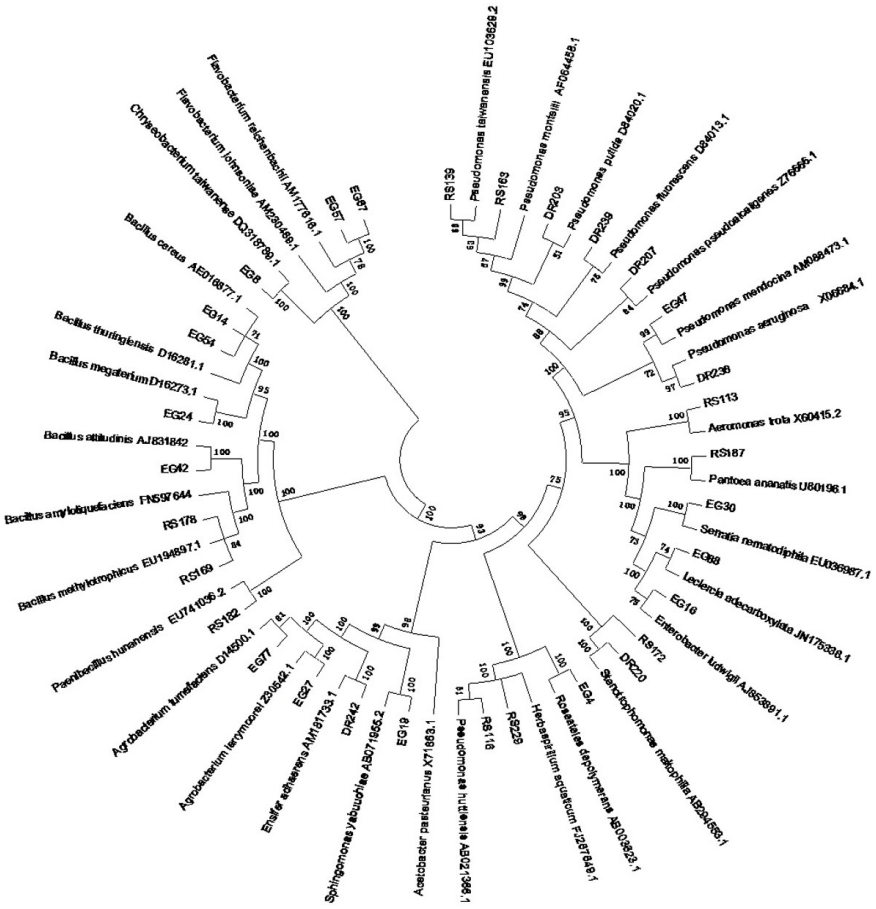

Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of endophytic bacteria and the closest type strain for each isolate.

Antibacterial effects of fresh ginger juice

The experimental results showed that only a few endophytic bacterial strains were inhibited by fresh ginger juice. Most of the strains were isolated from the seedling stage, including *Roseateles depolymerans*, *Chryseobacterium taiwanense*, *Enterobacter ludwigii*, *Agrobacterium larrymoorei*, *Pseudomonas fluorescens*, and *B. amyloliquefaciens*. These results indicate that most endophytic bacteria isolated from ginger may be used to internally colonize ginger. At the seedling stage of ginger, when the tuber is just beginning to grow, numerous endophytic bacteria may colonize the root and be transported into the plant because of relatively low gingerol levels. Gingerol content increases with the growth of ginger, resulting in the inhibition of certain endophytic bacteria. Gingerol levels are high during the stem and leaf vigorous growth stage and rhizome enlargement stage. Thus, endophytic bacteria obtained at these stages are not inhibited by fresh ginger juice.

Table 4. IAA production by endophytic isolates.

	Isolates	IAA production [$mg \cdot L^{-1} \cdot (OD_{600})^{-1}$]		Isolates	IAA production [$mg \cdot L^{-1} \cdot (OD_{600})^{-1}$]
P. fluorescens	EG81	88.96 ± 0.2	*A. tumefaciens*	EG1	16.14 ± 0.3
	DR239	81.62 ± 0.4		EG3	9.10 ± 0.2
	EG91	92.33 ± 0.7		EG6	11.00 ± 0.3
	RS95	80.83 ± 0.2		EG28	12.23 ± 0.3
	RS133	86.09 ± 0.3		EG48	12.46 ± 0.4
	DR224	99.07 ± 0.2		EG49	9.22 ± 0.2
	DR246	81.63 ± 0.3		EG62	15.86 ± 0.3
P. taiwanensis	RS139	28.15 ± 0.3		EG77	13.28 ± 0.3
	RS142	26.02 ± 0.4		EG79	18.33 ± 0.4
	DR216	27.58 ± 0.5		EG90	11.45 ± 0.6
P. putida	DR203	26.71 ± 0.4		RS193	5.64 ± 0.4
	DR208	27.11 ± 0.3		DR234	9.27 ± 0.5
P. mendocina	DR207	60.87 ± 0.2	*E. adhaerens*	DR241	45.19 ± 0.4
P. pseudoalcaligenes	DR236	45.99 ± 0.3		DR242	40.20 ± 0.5
	DR238	46.81 ± 0.3	*S. yabuuchiae*	EG19	46.62 ± 0.3
E. ludwigii	EG16	96.87 ± 0.4		RS157	48.91 ± 0.2
	EG51	114.52 ± 0.6		DR213	47.51 ± 0.3
	EG55	90.12 ± 0.2	*P. huttiensis*	EG36	45.05 ± 0.2
	EG76	99.21 ± 0.5		EG45	30.94 ± 0.2
	EG86	97.99 ± 0.3		RS116	37.56 ± 0.1
	EG87	94.68 ± 0.5		RS125	37.64 ± 0.2
	RS98	93.18 ± 0.4		DR240	52.15 ± 0.2
	RS101	104.49 ± 0.5		DR248	41.50 ± 0.1
	RS103	121.93 ± 0.3	*H. aquaticum*	DR229	85.55 ± 0.2
	RS108	105.16 ± 0.2	*R. depolymerans*	EG4	12.45 ± 0.2
	RS109	96.80 ± 0.3		EG7	15.18 ± 0.3
	RS111	162.90 ± 0.3		RS114	10.97 ± 0.2
	RS115	111.19 ± 0.2		RS162	17.75 ± 0.3
	RS132	103.92 ± 0.3	*F. reichenbachii*	EG67	27.25 ± 0.2
	RS153	123.40 ± 0.2	*F. johnsoniae*	EG57	72.50 ± 0.2
	RS161	148.48 ± 0.3		EG61	63.23 ± 0.2
L. adecarboxylata	EG88	77.84 ± 0.2	*C. taiwanense*	EG8	76.41 ± 0.3
	EG92	72.78 ± 0.3	*B. megaterium*	EG24	126.84 ± 0.2
S. nematodiphila	EG30	77.10 ± 0.2		RS136	120.80 ± 0.2
	EG31	73.64 ± 0.2		RS167	122.02 ± 0.2
	RS144	75.82 ± 0.2		RS168	123.46 ± 0.4
Pantoea ananatis	RS187	78.85 ± 0.2	*B. thuringiensis*	EG54	8.88 ± 0.2
A. pasteurianus	RS172	18.50 ± 0.2		EG82	3.95 ± 0.3
	RS175	20.11 ± 0.1		EG93	2.58 ± 0.2
S. maltophilia	DR220	15.45 ± 0.2		RS201	4.97 ± 0.3
	DR211	10.31 ± 0.2		DR202	8.76 ± 0.2
	DR212	10.21 ± 0.2		DR206	2.26 ± 0.1
A. trotsa	RS113	86.04 ± 0.2		DR209	3.62 ± 0.2
	RS150	91.69 ± 0.3		DR214	3.97 ± 0.4
A. larrymoorei	EG27	80.78 ± 0.2	*P. hunanensis*	DR182	17.32 ± 0.2

Data are reported as means ± SE from triplicate samples.

DISCUSSION

Endophytic bacteria are important constituents of the plant micro-ecological system, providing resources that promote crop growth and plant disease prevention (Forchetti et al., 2010). In this study, a large number of endophytic bacterium species from a diverse range of genera were found in ginger plants. Based on the clustering results of ARDRA and 16S rRNA gene sequencing, 107 isolates were separated into 16 different genera and at least 31 different species, representing a wide variety of endophytic bacteria. The results indicate that the numbers and types of endophytic bacteria present decrease as the host ginger grows, which supported the results obtained from the colony count and 16S rDNA sequencing data.

The most dominant genera were *Bacillus* and *Pseudomonas*, both of which were found in all 3 growth stages. *Chryseobacterium*, *Flavobacterium*, *Serratia*, and *Leclercia* were only found in the seedling stage. *Paenibacillus*, *Aeromonas*, *Acetobacter*, and *Pantoea* were only found in the stem and leaf vigorous growth stage. *Herbaspirillum*, *Ensifer*, and *Stenotrophomonas* were only found in the rhizome enlargement stage. The seedling stage yielded the highest number of endophyte species, whereas the rhizome enlargement stage yielded the least. Colony counting showed that the maximum density of endophytic bacteria appears during the seedling stage. In contrast, the minimum density of bacteria appeared in the rhizome enlargement stage. Chu et al. (2011) isolated 23 endophytes from a ginger tuber cultivar, 8 of which were sequenced and analyzed. In their study, the strains fell into the following genera: *Pseudomonas* spp, *Bacillus* spp, *Brachybacterium* spp, *Stenotrophomonas* spp, and *Rahnella* spp. In comparison, 23 different genera were identified in the current study. Distinct changes in the quantity of the endophytic population, with a peak occurring at the stem and leaf vigorous growth stage, were observed. This observation proves that colony counts are not consistent across different stages of growth. We also found that ginger leaves have the largest number of endogenous bacteria, followed by the roots, stems, and tubers. Fluctuations in the number of retrieved endophytic microorganisms seem to be influenced by both the host plant and the surrounding environment.

Ninety IAA-producing isolates and 17 antagonistic strains were isolated and screened from the ginger plants during the 3 growth stages. A significant change in bacterial diversity was observed among the growth stages. Six *Bacillus* spp (Table 4) were isolated in this study, with their sequences aligning with those of *B. amyloliquefaciens*, *B. methylotrophicus*, *B. thuringiensis*, *B. altitudinis*, *B. cereus*, and *B. megaterium*. The results showed that *B. cereus*, *B. methylotrophicus*, and *B. amyloliquefaciens* exhibit antimicrobial activities against *P. myriotylum* Drechsler. *B. altitudinis*, which also has a broad antibacterial spectrum, exhibited antimicrobial activity against *P. zingiberi* Hori (Xu et al., 2012). *B. thuringiensis*, which is extensively used in the biological control of insects, exhibited the lowest IAA production in this study (Vilas-Bôas et al., 2007). The most active IAA producer was *B. megaterium*, which produced 126.84 $mg \cdot L^{-1} \cdot (OD_{600})^{-1}$ IAA. This result supports a previous report by Ali et al. (2009), in which *B. megaterium* was observed to promote the growth of red clover plants, either individually or in combination, with *Rhizobium leguminosarum*.

P. aeruginosa and *P. monteilii* exhibited antimicrobial activity against *P. myriotylum* Drechsler and *P. zingiberi* Hori. Of these 2 bacteria, *P. monteilii* exhibited a higher level of activity. Based on published literature, we found that *P. aeruginosa* and *P. monteilii* also have broad antibacterial spectra. *P. monteilii* has antimicrobial activities against *Cylindrocarpon destructans* of ginseng, black spot of *Dioscorea nipponica* Makino, and *P. myriotylum* Drechsler of *Schisandra chinensis* (Zhai et al., 2012). *P. aeruginosa* exhibits antimicrobial activities against *Ralstonia solanacearum* of tobacco (Dong et al., 2011). Other *Pseudomonas* isolates from the present study, including *P. fluorescens*, *P. taiwanensis*, *P. mendocina*, *P. putida*, *P. pseudoalcaligenes*, and *P. huttiensis*, also produce IAA at varying levels. *P. putida* and *P. fluorescens* exhibit antagonistic effects on the tobacco mosaic virus (Wu et al., 2008; Guo et al., 2011).

Previous studies have reported that some IAA-producing endophytic bacteria, such as *R. depolymerans*, *Herbaspirillum aquaticum*, *Sphingomonas yabuuchiae*, and *Agrobacterium tumefaciens*, have associated nitrogen-fixation functions (Kanvinde and Sastry, 1990; An et al., 1999; Hu et al., 2007; Liu et al., 2011). We also screened *E. ludwigii* in ginger, which produced both IAA but also ACC deaminase, and showed antimicrobial activity against *E. coli* and *B.*

subtilis (Gong et al., 2011). Endogenetic bacteria with ACC deaminase play important roles in alleviating plants subject to various stressors, such as drought, water logging, salt, heavy metal, and pressure (Zahir et al., 2008). Other studies have also reported that *E. ludwigii* in vetiver grass has associated nitrogen-fixation functions, while *E. ludwigii* in cotton and tobacco exhibits antimicrobial activity against *Fusarium* and *Verticillium* (Li et al., 2009; Zhao et al., 2009).

Other endophytic bacteria in ginger that also produce IAA include *C. taiwanense*, *Flavobacterium johnsoniae*, *F. reichenbachii*, *A. larrymoorei*, *Aeromonas trotsa*, *Acetobacter pasteurianu*s, *Stenotrophomonas maltophilia*, *Serratia nematodiphila*, *Leclercia adecarboxylata*, and *Pantoea ananatis*. Members of the genus *Chryseobacterium* are relatively common, and have been reported to be present in asparagus lettuce, wheat, and ramie, among other plants (McSpadden Gardener and Weller, 2001; Young et al., 2005; Shao et al., 2010). The present study is the first to report *Chryseobacterium* as an endophytic bacterium of ginger. *F. johnsoniae* is found ubiquitously in nature including inhabiting plants, soil, and water, and plays a significant role in the natural material circulation process. They can degrade various bio-macromolecules, such as chitin, glucose, and protein, and play an important role in the natural recycling of substances.

The roles that the endophytic bacteria identified in ginger play toward promoting plant growth have yet to be determined; hence, further experiments are required. This paper revealed changes in endophytic bacterium density and distribution at different growth stages of ginger, in addition to analyzing the antistatic ability of these bacterial groups and their ability to produce IAA. This study serves as a reference for strain resources for the recorded biological agents, and provides a theoretical basis for their subsequent use.

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