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An Endophytic Bacterium Synthesizing Homologous Fragrant Compounds as Its Host Plant

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An endophytic bacterial strain from fresh flower of *Gardenia jasminoides*, ZZB08, was identified as *Bacillus vallismortis* based on morphologic, physiological, biochemical characteristics, and 16S rDNA sequence analysis. By method of SPME-GC/MS, 17 and 18 compounds with relative content (RT) more than 0.5% were respectively detected from flower and the culture of ZZB08, which covered a wide range of chemical types. Comparatively, five major volatile compounds of flower, linalool, a-farnesene, trans-b-ocimene, z-3-hexenyl tiglate and methyl tiglate, were also found in culture of the ZZB08 but with lower concentrations than that in the flower. This is the first report on the endophyte which could produce fragrant volatiles similar as its host.

Key words: *Bacillus vallismortis*; *Gardenia jasminoides*; endophytic bacterium; volatile; flower.

Gardenia jasminoides J. Ellis (Rubiaceae) is a popular ornamental plant with white and sweet fragrant flowers[1]. For a long time, flowers of *G. jasminoides* have universally been used for manufacturing of perfume[8]. However, relying on the flower uniquely as material limits the yield of perfume due to the flowering once a year of the plants. Finding alternative resource from microorganisms for perfume production is considered.

Endophytic bacteria live in plant tissues without causing substantive harm to their hosts[10]. Previous investigations indicated that endophytic bacteria exist in a variety of tissue types within a broad range of plants[16]. It has been hypothesized and partially demonstrated that some endophytes can synthesize the same or homologous chemicals of their plant hosts[18]. A typical example is the endophytic fungi from *Taxus* spp. could produce taxol[12]. Although numerous studies concerning bacterial endophytes from different tissues of various plants have been reported[11], rare attention was focused on that from flower. During a survey on the biodiversity of endophytic bacteria from fragrant flowers, ZZB08, a bacterial strain producing sweet flavor was isolated from flower of *G. jasminoides*. Here, we identified the ZZB08 and characterized its volatile compounds comparing with the host flower by method of solid-phase micro-extraction (SPME) combined with GC/MS.

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MATERIALS AND METHODS

Flower sampling and isolation of endophytic bacteria

Fresh followers of *G. jasminoides* were sampled in Kunming City in May, 2012. The samples were firstly washed thrice with sterile distilled water, then sterilized sequentially by 75% (v/v) ethanol and hypochlorite solution (3%

available Cl^-) for 30 sec, and finally washed thrice again with sterile distilled water. After surface sterilization, flower materials were homogenized in a mortar containing suitable sterile distilled water to obtain a concentration of 10^{-2}. After filtration to remove the tissue, a volume of 200 µL suspension was spread on a plate containing BPN (L^{-1}: beef extract 3 g, peptone 10 g, NaCl 5 g, agar 20 g, water 1,000 ml, pH 7.2). Ten plates for each sample were preformed. After 48 h incubation at 37 ºC, bacterial colonies were purified randomly, and those producing fragrant flavor were selected for further studies.

Bacterial identification

All physiological characteristics were tested with cells cultured using medium of Luria-Bertani (LB) with pH 7-8 at 28 °C. Gram staining was performed as described by Gregersen[4]. Oxidase activity was detected using API oxidase reagent according to the manufacturer's instructions. Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2. The oxidation of carbon source was performed using Biolog GEN III system (MicroPlate). Cell morphology and motility were observed by light microscopy (BH-2; Olympus) and transmission electron microscopy (H-7650; Hitachi). Growth at different pH (5-10, in increments of 1.0 pH units), temperatures (4, 10, 15, 20, 28, 37, 45, 50 and 55 °C) and sodium chloride regimes (0-12 % w/v, at intervals of 1%) was determined using LB agar plates incubated for up to 5 days.

Extraction of genomic DNA and amplification of the 16S rRNA gene were performed as described by Li *et al*. (13). The 16S rRNA gene sequences were aligned with representative bacterial sequences from the GenBank database by using ClustalX (20) and then manually adjusted. Distance matrices and phylogenetic trees were calculated by the Kimura two-parameter model (9) and neighbor-joining algorithms using the program MEGA (version 4) by bootstrap analysis of 1,000 replications (19). The tree was rooted using *Thermoactinomyces vulgaris* VTTE-062992 as outgroup.

Chemical analysis of bacterial culture and flower

Bacterium was cultured using BPN medium and their volatiles were extracted by SPME as described by Díaz *et al*. (3) with minor modification. Briefly, 75 ìM fibers (Supelco, Bellefonte, PA, USA) used for SPME were first equilibrated with helium at 250 ÚC for 15 min. Extractions were performed inside 15 mL Supelco SPME vials filled with 9 ml bacterial culture and under the conditions of 40 ÚC for 1 h. Volatiles from BPN medium were used as control. For flower, the SPME fiber was inserted directly into the vial containing one fresh flower, and extraction was carried out at room temperature for 12 h. After extraction, the SPME fiber was directly inserted into the front inlet of GC-MS (MS, HP 5973, GC/MS: Agilent Technologies, USA) and desorbed at 250 °C for 2 min. GC conditions were the same as Xu *et al*. (21). The volatile compounds were identified based on a comparison of the mass spectrum of the substance with GC/MS system data banks (Wiley 7 and NBS 75 k library).

RESULTS

Bacterial identification

Among the endophytic bacteria from flowers of *G. jasminoides*, a strain designated ZZB08 producing sweet flavor on BPN medium was obtained. Cells of ZZB08 are motile, gram positive, bacilli with size of 0.71-1.0×1.88-4.27 ìm, occur singly and in short chains. Agar colonies are opaque, smooth, circular, entire, and 1.0-2.0 mm in diameter after growth at 28°C for 48 h. Growth occurs at 10-50°C, pH 6.0-9.0 and NaCl concentrations of 0-5 %. The optimal growth pH, temperature and NaCl concentration were 27-35°C, pH 7.0-8.0 and 0-2 %, respectively. Catalase and oxidase are positive. Growth is aerobic. Nitrate is reduced to nitrite. Starch and casein are hydrolyzed. Citrate is utilized, but propionate is not utilized. The 16S rDNA sequence of strain ZZB08 had been deposited in GenBank under the accession number JQ765433. Sequence comparison *via* BLAST searches against sequences from the GenBank, EMBL or DDBJ databases revealed that strain ZZB08 had a close relationship with members of the genus *Bacillus*, which was classified in the family *Bacillaceae*. The ZZB08 was most closely related to *B. vallismortis* with sequence similarity of 99.25%. Moreover, in a neighbour-joining phylogenetic tree based on 16S rDNA sequences, strain ZZB08 formed a stable clade with *B. vallismortis* (Fig. 1).

On the basis of morphologic, physiological, biochemical characteristics, and 16S rDNA sequence analysis, strain ZZB08 was identified as *Bacillus vallismortis*.

Comparison of the volatile compounds between flower and strain ZZB08

By method of SPME-GC/MS, 17 and 18 compounds with relative content (RT) more than 0.5% were respectively detected from flower and culture of ZZB08 (Table 1). These 35 chemical constituents covered a wide range of acids, alkenes, alcohols, aromatics, benzenes, esters, heterocyclics, ketones, monoterpenoids, monoterpene alcohols and sesquiterpenes. Major volatiles of the flower were linalool (RT = 35.74%), a-farnesene (11.43%), trans-b-ocimene (10.17%) and z-3-hexenyl tiglate (9.68%), in which their total RT values accounted to 67.02% of the all volatiles. While, the abundant compounds of the ZZB08 were 2-butanone, 3-hydroxy- (36.82%), acetone (9.12%), 2-thiophenecarboxylic acid, undec-10-enyl ester (8.39%) and linalool (5.28%). The four

Table 1. Volatile components from flower of *G. jasminoides* and *B. vallismortis* ZZB08

S. No.	RT (min)	Compound	Group	Formular	Molecular Weight	Relative content (>0.50%)	
						Flower	ZZB08
1	2.24	trans-1, 4-hexadiene	alkenes	C_6H_{10}	82	1.47	-
2	2.56	cis-3-Hexenol	alcohols	$C_6H_{12}O$	100	0.69	-
3	2.59	methyl tiglate	esters	$C_6H_{10}O_2$	114	3.34	0.86
4	2.64	3-hexanol, 4-methyl-	alcohols	$C_7H_{16}O$	116	-	2.66
5	3.57	toluene	benzenes	C_7H_8	92	3.26	-
6	4.82	trans-b-ocimene	monoterpenes	$C_{10}H_{16}$	136	10.17	2.33
7	5.35	acetone	ketones	C_3H_6O	58	-	9.12
8	6.03	3-hexen-1-ol	alcohols	$C_6H_{12}O$	100	0.81	-
9	6.16	linalool	monoterpene alcohols	$C_{10}H_{18}O$	154	35.74	5.28
10	6.24	p-xylene	benzenes	C_8H_{10}	106	3.97	-
11	7.95	2,3-butanedione	ketones	$C_4H_6O_2$	86	-	1.13
12	9.03	oxetane,2,2-dimethyl-	heterocyclics	$C_5H_{10}O$	86	-	0.60
13	11.25	b-myrcne	monoterpenes	$C_{10}H_{16}$	136	5.24	-
14	11.74	z-3-hexenyl tiglate	esters	$C_{11}H_{18}O_2$	182	9.68	2.15
15	12.79	L-limonene	monoterpenes	$C_{10}H_{16}$	136	2.43	-
16	13.09	2-butanone,3-hydroxy-	ketones	$C_4H_8O_2$	88	-	36.82
17	13.42	cis-ocimene	monoterpenes	$C_{10}H_{16}$	136	1.28	-
18	14.82	trans-caryophyllene	sesquiterpenes	$C_{15}H_{24}$	204	1.46	-
19	15.63	methyl benzoate	aromatics	$C_8H_8O_2$	136	2.27	-
20	16.07	2,3-butanediol	alcohols	$C_4H_{10}O_2$	90	-	0.66
21	16.71	a-farnesene	sesquiterpene	$C_{15}H_{24}$	204	11.43	3.54
22	18.04	butanoic acid,3-methyl-	acids	$C_5H_{10}O_2$	102	-	1.27
23	18.55	hexanoic acid, 2-methyl-	acids	$C_7H_{14}O_2$	130	-	1.30
24	19.28	1,3,8-p-menthatriene	monoterpenes	$C_{10}H_{10}$	134	2.15	-
25	21.36	2-heptanone	ketones	$C_7H_{14}O$	114	-	1.47
26	26.77	butanoic acid, 3-methoxy-	acids	$C_5H_{10}O_3$	118	-	1.56
27	31.25	cis-3-hexenyl butyrate	esters	$C_{10}H_{18}O_2$	170	0.61	
28	34.23	2-furancarboxylic acid,nonyl ester	esters	$C_{14}H_{22}O_3$	238	-	1.74
29	34.87	2-thiophenecarboxylic acid, undec-10-enyl ester	esters	$C_{16}H_{24}O_2S$	280	-	8.39
30	52.09	1H-indene-1,3(2H)-dione,2-hydroxy-2-(9-methoxy-9H-fluoren-9-yl)-	aromatics	$C_{23}H_{16}O_4$	356	-	1.99

Note: - not identified; RT retention time

counted up to 59.61% of the volatiles produced by the bacterium.

Comparatively, five major volatile compounds of flower, linalool (RT = 35.74%), a-farnesene (11.43%), trans-b-ocimene (10.17%), z-3-hexenyl tiglate (9.68%) and methyl tiglate (3.33%) were also found in culture of the ZZB08 but with lower concentrations than that in flower, in which their RT values were 5.28%, 3.54%, 2.33%, 2.15% and 0.86%, respectively. Additionally, those compounds of ketones and acids produced by ZZB08 were not found in the flower sample.

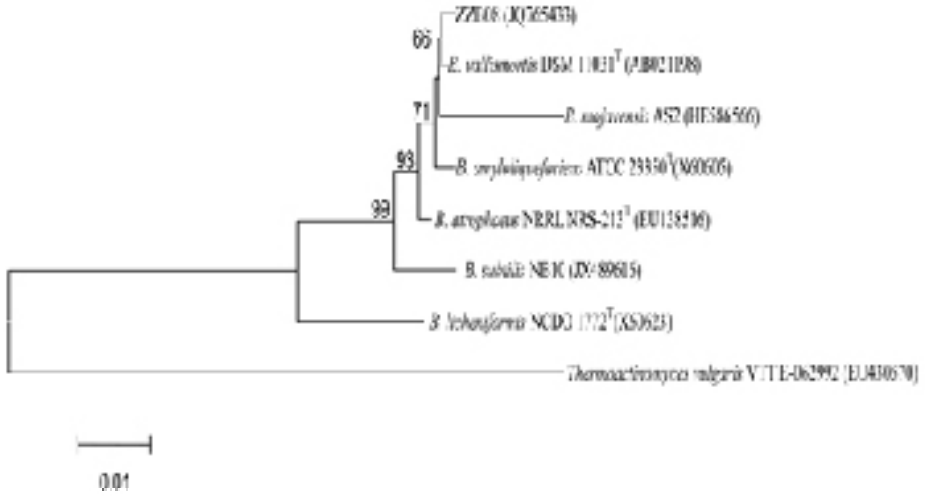

Fig. 1. Neighbour-joining tree derived from aligned 16S rDNA sequences, showing the position of strain ZZB08 in the genus *Bacillus*. *Thermoactinomyces vulgaris* was used as outgroup. Bar, 0.01 substitutions per nucleotide position

DISCUSSION

We identified a fragrant endophytic bacterium, *B. vallismortis* ZZB08, from fragrant flower of the plant *G. jasminoides*. *B. vallismortis* was firstly isolated from soil in Death Valley Californial and as a novel taxon was reported[17]. Strains of this species with different functions have previously been reported from other environments. *B. vallismortis* BIT-33 from seawater samples could produce cytotoxic compound and showed direct cytotoxic and apoptotic effects on colon cancer cells[7]. *B. vallismortis* C89 from the South China Sea sponge *Dysidea avara* could produce compounds of neobacillamide A and bacillamide C[22]. *B. vallismortis* BCCS 007 from the Maharla salt lake of Iran could produce lipase[5]. *B. vallismortis* Ace02 from the traditional Korean condiment Chungkook-jang should be used for prevention of dental caries as well as being an effective thrombolytic agent. (Kim *et al*., 2007). *B. vallismortis* JY3A from the polluted soil could remove 90.5% of pyrene[14]. As an endophyte, *B. vallismortis* ZZ185 was isolated from healthy stems of broadleaf holly *Ilex latifolia*, and exhibited a broad antifungal functions against phytopathogens by produced the compounds of bacillomycin mixtures[23]. Endophytic bacteria have been found in virtually every plant and tissue types studied. However, endophytes colonizing plant reproductive organs have been rarely investigated, especially for the flower[2]. To our knowledge, *B. vallismortis* ZZB08 was the first endophyte which could produce fragrant volatiles similar to that of its host.

As a famous fragrant plant, the volatile constituents from fresh flower of *G. jasminoides* had been investigated previously. By method of headspace-SPME-GC/MS, Liu and Gao (15) identified 54 volatile compounds from fresh flower of *G. jasminoides*, in which farnesene (RT=64.87%), cis-ocimene (29.33%) and linalool (2.74%) were the major constituents. By same method, Chaichana *et al*.[1] identified 23 compounds from fresh flower of *G. jasminoides*, in which the major chemical constituents were linalool (38.23%), farnesene (21.40%), z-3-hexenyl

tiglate (16.27%) and trans-b-ocimene (9.50%). By method of adsorption wire- GC/MS, Huang *et al*. (6) reported that fresh flower of *G. jasminoides* could generate 86 volatile constituents with the major compounds of linalool (43.05%), b-myrcene (8.32%), methyl benzoate (7.61%) and p-xylene (7.17%). In this study, 17 were detected from *G. jasminoides* flower with major volatiles of linalool (with RT of 35.74%), a-farnesene (11.43%), trans-b-ocimene (10.17%) and z-3-hexenyl tiglate (9.68%). The differences of volatile constituents and their relative contents among different investigations maybe related to the performance conditions used in headspace- SPME-GC/MS and the flower samples analyzed. However, based on the results above, linalool, farnesene, ocimene and z-3-hexenyl tiglate as the major constituents of *G. jasminoides* flower should be confirmed.

In this study, we identified 18 volatiles from then endophytic bacterium *B. vallismortis* ZZB08. Of them, five major volatile compounds of flower, linalool, a-farnesene, trans-b-ocimene, z-3-hexenyl tiglate and methyl tiglate, were also found in culture of the ZZB08. Though with lower concentrations of the major volatiles than that in the flower, the *B. vallismortis* ZZB08 showed the potential as the alternative of *G. jasminoides* flower for perfume manufacturing in future.

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