African Journal of Biotechnology Vol. 8 (21), pp. 5873-5882, 2 November, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Plasmids profiles, antibiotic and heavy metal resistance incidence of endophytic bacteria isolated from grapevine (*Vitis vinifera* L.)

Abdulla D. Altalhi

Biological science department, Faculty of Science, Taif University, Taif, Saudia Arabia.
E-mail: aaltalhi2001@hotmail.com.

Accepted 2 October, 2009

Little is known about the bacterial communities associated with the plant inhabiting desert ecosystem. In this study, the bacterial population associated with grapevine (*Vitis vinifera* L.) plant, growing desert soil was analyzed using the culture dependent approach. A total of 111 bacterial isolates were isolated from stems and leaves samples, collected from different locations and subjected to further analyses. Based on the identification methods, the bacterial isolates were grouped into 14 genera. The main genera are *Acetobacter*, *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Methylococcus*, *Xanthomonas*, *Vibrio*, *Bacillus*, *Micrococcus*, *Planococcus*, *Staphylococcus* and *Streptomyces.* Significant differences in the endophytic communities were observed between plants collected from different sites and also between plant stems and leaves. All the isolates were examined for plasmid DNA content and resistance to antibiotics (Ampicillin, Kanamycin, Tetracyclin) and heavy metals. Minimum inhibitory concentrations (MICs) of Cu, Cd, Hg, Mn, Ni and Zn for isolates were also determined. Resistance was most frequent to Ampicillin (57%), followed by Kanamycin (53%) and Tetracycline (26%). The highest MICs observed were 10 µg/ml for mercury, 50 µg/ml for Cu and Cd and 200 µg/ml for other metals. On a percentage basis, 18.48% of total strains from leaves were found to harbour plasmids, whereas, 11.83% of the roots isolates contained plasmids.

Key words: Endophytic bacteria, antibiotic resistance, heavy metals, plasmids.

INTRODUCTION

Bacterial endophytic species are common inhabitants of a wide range of plant species and reside either within cells (Jacobs et al., 1985), in the intercellular space (Patriquin and Dobereiner, 1978), or in vascular systems (Bell et al., 1995) of a plant. Microbial endophytes are typically defined as microorganisms that do not visibly harm the host plant but can be isolated from surface-disinfested plant tissues or the inner parts of plant organ (Hallmann et al., 1997). Bacterial endophytes seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Significant variations in the populations of both indigenous and introduced endophytes have been reported. These variations are attributed to plant source, plant age, tissue type and environment (Zinniel et al., 2002).

The potential to use plant-bacteria association or endophytes to remediate polluted soils has attracted considerable interest (Idris et al., 2004). Bacterial endophytes can stimulate contaminant disappearance by the accumulation and transformation of heavy metals and some xenobiotic compounds. Several authors have investigated the role of endophytes in phytoremediation and they have found that certain plant-bacterial associations can increase bioremediation processes (Burd et al., 2000).

While there have been many studies on the microbial diversity associated with the plants of terrestrial environment (Kobayashi and Palumbo, 2000), virtually little information is available about the microbial diversity associated with plants in desert environments with special attention to the bacterial endophytes of grapevine.

My research goal was to determine the prevalence,

properties, persistence and types of endophytic bacteria in grapevine. An additional goal was to identify and characterize the plasmid complement in several strains of the endophytic bacteria as a first step for determination of whether they have a role in the endophytic phenotype.

MATERIALS AND METHODS

Plant sample processing and isolation of endophytic bacteria

The host plant used in this study was grapevine (*Vitis vinifera* L.). This plant grows in the west Taif governate, Saudia Arabia. Grapevine plant was removed from soil with a trowel placed in plastic bags and immediately taken to the laboratory. Leaves and stems were washed in running tap water and graded by their size and surface appearance in order to exclude samples that showed symptoms of diseases or superficial damage. Endophytic bacteria were isolated from internal plant tissues using surface-trituration-plating technique. This technique involves immersing tissue samples in a sequence of 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl-) for 4 min, 70% ethanol for 30 s and rinsed three times in sterile distilled water (Kuklinsky-Sobral et al., 2004). To ensure that the surfaces were sterile, samples were imprinted on tryptic soy agar plates (10% TSA) and water from the final washing step was spread on tryptic soy agar plates. After surface disinfection, the leaves or stems tissues were cut and triturated in 20 ml of sterile 20 mM potassium phosphate buffer, pH 7 and allowed to stand at room temperature for 20 min, after which 100 µl of 10- fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}) of suspension in sterile buffer was plated on Plate Count Agar (PCA, Difco Laboratories, Detroit, MI) at full strength for colony forming units (CFU) enumeration. The duplicate dilution plates were incubated at 28°C, colony forming units (CFU) per gram were scored after 5 days because some slow growing bacteria took more than 3 days to appear. Representative bacterial colonies were selected from dilution plates based on colony size, shape, morphology and color and purified by restreaking onto fresh plates of the same medium used for primary inoculations. These colonies were also cultivated in 10% TSA, incubated at 28°C for 18 h and following each culture, were suspended in 20% glycerol solution and stored at -70°C.

Identification of bacteria

Colonies representing the most numerous members in each sample were subcultured on PCA by streaking on the same fresh medium and incubated at 30°C for 3 days. The identification of strains was carried out by using the classification given in the Bergey's Manual of systematic Bacteriology (Krieg and Holt, 1984). The strains were further identified with API 20 NE strips (biomerieux, Marcy l'Etoile, France).

Exoenzyme activity tests

The tests included (i) starch hydrolysis on starch plates (Claus, 1988); (ii) lipid hydrolysis using both egg yolk agar (Claus, 1988) and Tributyrin agar (iii) proteolysis as hydrolysis of skim milk (Claus, 1988) and gelatin (Biling, 1970); (iv) cellulose degradation (Farkas et al., 1985); (v) pectolysis with either polygalaturonate or sodium polypectate as substrates after the method of Collmer et al. (1988); and (vi) chitin hydrolysis as described by Zhou et al. (1999).

Antibiotic resistance test

The test isolates were spot-inoculated on nutrient agar plates incurporated with filter-sterilized ampicillin, kanamycin and tetracycline, at the rate of 100, 50 and 10 µg/ml respectively and incubated for 48 h at 30°C. The antibiotics resistance was recorded as positive if the test colony appeared on the plates, as compared to the control plate in which no antibiotic was added.

Motility test

Each isolate was spots-inoculated on the center of semi-solid nutrient agar plates (0.2% agar) and incubated at 30°C. The diffusion of colony was observed and recorded at 24 h (Elbeltagy et al., 2000).

Determination of minimum inhibitory concentrations (MIC) of heavy metals

The MIC of the metal for each isolate was determined by the plate dilution method as adopted by Summers and Silver (1972). The metals, Hg^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and zin^{2+} were used as $HgCl_2$, $CdCl_2$, $CuCl_2.2H_2O$, $PbCl_2$, $NiCl_2$ and $ZnCl_2$ in different concentrations ranging from 5 to 1000 ppm. Stock solutions of the metal salts were prepared in double distilled water and were added to sterilized nutrient agar plates. In each test, 5 µl of a liquid broth overnight broth culture (containing approximately 2 - 4 x 10^5 CFU) was applied onto duplicate agar plates containing the appropriate heavy metal salts and incubation was at 30°C for 3 days. The lowest concentration of the metals, which inhibits the bacterial growth, was considered as MIC. Since there is no defining concentration of metal ions which can be used to distinguish metal resistant bacteria and metal sensitive bacteria, the concentration used in this study has been employed in similar studies reported on soil bacteria (Trevors et al., 1985; Malik and Ahmed, 2002; Malik and Jaiswal, 2000).

Plasmid isolation

Several plasmid isolation methods and modification of these methods were used including: alkaline lysis (Ausubel et al., 1987), the method of Kado and Liu (1981), Brenner et al. (1993) and Hansen and Olsen (1978). The isolated plasmids were characterized by agarose gel electrophoresis according to standard procedure (Maniatis et al., 1989). The size estimates of the isolated plasmids were obtained by comparing their relative mobilities on agarose gel with standard molecular weight DNA marker.

RESULTS

Incidence of endophytic bacteria isolated from stems and leaves of grapevine (*Vitis vinifera* L.)

The endophytic bacterial communities of healthy looking leaves and stems of grapevine (*V. vinifera* L.) were assessed in surface disinfested plant parts upon cultivation in TSA medium. During this study, a total of 111 isolates of endophytic bacteria were recognized in both leaves and stems of grapevine. Preliminary characterization of these isolates indicated that studied parts of tested contained both gram-negative and gram-positive bacteria.

Table 1. Frequency (%) and genus diversity of endophytic bacteria recorded from young and old organs of Grapevine (*Vitis vinifera L.*).

Bacterial strains	Stems		Leaves	
	Young	Old	Young	Old
Gram-negative				
Acetobacter	0	1.47	2.32	4.41
Acinetobacter	5.88	0	0	0
Citrobacter	0	0	0	0
Enterobacter	2.94	0	0	4.41
Erwinia	0	0	0	4.41
Escherichia	0	0	0	0
Methylococcus	0	1.47	0	0
Vibrio	0	1.47	0	0
Xanthomonas	0	0	0	0
Gram-positive				
Bacillus	17.64	11.47	11.76	19.11
Micrococcus	0	0	0	0
Planococcus	0	0	1.47	1.47
Staphylococcus	0	0	2.94	0
Streptomyces	1.47	0	0	0

Identification of bacteria and frequency of occurrence

A more extensive phenotypic characterization was carried out with 111 different isolates of endophytic bacteria isolated from different organs (stems and leaves) of Grapevine plants collected from different locations. As whenever possible, the bacterial isolates were identified to the species level, but most isolates were only identified to the genus level.

The fourteen bacterial genera identified in a survey of 111 isolates; *Acetobacter*, *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Methylococcus*, *Xanthomonas*, *Vibiro*, plus gram-positive bacteria; *Bacillus*, *Micrococcus*, *Planococcus*, *Staphylococcus* and *Streptomyces* were the most predominant (Table 1). There appears to be differences in the frequency of occurrence of specific genera of bacteria between the plant organs, age and the water sources from which the plants were collected.

The results in Table 1 revealed that four genera (1 gram-negative and 3 gram positive) were identified in young leaves compared to five genera (3 gram-negative and 2 gram-positive) recorded in old leaves collected from different locations. Among gram-negative bacteria, *Acetobacter* was recorded from both young and old leaves with different frequencies (2.32 and 4.41%, respectively). However, *Enterobacteor* and *Erwinia* were recorded only in old leaves. Of gram-positive bacteria, *Bacillus* and *Planococcus* were found in both young leaves (frequency, 11.76 and 1.47% respectively) and old leaves (frequency, 19.11 and 1.47% respectively). However, *Staphylococcus* was isolated only from young leaves at a frequency of 2.94%.

On the other hand, three genera of endophytic bacteria (2 gram-negative and one gram-positive) were recorded in young stems compared to three genera (2 gram negative and one gram-positive) in old stems of grapevine plants collected from different locations. Two genera of gram-negative bacteria *(Acintobacter* and *Enterobacter*) were isolated from young stems and (*Acetobacter* and *Methylococcus)* were isolated from old stems. Species of *Bacillus* were the only Gram-positive isolates from young and old stems (Table 1).

The results presented in Table 1 show that three genera of endophytic bacteria were isolated from young leaves (2 gram-negative and 1 gram positive) compared to four genera were isolated from old leaves (3 gram-negative and 1 gram positive) of grapevine plants collected from irrigation canals. *Bacillus* and *Enterobacter* were isolated from young and old leaves with different frequencies. However, *Acintobacter* and *Xanthomonas* were found only in old leaves and *Acetobacter* in young leaves.

On the other hand, three genera were recorded in young stems (1 gram-negative and 2 gram-positive) compared to 4 genera (3 gram-negative and one gram-positive) in old stems of grapevine (*V. vinifera* L.) plants collected from different locations. Among gram- negative bacteria, only *Enterobacter* was isolated from young and old stems. However, *Citrobacter* and *Escherichia* were isolated only from old stems (Table 1). Gram-positive bacteria were represented by *Bacillus* sp. in both young and old stems, whereas, *Planococcus* was only recovered from young stems.

Table 2. Endophytic bacterial population density (CFU g^{-1} FW) recovered from leaves and stems of grapevine (*Vitis vinifera* L.).

Stems		Leaves	
Old	Young	Old	Young
7.7×10^3	1.1×10^4	4.7×10^4	2.4×10^3
5.2×10^4	1.15×10^3	1.2×10^3	1.34×10^3

Population density of endophytic bacteria

The number of colony-forming units per gram fresh weight (CFU) of culturable endophytic bacteria isolated from various plant tissues of grapevine (*V. vinifera* L.) collected from different locations (Table 2). The results showed that CFU value in old leaves (4.7×10^4) is much higher than in young leaves (2.4×10^3) of grapevine plants. In contrast, young stems (1.1×10^4) had higher CFU values than old stems (7.7×10^3).

On the other hand, the CFU value did not differ markedly between leaves and stems of Grapevine plants collected from different locations (Table 2). However, old

Table 3. Number of isolates reacted in screening.

Test	Stems		Leaves	
	Gram-positive	Gram-negative	Gram-positive	Gram-negative
Motility	41 (42)	11 (26)	21 (26)	5 (17)
Cellulase	0 (42)	0 (26)	0 (26)	0 (17)
Chitinase	0 (42)	0 (26)	0 (26)	0 (17)
Pectinase	0 (42)	0 (26)	0 (26)	0 (17)
Lipase	38 (42)	5 (26)	23 (26)	11 (17)
Amylase	2 (42)	1 (26)	2 (26)	0 (17)
Ampicilin	12 (42)	15 (26)	9 (26)	12 (17)
Kanamycin	25 (42)	14 (26)	18 (26)	11 (17)
Tetracycline	14 (42)	7 (26)	11 (26)	7 (17)

stems contained greater CFU values than young stems.

Activity of hydrolytic enzymes in endophytic bacterial population of Grapevine (*Vitis vinifera* L.)

A more extensive phenotypic characterization was carried out with 111 culturable bacterial isolates recovered from surface sterilized stems and leaves of grapevine plants collected from different locations. The *in vitro* activities of some hydrolytic enzymes of isolated strains were studied. The data presented in Table 3 revealed that all the tested strains were cellulase, pectinase, chitinase and protease negative. Amylase activity was only observed in *Vibrio, Micrococcus and Staphylococcus* strains. All the Gram positive strains except *Streptomyces* were lipase positive. In contrast, all Gram-negative strains recorded were lipase negative; however, *Enterobacter* and *Erwinia* were positive.

Antibiotic resistance

All the 111 endophytic isolates were screened for growth on LB agar amended with ampicillin 100 µg/ml, tetracycline 50µg/ml, or kanamycin 10 µg/ml. Analyses of resistance to various antibiotics are shown in Table 3. For gram-negative bacteria, resistant to ampicillin was most common among the strains isolated from plants (57%) followed by resistance to kanamycin (53%) and resistance to tetracycline (26%). Gram-positive strains demonstrated a similar pattern of antibiotics resistance. However, the isolated strains were more likely to be resistant to kanamycin than to ampicillin (Table 3)

Heavy metal resistance

All the isolates were examined for presence of plasmids and were also checked for tolerance to all metals studied. The tolerance of the plasmid-containing strains isolated from different organs of Grapevine (*V. vinifera* L.) collected from different locations expressed as MICs (The lowest concentration at which no growth was observed) is shown in Table 4.

Hg^{2+} resistance

Mercury was the most toxic since only two isolates, *Erwinia*13 isolated from old leaves and *Enterobacter* isolated from old stems, were able to grow in the presence of 10 ppm.

Pb^{2+} resistance

All the tested strains isolated from leaves and stems of Grapevine plants collected from different locations were resistant to high levels of Pb^{2+} with MIC values ranged between 800 and 1200 ppm.

Cu^{2+} resistance

Most of the tested bacterial isolates isolated from both organs are less tolerant to Cu^{2+} ions. However, *Enterobacter*26, *Escherichia*19, *Citrobacter* 54, *Vibrio*7 and *Bacillus* 61 grew in the presence of 50 ppm Cu^{2+} ions.

Cd^{2+} resistance

All of the tested strains isolated from different organs of plants collected from target sites showed the same sensitivity to Cd^{2+} ions and MIC was (25 ppm). However, *Enterobacter*18 and *Micrococcus*11 exhibited MIC (50 ppm).

Ni^{2+} resistance

Most of the tested strains isolated from both organs

Table 4. Plasmid-containing endophytic bacteria and their MIC[a] values against the metals isolated from different organs of Grapevine (*Vitis vinifera L.*).

Genera	Plant organs	Resistant pattern (µg/ml)						
		Zn^{2+}	Mn^{2+}	Ni^{2+}	Cd^{2+}	Cu^{2+}	Hg^{2+}	Pb^{2+}
Gram-negative								
Citrobacter 54	Old stems	75	400	100	50	50	-	1000
Enterobacter 47	Young leaves	250	200	350	50	40	-	1200
Enterobacter 23	Old leaves	200	200	300	50	25	-	1200
Enterobacter 18	Old leaves	200	200	300	25	30	-	1000
Erwinia 13	Old leaves	300	200	300	50	25	10	1200
Vibrio 7	Young stems	100	50	70	50	50	-	1000
Enterobacter 26	Old stems	50	400	70	50	50	10	1000
Escherichia 19	Old stems	60	400	80	50	50	-	1000
Gram-positive								
Bacillus 3	Young leaves	300	250	200	50	25	-	1000
Bacillus 9	Old leaves	200	250	200	50	30	-	1000
Bacillus 14	Old leaves	200	100	200	50	30	-	800
Bacillus 42	Old leaves	300	200	200	50	25	-	800
Micrococcus 11	Young stems	300	100	300	25	40	-	1000
Bacillus 61	Old stems	300	100	200	50	50	-	800
Bacillus 28	Young leaves	300	200	250	50	40	-	800
Bacillus 60	Old leaves	300	200	200	50	25	-	800

exhibited MIC ranging between 200 and 350 ppm. However, *Citrobacter*54 and *Vibrio*7 were less resistant to Ni^{2+} ions with MIC values of 100 and 70 ppm, respectively. On the other hand, bacterial strains isolated from different organs of grapevine plant were tested for nickel resistance, *Bacillus* strains showed the same response to Ni^{2+} ions with MIC 200 ppm, whereas, the MIC for *Micrococcus*11 was 300 ppm. However, *Enterobacter*26 and *Escherichia*19 were sensitive to Ni^{2+} ions.

Mn^{2+} resistance

Most of the tested strains isolated from leaves and stems had similar MICs to Mn^{2+} ions (200 ppm). However, *Citrobacter*54 was more resistant to Mn^{2+} and exhibited MIC of 400 ppm, whereas, *Vibrio* was sensitive to Mn^{2+} (50 ppm). On the other hand, gram-positive bacteria isolated from different plant organs responded differently to the presence of Mn^{2+} ions. *Bacillus*3 and *Bacillus*9 strains had the same MIC (250 ppm), while the MICs for *Bacillus*14 and *Bacillus*42 were 100 ppm and 200 ppm respectively. The MIC for *Micrococcus*11 was 100 ppm (Table 4).

Zn^{2+} resistance

All the tested strains recovered from different organs of plant, collected from different target sites exhibited MIC which ranged between 200 to 300 ppm. However, *Citrobacter*, *Enterobacter* and *Escherichia* were sensitive to Zn ions and the MICs were 75, 50 and 60 ppm, respectively.

Plasmids incidence

Although the aforementioned parameters strongly suggest the presence of plasmid DNA in the bacterial isolates, yet remains confirmation of this suggestion. In present investigation all the metals and antibiotic resistant strains were screened for the presence of plasmid DNA. Figure 1 shows a typical electrophoretic seperation of the plasmids in some of the isolates having more than one plasmid. Approximate size and in some cases, size ranges, for the plasmids were determined by comparing their mobilities with those of the known multiple plasmid in *Shigella* sp.

The results in Table 5 show the number and type of plasmid possessed by endophytic bacterial strains isolated from leaves and stems of plants collected from different locations. Among Gram-negative bacteria, *Enterobacter* 47 and *Erwinia* 13 harboured two large plasmids, whereas, *Enterobacter*23 and *Enterobacter*18 possessed one large plasmid. In the case of *Citrobacter* 54, an additional small plasmid was observed. However, *Vibrio7*, recorded from young stems possessed only two small plasmids. In addition, Gram-positive bacteria represented by *Bacillus* species harboured only large plasmids.

On the other hand, plasmid screening of bacterial

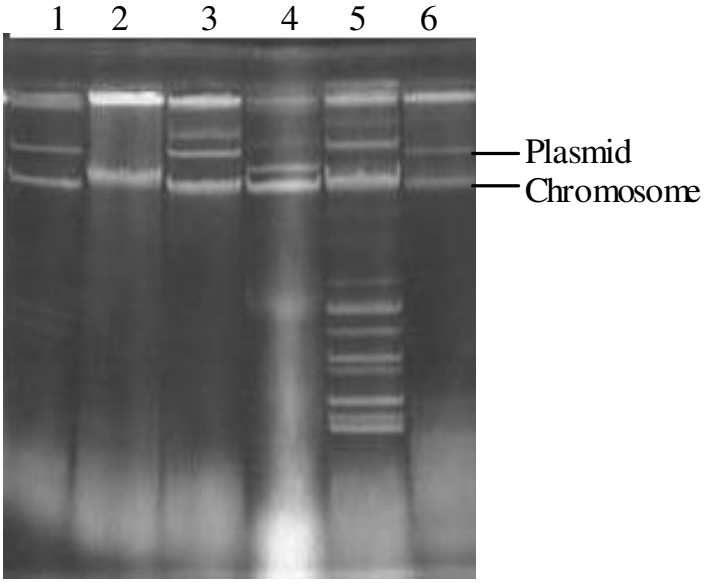

Figure 1. Agarose gel electrophoretic profiles of plasmids DNA from five distinct bacterial isolates; (1) *Citrobacter* 54, (2) *Bacillus* contains no plasmids, (3) *Escherichia* 1, (4) *Enterobacter* 26, (5) *Shigella flexneri* 49 used as size standard and (6) *Enterobacter* 23.

Table 5. Plasmid profiles of endophytic bacterial strains recorded from leaves and stems of grapevine (*Vitis vinifera L.*).

Strain	Plant organ	Plasmid profiles	
		Large	Small
Gram- negative			
Citrobacter 54	Old stems	1	1
Enterobacter 47	Old stems	2	-
Enterobacter 23	Young leaves	1	-
Enterobacter 18	Old leaves	1	-
Erwinia 13	Old leaves	2	-
Vibrio 7	Young stems	-	2
Enterobacter 26	Old stems	2	2
Escherichia19	Old stems	2	-
Gram - positive			
Bacillus3	Young leaves	1	-
Bacillus 9	Old leaves	1	-
Bacillus14	Old leaves	1	1
Bacillus 42	Old leaves	1	-
Micrococcus11	Young stems	1	-
Bacillus 61	Old leaves	1	-
Bacillus 28	Old leaves	1	-
Bacillus 60	Young stems	1	-

strains isolated from different plant organs and collected from different locations revealed that all plasmid-containing strains harboured large plasmids (Table 5). Among gram- negative bacteria, *Escherichia* 19 harboured two large plasmids whereas; *Enterobacter* 26 possessed two large and two small plasmids. Among gram-positive strains, *Bacillus* sp and *Micrococcus* 11 possessed only one large plasmid. However, *Bacillus* 14

Table 6. Distribution of plasmid patterns among endophytic bacterial strains isolated from grapevine (*Vitis vinifera L.*).

Organs age	Plant organ	Total no. of isolates	Total number of isolates containing plasmids	
			No.	%
Old	Leaves	39	6	15.38
	stems	29	3	10.34
	Total	68	9	13.23
Young	Leaves	20	4	20
	stems	23	3	13
	Total	43	7	16.27

Table 7. Distribution of plasmids (large / small or both) in endophytic- plasmid containing isolates.

Organs age	Plant organ	Total No. of strains	Large		Small		Large and small	
			No.	%	No.	%	No.	%
Old	Leaves	6	6	100	0	0	0	0
	Stems	3	2	66.6	1	33.3	1	33.3
	Total	9	8	88.8	1	11.1	1	11
Young	Leaves	4	4	100	1	25	1	25
	Stems	3	3	100	1	33.3	1	33.3
	Total	7	7	100	2	28.57	2	28.57

contained one large and one small plasmid.

Distribution of plasmid patterns among endophytic bacterial strains isolated from grapevine (*Vitis vinifera L.*)

On the percentage basis 9 (13.23%) out of 111 isolates recovered from old stems and leaves collected from different sites were found to harbour plasmids (Table 6). Only 3 (10.34%) strains out of 29 isolates recorded from stem tissues contained plasmids, whereas only 6 (15.38%) isolates out of 39 isolates recorded from leaf tissues possessed plasmids. On the other hand, 7 (16.27 %) out of 43 endophytic bacterial isolates obtained from young organs collected from target sites; 4 (20%) out of 20 isolates from leaf tissues possessed plasmids compared to 3 (13 %) out of 23 isolates from stem tissues. Moreover, 8 isolates (88.8%) out of 9 isolates harboured large plasmids whereas one isolate (11%) among these isolates contained small plasmid from old organs of plants collected from different sites. On the other hand, 7 isolates (100%) of total strains possessed plasmids harboured large plasmids whereas, only 28.57% of these isolates possessed small plasmids from plants collected young organs (Table 7).

DISCUSSION

Endophytic bacterial strains were defined as isolates that were obtained from surface-sterilized plants, displayed differentiable colony morphologies and were recovered from initial survey of grapevine (*V. vinifera* L.) plant.

It is well established that plant bacterial endophytes are to be found in most healthy plant tissues (Frommel et al., 1993; McInory and Kloepper, 1995; Sturz, 1995). In this study, we isolated more than hundred bacterial strains from stems and leaves of Grapevine (*V. vinifera* L.) plant collected from different locations at Taif governate. Similarly, other workers have reported isolation of indigenous endophytic bacteria from grapevine (Bell et al., 1995). To my knowledge, this study is the first to describe indigenous bacterial endophytes isolated from grapevine (*V. vinifera* L.) in Saudia Arabia to evaluate populations of potentially endophytic bacteria, and a total of 111 isolates were collected over 1-year period.

There is a significant variation in the types of indigenous bacteria isolated from diverse host plant species (Zinniel et al., 2002). However, there is similarity in the types of indigenous bacteria isolated from different parts of the same host plant (Mocali et al., 2003). Preliminary characterization of endophytic bacteria in this study showed that a slight variation in the type of indigenous bacteria isolated from stems and leaves. Several factors may explain these differences, including plant age and tissue type (Kobayashi and Palumbo, 2000).

Morphological and biochemical characterization of these bacteria indicated that Gram-negative and gram-positive bacteria were recovered from stems and leaves of Grapevine (*V. vinifera* L.) plant (Kobayashi and

Palumbo, 2000). Furthermore, gram-negative bacteria were more diverse than gram-positive bacteria either those isolated from tissues of stems or leaves. In this context, similar results were obtained with other plant species for the diversity of Gram-negative bacteria isolated from different parts of the plant (Mocali et al., 2003). Interestingly, Gram-positive bacteria (61.73%) were isolated more frequently than gram-negative bacteria from grapevine plant tissues. Earlier workers have reported the predominance of gram positive bacteria in the tissues of various plants (Lalande et al., 1989; Leifert et al., 1989). In contrast, other investigators have reported the predominance of gram negative bacteria (Elbeltagy et al., 2000). However, Zinniel et al. (2000) reported an equal presence of gram-negative and Gram-positive bacteria.

The population densities of endophytic bacteria obtained in this study using TSA medium ranged from 1.15 x 10^3 to 5.2 x 10^4 colony forming units/g (CFU/g) of fresh tissues. Similar-sized populations were isolated from other host plants (Kobayashi and Palumbo, 2000; Zinniel et al., 2002). Furthermore, the host plant has an optimum carrying capacity of endophytic populations, which fluctuates depending on the plant age and different environmental factors (Hallmann et al., 1997). In present investigation, endophytic population was highest in the old tissues (Kuklinsky-Sobral et al., 2004).

The nature of endophytes in Grapevine plant also agrees with other findings of previous studies in which the taxonomic status of endophytes was determined (Gange et al., 1987; Lalande et al., 1989; Jacobs et al., 1985; McInroy and Kloepper, 1995; Mahaffee and Kloeppr, 1997; Kuklinsky-Sobral et al., 2004; Fisher et al., 1992; Bell et al., 1995; Sturz et al., 1997; Zinniel et al., 2002), members of the genera *Bacillus*, *Acinitobacter, Vibrio, Escherichia, Staphylococcus, Streptomyces, Micrococcus*, *Erwinia*, *Enterobacter* and *Xanthomonas* were recorded. Such is the case with grapevine (*V. vinifera* L.), endophytes where all of the above genera were recorded.

The plant- associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the microbial communities that colonize, stems branches and leaves (Kuklinsky-Sobral et al., 2004). Some of these factors are plant tissue type (Mocali et al., 2003), the habitats type and other environmental factors (Dalmastri et al., 1999). In the present study, endophytic bacteria were isolated from leaves and stems of grapevine; some genera such as *Erwinia, Planococcus* and *Staphylococcus* were recorded from leaf tissues and missed from the stem tissues collected from the same location. However, *Acinetobacter* and *Xanthomonas* were recorded from both tissues. Similarly *Acetobacter, Acinetobacter, Vibrio and Streptomyces* were recorded from stem tissues and never from plant leaves, whereas, *Escherichia* and *Micrococcus* were recovered from stems and were absent in plant stems. This is in agreement with previous results indicating the population fluctuation in plants grown in different sites (Picard et al., 2000).

It has been previously shown that fluctuation of endophytic communities depends also on the type of plant organ (Mocali et al., 2003). In the current study, certain endophytic genera were limited to stem tissues such as *Vibrio, Escherichia, Micrococcus* and *Streptomyces*, whereas other genera such as *Xanthomonas* and *Staphylococcus* were specific to leaf tissues. This could be related to the different environments involved; the leaves which are a source of nutrients or roots which are a nutrient sink (Mocali et al., 2003).

Gardner et al. (1982) observed that fluctuation of endophytic bacteria not only depends on the type of organ but also on the age of the plant organ. Siciliano et al. (1998) also suggested that young tissues had morphological structure or chemical composition that affected the ability of certain bacteria to colonize these tissues. In the present study, *Staphylococcus, Vibrio* and *Streptomyces* were detected in young tissues whereas certain genera such as *Erwinia, Xanthomonas, Escherichia*, *Methylococcus* and *Citrobacter* were isolated from old tissues.

To evaluate the function and persistence of endophytic bacteria in plants, hydrolytic enzymes and motility were assayed. Hydrolytic enzymes, pectinase and cellulase may play role in the mechanisms by which endophytic bacteria penetrate into and persist in the host plant (Hallmann et al., 1997). However, release of plant cell wall constitutive degrading enzymes by endophytic bacteria is undesirable since this would confer pathogencity (Collmer et al., 1988). In this study, *in vitro* activity of extra-cellular hydrolytic enzymes was investigated. All isolates recovered during this study were pectinase, cellulase and chitinase negative. More than (70%) of the isolated strains were motile. Thus finding is in accordance with of Elbeltagy et al. (2000). The motility of these endophytes may confer advantages for intercellular entrance and spreading into host tissues (Hallmann et al., 1997).

The potential to use plants to remediate polluted sites has attracted considerable interest over the last few years (Zaurov et al., 2001; Salt et al., 1995; Siciliano et al., 1998). Several authors have investigated the role of microorganisms in phytoremediation and found that certain plant-bacterial associations (endophytic bacteria) can stimulate disappearance of contaminants by metal accumulation (Barac et al., 2004; So et al., 2003; Zaurov et al., 2001) and by extracellular transformation (Siciliano et al., 1998; Garcia, 1987). From the standpoint of environmental pollution, heavy metals and metalloids are extremely toxic because of their relative accessibility to biological systems (Taylor, et al., 1989). The ability of bacteria to adapt through mutation and selection to the presence of toxic metal can be used to indicate whether certain metals are present or have been present in a

particular environment (Taylor et al., 1989). Therefore, we examined the natural metal tolerance levels of bacterial community isolated from Grapevine (*V. vinifera* L.). In the current study, some isolates susceptible to various concentrations of heavy metals were obtained. The MICs of endophytic bacterial strains isolated from leaves and stems of Grapevine (*V. vinifera* L.) plants. Mercury was the most toxic since 99% of the isolates were inhibited by only 10 µg/ml. Similar observations have been reported earlier for mercury toxicity to bacteria (Trevors et al., 1985).

Large numbers of strains isolated from plants collected from different locations were resistant to lead (100%). The high resistance to lead could be attributed to the water lead pollution or lead accumulation by the plant tissue. Nearly, all tested strains isolated from different plant organs collected from different locations exhibited resistance to nickel, zinc and manganese. Increased industrialization has resulted in environmental contamination by these metals in many aquatic systems (Gazso, 2001; Sabry et al., 1997; Taylor et al., 1989). The tested strains isolated from plants collected from different locations showed similar sensitivity to Cd^{+2} and Cu^{+2} ions. Finally, there was no difference between the minimum inhibitory concentration exhibited by isolates from different tissues. Therefore, the similarity of heavy metals susceptibility among the tested strains might be attributed to the capacity of tolerance of metal level accumulated by the plant itself (Idris et al., 2004). This hypothesis needs further study.

The lack of a well-established genetic system is a major obstacle in the elucidation of the mechanism of endophytes or plant-microbe interactions. There is a paucity of genetic markers for endophytic bacteria. Some of the most useful genetic markers include resistance to antibiotics, antimetabolites and resistance to heavy metals (Van Elsas et al., 2003). Plasmid incidence and characterization among endophytic bacteria are useful in genetic studies and are also useful in the development of cloning vectors.

Examination of the plasmid content and establishing the plasmid profiles is the first step of genetic investi-gation for diversity among endophytic bacterial popula-tion. In the present study, several kinds of plasmids and a diversity of plasmid profiles were found among endophytic bacteria isolated from different organs of grapevine plants collected from different locations. The plasmid frequency in bacteria isolated from leaves and stems of plants collected from different locations were variable, 9 (13.23%) out of 68 isolates harboured plasmids. Only 3 (10.34%) out of the 29 isolates recovered from stem tissues contained plasmids, compared to 6 (15.38%) out of 39 isolates recovered from leaf tissues possessed plasmids. Furthermore, the majority of plasmids recovered from plants collected from both sites were large enough (> 50 kb) to carry genes for conjugal transfer (Piotrowska-seget et al., 2005) suggesting the possibility of such transfer in this environment.

Modification of plants, to acquire organisms with improved genetic capabilities and tolerance to different environmental conditions is generally carried out by plant breeding and by integrating foreign DNA into plant genomes to produce transgenic plants (Barac et al., 2004). Although successful for certain plants, these methods are costly and are dependent on the plant variety being studied and takes several years to reach market. As an alternative approach, beneficial endophytes have been used to express and secrete useful products without requiring integration of foreign DNA into the plant genome (Barac et al., 2004). Moreover, endophytic bacteria have a multitude of applications to enhance agricultural production; e.g. wheat growth was found to be enhanced through production of phytohormones increased resistance of cotton to diseases nitrogen fixation in rice and wheat and increasing potato tuber formation under heat stress condition (Hallmann et al., 1997).

Finally, this study demonstrated the occurrence and diversity of culturable endophytes in grapevine (*V. vinifera* L.). The roles of these endophytes in this habitat remain to be elucidated. Future work will address the effect of selected bacteria on plant growth and the uptake of heavy metals by the plant as well as the mechanisms involved.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JA, Smith JA, Struhi K (1987). Current protocol in molecular biology. Greene Publishing Associates, New York.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, Vander der Lelie D (2004). Engineered endophytic acteria improve phytoremediation of water-soluble volatile, organic pollutant. Nat. Biotechnol. 22: 583-588.

Bell CR, Dickie GA, Harvey WLG, Chan JWYF (1995). Endophytic bacteria in grapevine. Can. J. Microbiol. 41: 46-53.

Biling E (1970). *Pseudomonas viridiflava* (Burkholder 1930; Clara 1934). J. Appl. Bacteriol. 33: 492-500.

Brenner V, Hernandez BS, Focht DD (1993). Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. Appl. Environ. Microbiol. 59: 2790-2794.

Burd GI, Dixon DG, Glick BR (2000). Plant growth promoting bacteria that decrease heavy metal toxicity in plants. Can. J. Microbiol. 46: 237-245.

Claus WG (1988). Understanding Microbs; a Laboratory text book for microbiology. 1st ed. W.II Freeman Co, New York.

Collmer A, Reid JL, Mount MS (1988). Assay methods for pectic enzymes. Methods. Enzymol. 161: 329-335.

Dalmastri C, Chiarini L, Cantale C, Bevivino A, Tabacchiono S (1999). Soil type and maize cultivar affect the genetic diversity of maize root-associated *Burkholderia cepacia* populations. Microb. Ecol. 38: 273-284.

Elbeltagy A, Nishioka K, Suzuki H, Sato T, Sato YI, Morisaki H, Mitsui H, Minamisawa K (2000). Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. Soil Sci. Plant Nutr. 46: 617-629.

Farkas V, Liskova M, Biely P (1985). Noval media for detections of Microbial procedures of cellulose and xylanase. FEMS Microbiol. Lett. 28: 137-140.

Fisher PJ, Petrini O, Lappin-scott HM (1992). The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New phytol.

122: 299-305.
Frommel MI, Nowak J, Larzorovits G (1993). Treatment of potato tubers with a growth promoting *Pseudomonas* sp.: Plant growth responses and bacterium distribution in the rhizosphere. Plant Soil, 150: 51-60.
Gange S, Richard C, Rousseau H, Antoun H (1987). Xylem-residing bacteria in alfalfa roots. Can. J. Microbiol. 33: 966-1000.
Gardner JM, Feldman AW, Zablotowic RM (1982). Identity and behavior of xylem-residing bacteria in rough lemon roots of Florida citrus trees. Appl. Environ. Microbiol. 43: 1335-1342.
Gazso LG (2001). The key microbial processes in the removal of toxic metals and radionuclide from the environment. CEJOEM, 7: 178-185.
Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997). Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43: 895-914.
Hansen JB, Olsen R (1978). Isolation of large bacterial plasmid and characterization of the p2 incompatibility group plasmids pMG1 and pMG2. J. Bacteriol. 135: 227-238.
Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004). Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi geoesingense*. Appl. Environ. Microbiol. 70: 2667-2677.
Jacobs MJ, Bugbee WM, Gabrielson DA (1985). Enumeration, location and characterization of endophytic bacteria within sugar-beet roots. Can. J. Bot. 63: 1262-1265.
Kado CI, Liu SJ (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365-1373.
Kobayashi DY, Palumbo JD (2000). Bacterial endophytes and their effects on plants and uss in agriculture. In: Bacon CW and White JF (eds); Microbial endophytes. Marcel Dekker, Inc., N. Y., pp. 199-233.
Krieg NR, Holt JG (1984). Bergey's Manual of Systematic Bacteriology, Vol. 1, eds Baltimore: Williams and Wilkins. ISBN 0-68304108-8.
Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Kleiner AAK, Azevedo LJ (2004). Isolation and characterization of Soybean-associated bacteria and their potential for plant growth promotion. Environ. Microbial. 6: 1244-1251.
Lalande R, Bissonnette N, Coutlee D, Antoun H (1989). Identification of rhizobacteria from maize and determination of their plant growth promoting potential, Plant Soil, 115: 7-11.
Leifert C, Waites WM, Nicholas JR (1989). Bacterial contaminants of Micropropagated plant cultures. J. Appl. Bacteriol. 67: 353-361.
McInory JA, Kloepper JW (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil, 173: 337-342.
Mahaffee WF, Kloepper JW (1997). Temporal changes in the bacterial communities of soil, rhizosphere and endorhiza associated with field grown cucumber (*Cucumis sativus* L.). Microb. Ecol. 34: 210-223.
Malik A, Ahmed M (2002). Seasonal variation in bacterial flora of the wastewater and soil in the vicinity of industrial area. Environ. Monitor. Assess. 73: 263-273.
Malik A, Jaiswal R (2000). Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. Word. microbial. Biotech. 16: 177-182.
Maniatis T, Fritsch EF, Sambrook J (1989). Molecular cloning: A Laboratory Manual, New York: Cold Spring Habor. ISBN 0-87969309-6.
Mocali S, Bertelli E, Cello F, Mengoni A, Sfalanga A, Viliani F, Caciotti A, Tegli S, Surico G, Fani R (2003). Fluctuation of bacteria isolated from elm tissues during different seasons and from different plants organs. Res. Microbiol. 154: 105-114.
Patriquin DG, Dobereiner J (1978). Light-microscopy observation of tetrazolium-reducing bacteria in endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. 24: 734-742.
Picard C, Di Cello F, Ventura M, Fani R, Guckert A (2000). Frequency and biodiversity of bacteria producing 2,4-diacetylphloroglucinol isolated from the maize rhizosphere at different plant growth stage. Appl. Environ. Microbiol. 66: 948-955.
Piotrowska-Seget Z, Cycon M, Kozdroj J (2005). Metal tolerant bacteria occurring in heavily polluted soil and mine spoil. Appl. Soil Ecol. 28: 237-246.
Sabry SA, Ghozlan HA, Abou-Zeid DM (1997). Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. J. Appl. Microbiol. 82: 245-252.
Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I (1995). Phytoremediation: a novel strategy for removal of toxic metals from the environment using plants. Biotechnology, 13: 468-474.
Siciliano SD, Theoret CM, Freitas JR, Hucl PJ, Germida JJ (1998). Difference in the microbial communities associated with roots of different culturves of Canola and Wheat. Can. J. Microbiol. 44: 844-851.
Summers AO, Silver S (1972). Mercury resistance in a plasmid bearing strain of *Escherichia coli*. J. Bacteriol. 119: 242-249.
Sturz AV (1995). The role of endophytic bacteria during seed piece decay and potato tuberization. Plant Soil, 175: 257-263.
Sturz AV, Christie BR, Matheson BG, Nowak J (1997). Biodeiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their infuence on host growth. Biol. Fertil. Soils, 25: 13-19.
Trevors JT, Oddie KM, Belliveau BH (1985). Metal resistance in bacteria. FEMS Microbio. Lett. 32: 39-54.
Taylor G, Pahlsson AM, Benglsson G, Baath E, Tranvik L (1989). Heavy metal ecology and terrestrial plant, microorganisms and invertebrates. A Review, Water Air Soil Pollut. 47: 189-215.
Van Elsas JD, Turner S, Bailey MJ (2003). Horizontal gene transfer in the phytosphere. New phytologist.157: 525-537.
Zaurov DE, Bonos J, Murphy JA, Richardson M, Belanger FC (2001). Endophytic infection can contribute to aluminum tolerance in Fine fescues. Crop Sci. 4: 1981-1984.
Zhou SN, Yang CY, Huang L, Cai CH, Lin YC (1999). Isolation and charaterization of chitinase from a marine bacterium *Vibrio* sp. World J. Microbiol. Biotechnol. 15: 745-746.
Zinniel KD, Lambrecht P, Harris NB, Feng Z, Kuczmarshki D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002). Isolation and characterization of endophytic bacteria from agronomic crops and Prairie plants. Appl. Environ. Microbiol. 68: 2198-2208.