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Table of Contents: Volume 10 Number 18 14 May, 2016

ARTICLES

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Full Length Research Paper

Incidence of zoonotic *Campylobacter jejuni* in fast meal meat, grill chickens and symptomatic Egyptians

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***Campylobacter jejuni* is one of the most important foodborne gastroenteric zoonosis. Most strains of *C. jejuni* produce a toxin (cytolethal distending toxin) that hinders the cells from dividing and produces diffuse bloody edematous exudative enteritis. The common routes of transmission are fecal-oral, person-to-person and the eating of raw or undercooked chickens or meat. This study recognizes the incidence of zoonotic *C. jejuni* in under cooked chickens and meat meals along with persons in contact. We examined 640 grilled chickens and 733 fast meat meals, plus 93 of symptomatic consumers and handlers were collected from five Egyptian governorates (Fayuom, Cairo, Qaluobia, Bin-suef and Assuit) from different restaurants through culture-based methods for detection of *Campylobacter* motility. Also, molecular tools were used for genetic amplification by PCR using specific primers of *hipO* gene. Contamination with *C. jejuni* was recorded in 21.5% in chickens (16.6% in grill tissues and 26.2% in raw visceral organs) and 16% in fast meat meals (18.2% Offal, 15.2% Sausages, 20.4% Hamburger, 13.2% Kofta and 14.5% Shawarma), plus 19.4% in Egyptian personnel's (25.8% in handlers and 19.4% in symptomatic consumers). The polymerase chain reaction (PCR) showed identical fingerprints of *Campylobacter parvum* at 344 bp, signifying the high possibilities of zoonotic hazards. Dissimilar incidence of chickens, meat and humans were verified with reference to different governorates, but Assuit recorded higher percentages sequence to hot weather. The collected documents in this study can offer a base for the progress of public health requisites for advances in food safety measures.**

Key words: *Campylobacter jejuni*, chickens, fast meat meals, consumers, polymerase chain reaction (PCR), Egypt.

INTRODUCTION

Campylobacter jejuni is gram-negative worldwide opportunistic bacteria, inducing one of the most notifiable gastroenteric foodborne zoonosis, due to the superior levels of human consumption. *C. jejuni* affecting about 2.4 million people, with up to 15% of all human diarrheal cases every year (Marler, 2015). It has been confirmed in

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Table 1. Collects of different chicken meat samples into five regions.

Governorates	Grill chickens			Fast meat meals						Symptomatic in contact		
	Tissues	Visceral organs	T	Shawarma	Kofta	Hamburger	Sausages	Offals & liver	T	Consumers	Food handlers	T
Total samples	457	183	640	165	144	103	178	143	733	62	31	93
Fayuom	R97	40	137	36	35	24	39	33	167	16	7	23
Cairo	116	54	170	47	41	31	46	39	204	21	10	31
Qaluobia	82	35	117	34	38	25	38	35	170	12	5	17
Bin-suef	85	28	113	27	21	12	31	26	117	8	5	13
Assuit	77	26	103	21	9	11	24	10	75	5	4	9

various animal reservoirs, but poultry and their products have been recognized as the main source especially free-range ones (Humphrey et al., 2007). Most strains of *C. jejuni* have opportunistic characters and produce a cholera-like enterotoxin that hinders the cells from dividing, simulate watery diarrhea, fever and abdominal cramping (USDA, 2008). Bacterial infection is the main cause of disease of Guillian Barre Syndrome (GBS), so the most important human complication as acute demylenating disease of peripheral nervous system, paralysis of the limbs which lasts for several weeks, also, include toxic megacolon, dehydration and sepsis specially in children (<1 year of age) and immune-compromised patients (Allos, 2001; Yuki, 2001; Butzler, 2004).

Consumption or even handling of polluted raw or under cooked chickens or meat mostly lead to acute diarrhea (Yazdanpanah et al., 2000). Human incubation period is usually 2 to 5 days and untreated persons may shed the organisms for as long as 7 weeks (Heymann, 2004). In 2013, the UK's Food Standards Agency (Wagenaar et al., 2013) warned that two-thirds of all raw chicken bought from UK shops was contaminated with *Campylobacter*, affecting an estimated half a million people annually and killing approximately 100%, because of the “improper handling of foods by consumers and food service employees Wagenaar et al. (2013). Wadl et al. (2010) assurance that *C. jejuni* having different persistence and growth rate within meats that exposed to dissimilar cooked methods.

Polymerase chain reaction (PCR) targeting *hipO* gene was used previously for identification of *C. jejuni* in chickens, meat and human samples (Khalifa et al., 2013). The first *Campylobacter* genome to be sequenced was *C. jejuni* by Parkhill et al. (2000). The aim of the current study is to identify the possible zoonotic hazard of *C. jejuni* through under cooked grill chickens and the common Egyptian fast meat meals (Shawarma, Kofta, Hamburger, Sausages and Offal) along with Egyptian consumers and handlers.

MATERIALS AND METHODS

The practical work was done in Zoonotic Diseases Department, National Research Center, Egypt, from July 2013 up to January 2014. Samples collection is specified in Table 1.

Chicken samples

A total of 640 chicken samples, 457 grill tissues (core portions) from different restaurants and 183 raw visceral organs from poultry stores were collected from four Egypt governorates (Fayuom, Cairo, Qaluobia, Bin-suef and Assuit).

Fast meat meals samples

A total of 733 quick meat meals samples were collected as 165 Shawarma, 144 Kofta, 103 Hamburger, 178 Sausages, 143 Offals and liver from the same governorates mentioned earlier.

Human samples

Stool samples were collected from 93 persons, 31 were chickens handlers employees, from different markets and restaurants, and 62 were symptomatic consumers with history of food poisoning from poultry origin collected from the governmental hospitals or health unites from the same governorates mentioned earlier (Table 1).

All samples were collected in each plastic bag within 2 h. Placed in a cool box and transported immediately to the Laboratory, where they were processed within 2 to 4 h. The processing varied according to the type of sample placed into tubes containing 3 ml physiological saline (0.85% Nacl) and left to stand for 5 to 10 min to suspend before further processing (Makela et al., 2009).

Isolation and identification

About 10 g of each sample was homogenized in sterile thioglucolate broth. Broth samples were incubated at 42°C for 48 h. Under microaerobic condition (5% O_2, 10% CO_2 and 85% N_2). A loopful of enrichment broth were plated on semisolid thioglucolate broth (Oxoid) and incubated in microaerophilic atmosphere at 25, 37 and 42°C for 48 to 72 h in accordance with Iraola et al. (2012).

Microscopic examination of suspected colonies of *Campylobacter* were stained with Gram's stain and identified under phase contrast microscope using (1000×) magnification power (Smibert, 1984). For detection of characteristic comma, S-shape and spiral motility characters of the isolated *Campylobacter* organisms and deep stab growth are typical growth ring test. According to Hald et al. (2008), suspected colonies plated onto blood agar plates. *Campylobacter* isolates were subcultured and identified by biochemical tests (Acha

Table 2. Results of biochemical tests of *C. jejuni* isolated from food samples and human in contact.

Test isolate	Oxidase reduction	Catalase reduction	Motility	H2S production	Growth in 1% glycine	Growth in 3.5% NaCl	Heat tolerance to 42°C	Sodiun hippurate hydrolysis	Sensitive to nalidixic acid	Sensitive to cephalothin
C. jejuni	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	S	R

+ve= positive result; -ve= negative result; S= sensitive result; R= rare.

Table 3. Different results of isolated percentages of chicken, meat and human samples reference to varied governorates.

Governorates	Samples	Fayuom (%)	Cairo (%)	Qaluobia (%)	Bin-suef (%)	Assuit (%)	Total (%)
Chickens	Grill tissues	16 / 97 (16.5)	21/116 (18.1)	13/82 (15.9)	11/85 (12.9)	15/77 (19.5)	76/457 (16.6)
	Raw visceral organs	11/40 (27.5)	17/54 (31.5)	7/35 (20)	6/28 (21.4)	7/26 (26.9)	48/183 (26.2)
	Total	27/137 (19.7)	38/170 (22.4)	20/117 (17.1)	17/113 (15)	22/103 (21.4)	124/640 (19.4)
Fast meat meals	Shawarma	6/36 (16.7)	7/47 (14.9)	5/34 (14.7)	2/27 (7)	4/21 (19)	24/165 (14.5)
	Kofta	4/35 (11.4)	4/41 (9.8)	5/38 (5.7)	3/21 (14.3)	3/9 (33.4)	19/144 (13.2)
	Hamburger	5/24 (20.8)	7/31 (22.6)	3/25 (12)	2/12 (16.7)	4/11 (36.4)	21/103 (20.4)
	Sausages	6/39 (15.4)	7/46 (15.2)	5/38 (13.2)	4/31 (12.9)	5/24 (20.8)	27/178 (15.2)
	Offals & liver	7/33 (21.2)	5/39 (12.8)	8/35 (22.9)	3/26 (11.5)	3/10 (30)	26/143 (18.2)
	Total	28/167 (16.8)	30/204 (14.7)	26/170 (15.3)	14/117 (12)	19/75 (25.3)	117/733 (16)
Symptomatic individuals	Consumers	3/16 (18.8)	4/21 (19)	2/12 (16.7)	1/8 (12.5)	2/5 (40)	12/62 (19.4)
	Food handlers	2/7 (28.6)	1/10 (10)	1/5 (20)	2/5 (40)	2/4 (50)	8/31 (25.8)
	Total	5/23 (21.7)	5/31 (16.1)	3/17 (17.6)	3/13 (23.1)	4/9 (44.4)	20/93 (21.5)

et al., 2004).

For DNA extraction, colonies were suspended in 500 µl of PBS, pH 7.2, washed 3 times in PBS. The cell suspension was centrifuged for 10 min at 800 ×g, then the supernatant was discarded carefully and the pellet was dried and stored at -20°C till use.

Molecular characterization of *C. jejuni*

Isolation of DNA

For extraction of DNA, bacterial pellets were re-suspended in 200 µl PBS and DNA was extracted using QIAamp DNA Mini Kit (Cat No.51304, Qiagen) according to manufacturer's instructions. The DNA pellet was dissolved in 50 µl of elution buffer. Extraction of genomic DNA from *C. jejuni* as mentioned earlier for use as a positive control.

DNA amplification reaction

PCR reaction contained 5 ul template DNA and 1 µl *hipO* primers (0.3 µM) (Wang et al., 2002). CJF (ACTTCTTTATTGCTTGCTGC) and CJR (GCCACAACAAGTAAAGAAGC) were performed in a total reaction volume of 50 µl containing 25 µl Taq PCR master mix (ViVantis Co., Malaysia). Thermocycler conditions were 95°C for 6 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s and finally 72°C for 7 min. Positive controls was incorporated with each set of test samples and subjected to PCR assays. The PCR amplified products were loaded onto gels of 1.5% agarose gel and stained with RedSafe™ Nucleic Acid Staining Solution Cat. No. 21141, iNtRON Co.) and visualized under UV trans-illuminator against 100 bp DNA ladder (Khalifa et al., 2013).

RESULTS

A total of 640 grill chickens and 733 fast meat meals, plus 93 of symptomatic consumers and handlers were examined from five Egyptian governorates (Fayuom, Cairo, Qaluobia, Bin-suef and Assuit) from different restaurants (Table 1). Through culture-based methods, the biochemical estimation of *C. jejuni* carried out by deep stab growth, typical growth ring test on semisolid thioglucolate broth at 37°C under microaerophilic conditions (5% O_2, 10% Co_2 and 85% N_2), and examined after 24 h under phase contrast microscope (Table 2) for characteristic *Campylobacter* motility and identification of characteristic comma, S-shape. Also, growth colonies were observed onto blood agar plates.

Table 3 and Figures 1 and 2 represented *C. jejuni* contamination values; 21.5% in chickens (16.6 in grill tissues, 26.2 in raw visceral organs) and 16% in fast meat meals (18.2% Offal, 15.2% Sausages, 20.4% Hamburger, 13.2% Kofta and 14.5% Shawarma), plus 19.4% in Egyptian personnel's (25.8% in handlers and 19.4% in symptomatic consumers). Dissimilar incidence

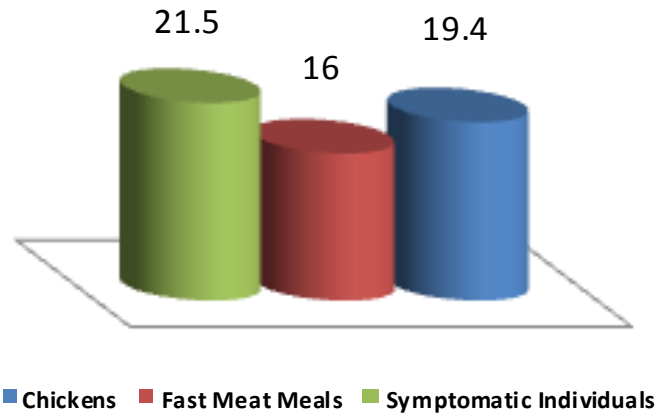

Figure 1. Total incidence of *C. jejuni* in chickens, meat meals and individuals reference to the overall studded governorates.

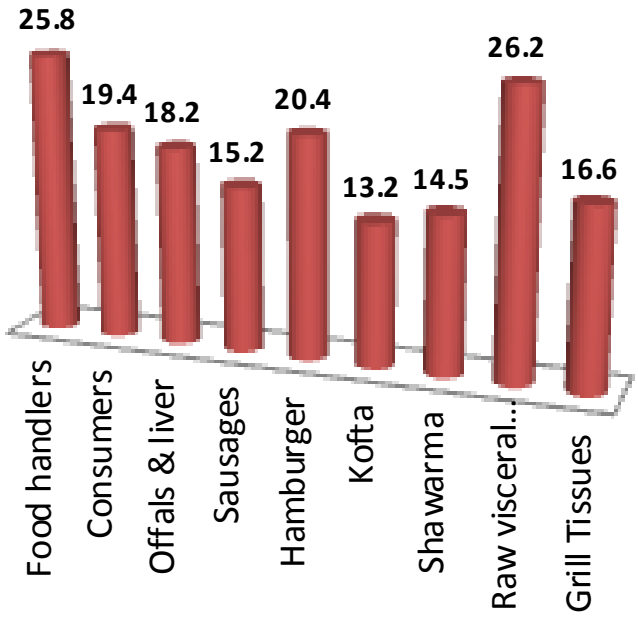

Figure 2. Overall percentages of *C. jejuni* in each food Organs.

of chickens, meat and humans were verified with reference to different governorates (Table 3 and Figure 3), Fayuom (21.7, 16.8 and 19.7), Cairo (16.1, 14.7 and 22.4), Qaluobia (17.6, 15.3 and 17.1), Bin-suef (23.1, 12 and 15) and Assuit (44.4, 25.3 and 21.4).

PCR using specific primers of *hipO* gene is in accordance with Wang et al. (2002). PCR confirmed all the bacteriologically positive isolates with a PCR product of 323 bp (Figure 4).

DISCUSSION

Chickens having up to 100% asymptomatic carriers of *C. jejuni* in their intestinal tracts and may harbor up to 10^9 bacteria per 25 g, which rapidly spread among other chickens. This much exceeds the human infectious dose of about 10^3 bacteria (Humphrey et al., 2007). The present study identify overall positive chicken samples which harbor *C. jejuni* to be 19.4% (Table 3 and Figure 1) and might referred to the original intestinal contamination during bird evisceration (Moore et al., 2005). Our result was lower than that of Khalifa et al. (2013) (36%) and El-Tras et al. (2015) (23.5%); the prevalence differences can be attributed to isolation methods, sample types and size in addition to seasonal and regional variations (Allos, 2001; Shimaa et al., 2015). Varied values with different types of chicken samples were recoded (Figure 2).

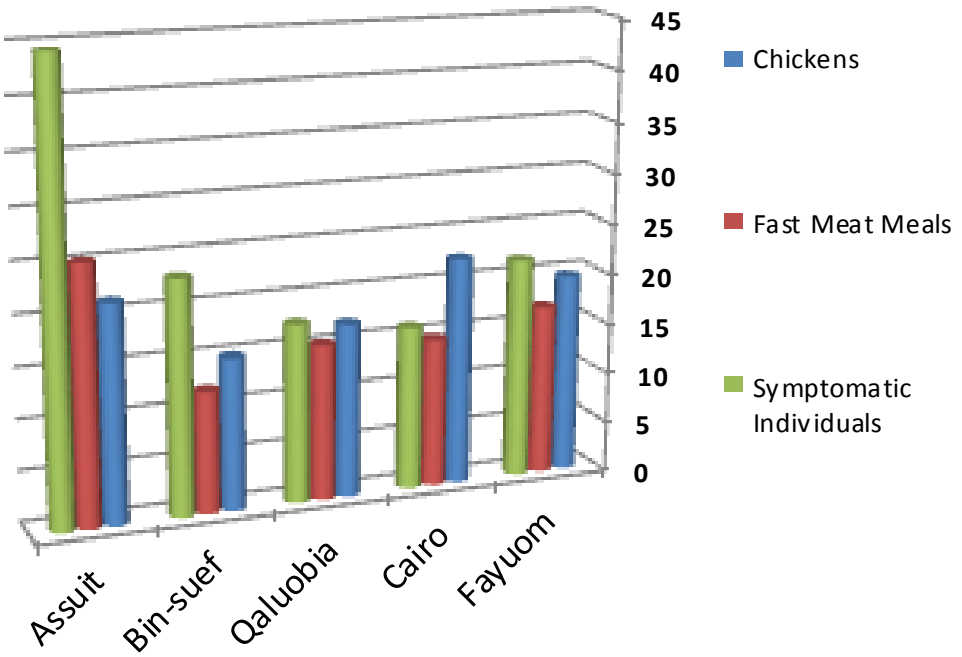

Figure 3. Total incidence of *C. jejuni* in chickens, meat meals and individuals reference to each studded governorates.

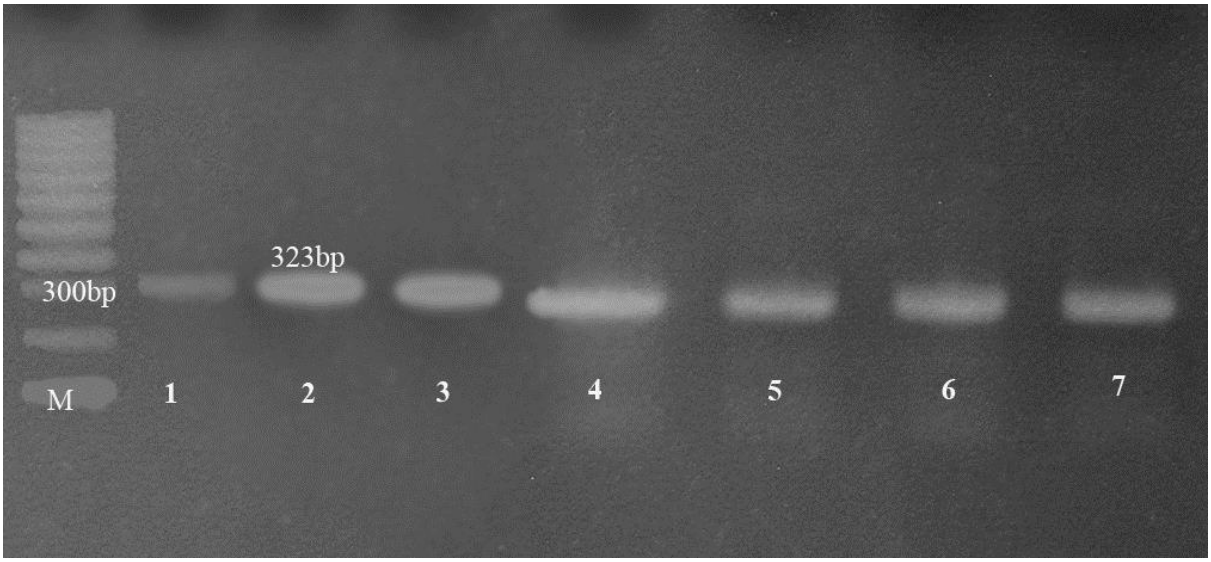

Figure 4. PCR amplification of the 323 bp products of DNA extracted from *C. jejuni*. Lane M: a 100 bp molecular size ladder. Lane 1, positive control; Lanes 2-4, are *C. jejuni* isolates from chicken samples, and 5-7, human samples respectively.

However, the raw offal and liver showed higher values (26.2%) than the barbeque chickens (16.6%). Visceral organs and grill tissues frequently polluted via either initial contamination from farm origin or pollution during processing via preparing utensils or food handlers. Also, most chicken farms do not have security fence to prevent penetration of other wildlife which are good carriers of *Campylobacter* including rats, exotic birds and insects. Furthermore, poor hygiene measures during process of slaughter possibly contaminate poultry carcasses. USDA researchers confirmed that the most retail chicken is contaminated with *C. jejuni* with an isolation rate of 98% for trade chicken meat; where *C. jejuni* counts often exceed 10^3 per 100 g, skin and offal have particularly

high levels of contamination (USDA, 2008). Also, dissimilar incidence was recorded for *C. jejuni* concerning under-cooked barbequed chickens which may be due to the varieties of treatment methods which differ in temperature degrees and pH or spices (Wang et al., 2013). However, socio-economic difference via different governorates was a non-negligible factor represented in different hygienic measures applied during preparation or cooking. Cleaning and disinfection of water-line between farms flocks may help to reduce the risk of chicken *Campylobacter* colonization (Newell and Fearnley, 2003).

The contaminated or under cooked quick meat meals are one of the major cause of *Campylobacter enteritis* in human due to *C. jejuni* (Heymann, 2004). Due to the varieties of cooking methods which differ in temperature degrees and courses, in addition to varied pH and salt concentration. So, the temperature within core parts along with short cooking time could be sustain the favorable media for *Campylobacter* persistence and survival and may be multiplied with lowering heating degrees (Haan et al., 2010). The current study established this concept through verified dissimilar incidence of *C. jejuni* in different types of examined quick meat meals; offal and liver sausage, hamburger, kofta and shawarma were recorded with varied values: 18.2, 15.2, 20.4, 13.2 and 14.5%, respectively, while the overall rate of isolation was 16% (Table 3 and Figure 1). This result was lower than that recorded in quick meat meals samples; 59% by Ledergerber et al. (2003), 40% by Altekruse et al. (1999), 54% by Ekdahl and Andersson (2004) and 20% by Lutgen et al. (2009). The difference may be related to the varied types of processed meat and that Egyptian consumers usually prefer the well done meat.

The current study approve overall (21.5%) zoonotic hazard within Egyptian individuals. The handler employees recorded 25.8% higher percentages than the symptomatic consumers (19.4%) (Table 3 and Figure 2), denoting that human manipulate and contact withdraw chickens or contaminated meat represent higher risk than consuming cooked meals. Shedder poultry and the polluted ones during slaughter or carcass dressing possible maximize infect of handlers and in contact especially those having skin abrasions. Furthermore, poor hygienic measures exploit the common routes of transmission from polluted chickens or droplets of polluted water and spread by fecal-oral, person-to-person (Barakat et al., 2015). In contrast, Shimaa et al. (2015) detected higher infection percentage within Egyptian consumers than handlers. The difference in results may be attributed to the number of examined human samples and/or the food varieties. However, study of 156 human and 682 chicken strains validated equal strains in 70.7% of families of human isolates from diarrhoeal and non-diarrhoeal cases were identical to a household chicken isolate (Oberhelman et al., 2003). Other studies were in line with our results, where a survey in Cairo, Egypt determined the prevalence, seasonality, and household risk factors for *Campylobacter*-associated diarrhea in children; *Campylobacter* species were more prevalent when associated with keeping fowl in the home (Pazzaglia et al., 1993). The organism is isolated from infants and young adults more frequently than from persons in other age groups (CDC, 2014). The high occurrence of *C. jejuni* in ready to eat quick meat meals in Egyptian restaurants, points toward the supervision defects of Egyptian health authorities concerning the suspected carrier shedders from at risk groups including meat and poultry handlers, farmers, restaurants staff, butchers and transported workers. Also, the results ensure the leak of sanitary measures during slaughtering or preparing poultries and animals that pollute products over the permissible limit than that of about (10^3) *C. jejuni* bacteria.

The benefit of salt and spices not only improve the edible test, but minimize bacterial growth within the permissible limit sequence to their bacteriostatic effects. Thus, salt and spices must be industrial applied during chilling storing phase for 2 h prior to meat or poultry cooking. Also, adding natural and lemon juice with salt concentration up to 7% of bacteriostatic action (Pham et al., 2010). However, *C. jejuni* polluted fast meat meals or chickens reflect either initial bacterial contamination or improper application of naturals and spices all through chilling store phase (Coker et al., 2002; Mauer et al., 2006).

Different governorates had recorded clear dissimilar values of *C. jejuni* contaminated chicken and meat samples (Table 3 and Figure 3). This variation may be due to warm or cold weather in addition to population behaviors (Lengerh et al., 2013). Higher temperature and humidity enhance *Campylobacter* growth (Refregier-Petton et al., 2001; Bouwknegt et al., 2004). The reason is still debated, but may indicate a possible relationship between temperature and *Campylobacter* survival and transmission as stated by Patrick et al. (2004). Also, insects frequently involved in summer season may be an important source of *Campylobacter* via mechanical transmission, where flies, cockroaches and other insects passed through the ventilation system into the chickens' house and the invasion of insects correlated with the outdoor temperature (Hald et al., 2008). So, in the current study, Assuit governorate with hot weather recorded the higher values of both human exposure and contaminated chicken and meat samples, than the others moderate weather Egyptian governorates (Figure 3).

Culture-based methods are time consuming and expensive, requiring filtration, selective enrichment, isolation and biochemical confirmation (Table 2). The application of molecular tools, such as PCR (Figure 4), may help to avoid some of the limitations of current methods, where the *hipO* gene is specific for *C. jejuni* strains (Sinha et al., 2004).

This study aimed to identify the zoonotic *C. jejuni*,

through culture-based methods and genetic characteristics of collected isolates from Egyptian under cooked ready-to-eat chickens and fast meat meals incriminated in high infection rate within Egyptians consumers and handlers' employees', reflect on advances in food safety events and provides background for the design of firm efficient control strategies.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Efficacy of plasmid curing agent on *Streptomyces longsporesflavns*

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Plasmid-specified traits like lactose metabolism and L-asparginase production could be eliminated from *Streptomyces longsporesflavns* culture during production of fermented product (Microbial succession). In this study we mainly focus on plasmid profiling and plasmid characterization from *S. longsporesflavns* isolated from marine soil. Then we observe the efficacy of plasmid curing agent on *S. longsporesflavns*. Plasmid-free strains and cured derivatives harboring only a single plasmid (6.2 Kbp) were also obtained. Treatment of *S. longsporesflavns* with novobiocin at concentrations of 2.4 µg/ml could produce a large number of chloramphenicol^{-} variants at a very high frequency (4.6%). These curing data confirmed the novobiocine act as an effective curing agent for *S. longsporesflavns*.

Key words: Antibiotic resistance, curing agent, *Streptomyces longsporesflavns*, plasmid profile, plasmid curing.

INTRODUCTION

Streptomyces sp is a commercially important actinomycets variety of wide applications, both in food industry and as a probiotic agent for the improvement of human health, antileukaemia agent by secreting L-asparginase (Cebeci and Gürakan, 2003). To improve the strain characteristic in food industries, genetic modification of *Streptomyces* strains are normally targeted toward the improvement or augmentation of specific strain characteristics, such as bacteriocin and L-asparginase. Hence bacteriocin producting ability is mediated by plasmids; it is readily lost by plasmid curing agents. For example, the gene for bacteriocin production is associated with the plasmid DNA in *L. plantarum* (Klaenhammer, 1978).

L-asparaginase belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonium. Asparagine is an amino acid required by cells for the production of protein. Asparagine is not an essential amino acid in normal cells and they synthesize this amino acid by the catalytic activity of asparagines synthetase from aspartic acid and glutamine. However, neoplastic cells

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cannot produce L-asparagine due to the absence of L-asparagine synthetase and they depend on cellular pools of L-asparagine for their growth. Tumor cells, more specifically, lymphatic tumor cells require huge amounts of asparagines for their rapid and malignant growth. L-asparaginase exploits the unusually high requirement tumor cells have for the amino acid asparagines (Sieo et al., 2005; Labeda et al., 2014) or *L. pentosus* (Whitehead et al., 2001). This enzyme has been isolated, purified and experimentally studied in detail as an antileukaemia agent in human patients and observed its high potential against childhood acute lymphoblastic leukaemia during the induction of remission or the intensification phases of treatment. Acute lymphoblastic leukaemia (ALL) is a malignant transformation of a clone of cells from the bone marrow where early lymphoid precursors proliferate and replace the normal cells of the bone marrow. It can be distinguished from other malignancies of lymphoid tissue by the immuno-phenotype of the cells.

Therefore, this study was undertaken to ascertain whether the loss of chloramphenicol (Chlo) resistance and bacteriocin production has any correlation with the loss of plasmid. This study also focuses on selecting an effective curing agent for *Streptomyces longsporesflavns*.

MATERIALS AND METHODS

Isolation and identification

Among ten production strains, the potential strain was *S. longsporesflavns* based on its physiological and biochemical characteristic. The colonies were cream, beige, little sticks and smooth round. The strain was gram positive rod. In liquid MRS broth produced uniform turbidity. It was homo-fermentative and it showed positive reaction in the fermentation of galactose, glucose, fructose, mannitol, lactose, sucrose and maltose but not with rhamnose. It did not produce catalase and amylase. It was resistant to bilary salt and produces H_2S. Future confirmation was done by MIS.

Plasmid profiling

Isolation of plasmid

Plasmid was isolated according to the method of O'Sullivan et al. (1993), Klaenhammer et al. (1978) and Maniatis et al. (1982). *S. longsporesflavns* was cultured in SD broth for 16 to 18 h at 37°C. 5 to 10 ml of overnight culture or 6.0×10^{-3} was centrifuge at 5,000 rpm for 5 min. The pellet was resuspended in 200 µl of sol A (25% Sucrose containing Lysozyme-30 mg/ml) and incubated at 37ºC for 15 min. After incubation 400 µl of sol B (3% SDS gm and 0.2N Sodium hydroxide) was added, mixed vigorously and incubated at 7 min. Then 300 µl of ice cold solution C was added (3 M Sodium acetate (pH-4.8), mixed vigorously and immediately centrifuge at 14,000 rpm for 15 min. Then, the supernatant was transferred to a fresh 1.5 ml centrifuge tube and 650 µl of isopropanol was added. It was centrifuge at maximum 15 min (4°C). Collected pellet was resuspended in 320 µl of sdH_2O, 200 µl of sol E (7.5 M Ammonium acetate containing ethidium bromide-0.5 mg/ml) and 350 µl of sol F (phenol and chloroform-1:1). It was mixed and centrifuge at maximum 5 min (4°C). The supernatant was transferred into fresh centrifugation tube and 1 ml of ethanol (-20°C) was added. It was centrifuge and the pellet was washed with 70% ethanol thrice and then the pellet was resuspended in 40 µl Tris EDTA buffer.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed by a horizontal gel electrophoresis system described by Maniatis et al. (1982), Thoma et al. (2012) with 0.6% agarose (Genei, bangloure, India) at 100 V for 90 min and Tris Acetate EDTA (TAE) (pH 8.0) as the running buffer. After the gel was stained in 0.5 pg ethidium bromide/ml, it was photographed by a MP-4 Polaroid camera (Biorad).

Detection of antibiotic resistance phenotypes

Disc diffusion method

Antibiogram was done according to Kirby-Bauer method (Jones et al., 1985). Small discs containing different antibiotics or impregnated paper discs were dropped in *S. longsporesflavns* culture MHA agar plate, a nutrient rich environment in which *S. longsporesflavns* can grow. The inoculated plates were incubated at 37°C. The antibiotic will diffuse in the area surrounding each tablet and a disc of *S. longsporesflavns* lysis will become visible. Since the concentration of the antibiotic was the highest at the centre and the lowest at the edge of this zone, the diameter is suggestive for the minimum inhibitory concentration or MIC.

Here the antibiotics used were amikacin, ampicillin and chloramphenicol. If the diameter of zone of inhibition is greater than 10 mm, it is considered as resistant and if it is less than 10 mm it is considered as sensitive.

Minimal inhibitory concentration for chloramphenicol

Minimal inhibitory concentration is regarded as the lowest concentration of antimicrobial agent which inhibits growth. Chloramphenicol was the antibiotics used in this procedure. The concentrations of these tested antibiotics ranged from 3.125 to 200 g/ml. An appropriate amount of antibiotic solution was separately added to the SD broth, in order to obtain the desired antibiotic concentrations. Tubes containing SD broth without antibiotics were used as a control. Each of the tubes were inoculated with a 16-h *S. longsporesflavns* culture at a 1% concentration (v/v), and were anaerobically incubated for 24 h at 39°C, which represents the optimal growth conditions of these strains. We also determined the minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic sufficient to completely inhibit the growth of the organism. As recommended by Jones et al. (1985), the observation of a faint haziness in the growth medium was not considered to represent growth, whereas the observation of definite turbidity was considered growth, as the drug had clearly failed to completely inhibit growth at that particular concentration. In addition to visual observations, we also evaluated bacterial growth with a spectrophotometer (CECIL, USA).Turbidity of less than 0.15 at an OD of 600 nm was considered "no growth", and turbidity of 0.15 or greater was considered "growth". All experiments were conducted three times, each with triplicate.

Bacteriocin assay

Bacteriocin production in the parent cultures and its cured

derivatives were determined by the method of Castaman et al. (1993) and Fons et al. (1997). The cultures were spot inoculated on M17 338 plates and incubated at 32°C for 16 h. Agar was detached from the edges of the petri plate and turned over onto the lid. The back surface of agar was flooded with 7 ml soft Elliker agar or BHI agar containing 0.7 ml of 16-hold indicator culture. Zones of inhibition around the test culture were measured after incubation for 24 h at the optimum growth temperatures of the respective indicator strains.

Plasmid curing using chemical agents such as acriflavine, ethidium bromide, novobiocin and SDS

S. longsporesflavns was subjected to plasmid curing according to the method of Sieo et al. (2005). The chemical agents used for plasmid curing of *S. longsporesflavns* were acriflavine, ethidium bromide, novobiocin and SDS. Prior to curing we determined the sublethal concentrations of the four curing agents for *S. longsporesflavns*. The sub lethal concentration was defined as the highest concentration allowing for the detectable, reduced growth of a *S. longsporesflavns*. The sublethal concentrations of SDS, acriflavin and ethidium bromide for *S. longsporesflavns* were found to be in the range of 1.8 to 40.0, 5 to 11, 12 to 28 and 2.5 to 6.5 µg/ml, respectively.

S. longsporesflavns were subcultured every 24 h in SD broth containing a sublethal concentration of the respective curing agents. At appropriate intervals (6, 12, 18 and 24 days) the cultures were serially diluted then plated onto the SD agar. After 48 h of incubation under anaerobic conditions at 39^{o}C, the emergent colonies were duplicated onto fresh SD agar containing 100 µg/ml of chloramphenicol. Colonies that failed to grow on the SD antibiotic plates after incubation were considered to have been cured and their duplicates on the SD agar were extracted and maintained in SD broth for further analysis. Respectively bacteriocin assay was performed and colonies that fail to produce inhibitory zone were also selected for further analysis. The curing rate (%) was calculated according to the following formula,

Curing rate (%) = (B/A) * 100

Where, A = number of colonies formed on MRS agar; B = number of colonies formed on MRS antibiotic agar.

Plasmid analysis

Ten ml of six to eight h *S. longsporesflavns* cultures which failed to grow in antibiotic agar plate were used for plasmid extraction. The cells were harvested by centrifugation at 3000 × g at 4°C. 200 µl of freshly prepared lysozyme soln was added to the cell pellet. The subsequent plasmid extraction procedure was conducted according to the method described by O'Sullivan et al. (1993).

RESULTS

S. longsporesflavns strains showed antagonistic activity against *S. longsporesflavns*. Among the strains tested, the *L. bulgaricus* strain was susceptible high; hence it was selected as indicator strain. Among ten pathogens tested *S. longsporesflavns* premier zone of clearance was noted as 3.1 mm (Table 1).

Table 1. Inhibitory activity of bacteriocin.

Test organism	Zone of clearance (mm)	Result
Bacillus subtilis	2.1	(+)
Staphylococcus aureus	2.9	(+)
Lactobacillus bulgaricus	3.1	(+)
Salmonella typhimurium	2.1	(+)
Salmonella paratyphi 'B'	2.5	(+)
Escherichia coli	2.5	(+)
Klebsiella pneumonia	3.0	(+)
Serratia marcescence	2.3	(+)
Pseu aeroginosa	2.3	(+)
Vibrio cholera	1.2	(-)

Positve (+) :Diameter of zone>2 mm; Negative (-) : Diameter of zone<2 mm.

Plasmid curing experience

In plasmid curing study we use two types of plasmid curing agent as novobiocin and ethidium bromide. Among the two agents we tested, novobiocin and ethidium bromide have curing activity of 4.6 and 3.3%, respectively. Of these curing agents, novobiocin acts as effective curing agent; it could be successfully cured, of their bacteriocin production (20.3 kbp plasmid) and ethidium bromide was able to cure the chloramphenicol resistance plasmid (6.4 kbp) (Figures 1 and 2). Novobiocin failed to eliminate bacteriocin production, even after prolonged sub-culturing (every 24 h for 28 h) in sublethal concentration of individual curing agent. This plasmid cured mutated strains, named *S. longsporesflavns* M which has been used for future studies.

Confirmation of cured derivatives

Confirmation of cured derivatives, antibiotic susceptibility was performed against *S. longsporesflavns.* In the present studies *S. longsporesflavns* M was sensitive against chloramphenicol. Whereas in bacteriocin assay it was negative against indicator strains (*L. bulgaries*).

Optimization of physicochemical parameter

Growth characteristics of stress-treated cultures of S. longsporesflavns

In the present study, an attempt was made to assess the stress tolerant capacity of *S. longsporesflavns* from marine ecosystem, such as temperature, salinity and pH.

Physicochemical parameters of the human marine varied according to the food habitat variations which in turn influenced the nature of the marine ecosystem. To

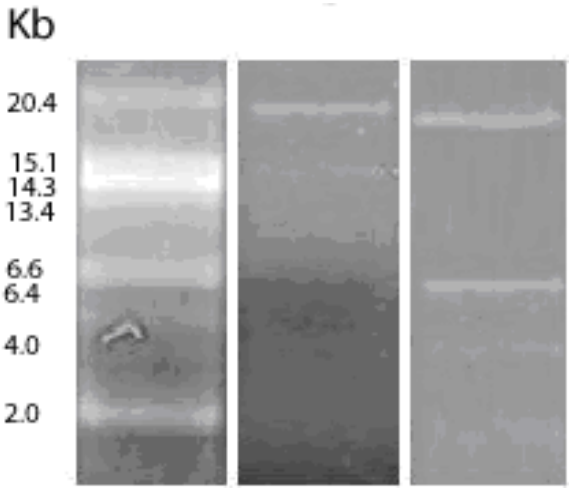

Figure 1. Plasmid profiles of wild type strains of *Streptomyces longsporesflavns* and their cured derivative (*S. longsporesflavns* M). Lane 1; super coiled standard DNA. Lane 2. *S. longsporesflavns* - cured derivatives (*S. longsporesflavns* M). Lane 2. *S. longsporesflavns* - wild strains.

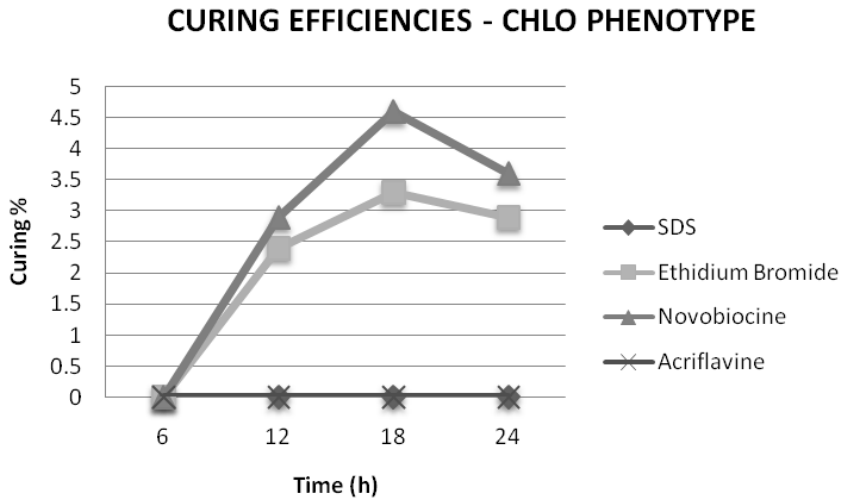

Figure 2. Curing efficiencies of Bac and Chlo phenotypes in *Lactobacillus acidophilus* MTCC447 after 24 h incubation.

assess the stress tolerant capacity of *S. longsporesflavns* fr, we select the ecosystem based on the intestinal physiology, such as temperature, salinity and pH.

Temperature (°C)

In the present investigation, the highest marine atmospheric temperature (AT) (60°C) was noticed and lowest (27°C) in Indian marine physicology was recorded. During the study, the average atmospheric temperature recorded was 28.95°C (Figure 3).

Based on this observation we select the temperature stress, such as the highest marine atmospheric temperature, 60°C and the lowest, 30˚C.

The growth pattern was determined in different heat shock at 35, 40, 45, 50, 55 and 60°C during the exponential growth phase as monitored by OD_{610} (Figure 1). Heat shock at 45°C for 30 min did not influence the growth rate of *S. longsporesflavns*. Heat shock at 50°C retarded the growth slightly (20% growth rate depressed) and the heat shock at 55°C retarded the growth rate severely (100% growth rate depressed). At 2 h after heat shock, the OD_{610} value of the control and 50°C-shocked cultures were 0.8 and 0.7 respectively,

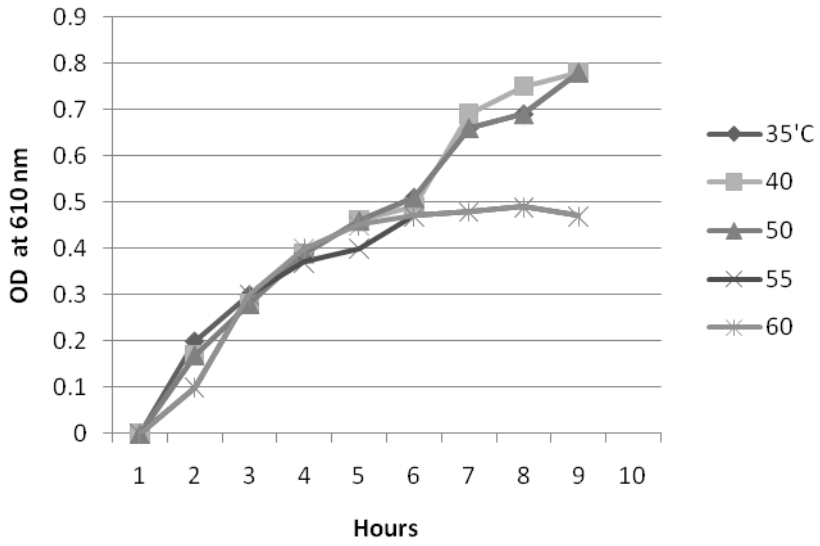

Figure 3. Growth characteristics of stress-treated cultures of *Streptomyces longsporesflavns.*

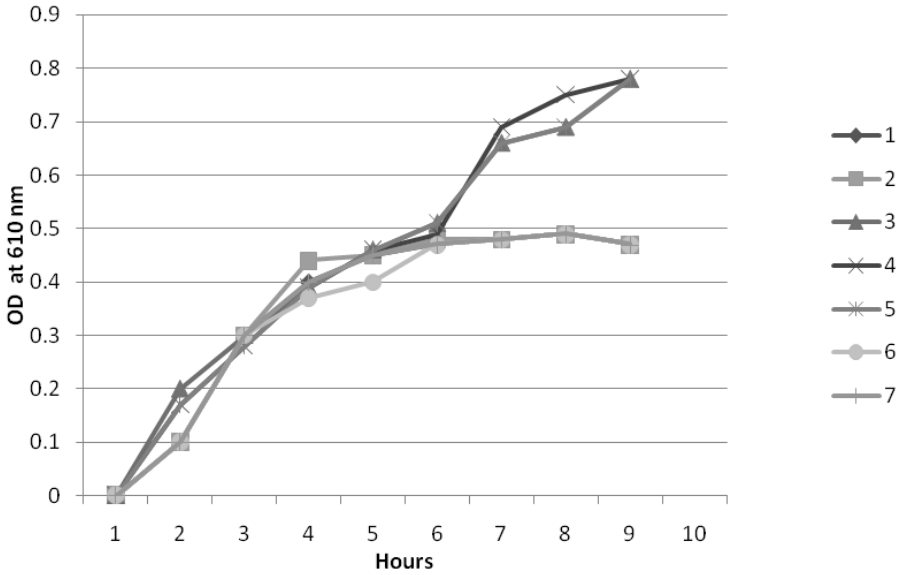

Figure 4. Growth characteristics of stress-treated cultures of *Streptomyces longsporesflavns.*

whereas the OD_{610} of the 55°C-shock culture was only 0.54 (Figure 3).

Salinity

S. longsporesflavns are often exposed to changes in the solute concentrations of their natural habitats like marine ecosstem. Nevertheless, their cytoplasmic solute concentration needs to be relatively constant (Kekessyd et al., 1970). A sudden increase in the osmolarity of the environment (hyperosmotic stress) results in the movement of water from the cell to the outside, which causes a detrimental loss of cell turgor pressure, changes the intracellular solute concentration and changes the cell volume. In the present investigation highest marine atmospheric osmolality (3.2±3 mmol/kg) was noticed and lowest (1.32±4 mmol/kg) in Indian human intestinal physicology was recorded. During the study, the average atmospheric osmolality recorded was 2.95±1 mmol/kg (Karthikeyan et al*.,* 2009). Based on this observation we select the temperature stress, such as the highest marine atmospheric osmolality, 1.0 and the lowest, 0.1 mol/kg.

In the present investigation, osmotic shock with 0.3 M NaCl did not affect growth (Figure 4), but higher salt

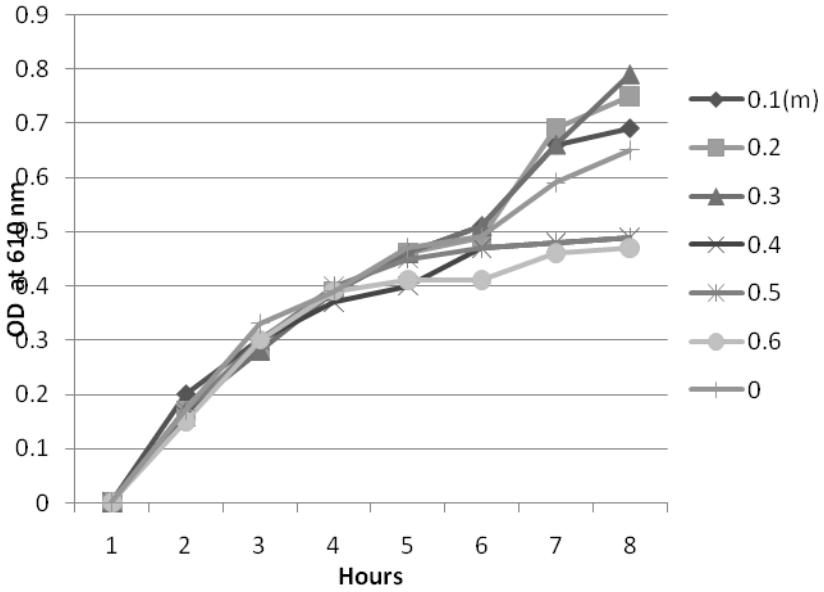

Figure 5. Growth characteristics of stress-treated cultures of *Streptomyces longsporesflavns*

concentration (0.4, 0.5, 0.6 and 0.7 M) reduced in the growth rate in the range of 12 to 40%. The osmolality of the MRS broth without any salt addition was 390± 2 mmol/Kg, whereas addition of 0.6 M NaCl raised this value to 1,498± 2 mmol/kg.

pH stress

pH is an important environmental stress which occurs in LAB during fermentation of foods and beverages. The pKa of lactic acid is 3.86 and in its undissociated form it enters the cells by a carrier-mediated electroneutral process. At cytoplasmic pH, lactic acid dissociates which determines the stationary phase of growth, also if nutrients are still available (Roberts et al., 1968; Ramesh et al*.,* 1987). Probiotic lactobacilli strains are exposed to extreme acid stress when they reach the stomach where hydrochloric acid is present. Several mechanisms regulate the homeostasis of pH_i. The proton-translocating ATPase is the most important for fermentative bacteria.

pH stress was selected based on marine ecosystem, the highest marine atmospheric pH was noticed (Sinha et al., 1981) and lowest in Indian human marine physiology was recorded. Based on this observation we select the pH stress, such as the highest intestinal pH 10 and the lowest, 2 (Figure 5).

The growth pattern of *S. longsporesflavns* was determined in different pH shock at 3, 4, 5, 6, 7 and 8PKa during the exponential growth phase as monitored by OD_{610} (Figure 4). pH shock at 4 for 30 min did not influence the growth rate of *L.acidophillus* MTCC447. pH shock at 2 and 3 retarded the growth slightly (20% growth rate depressed) and the pH shock at 7 retarded the growth rate severely (100% growth rate depressed).

DISCUSSION

S. longsporesflavns and antibiotic mutant

We assayed *S. longsporesflavns* strain with regard to their susceptibility to three antibiotics, namely chloramphenicol, erythromycin and tetracycline. This study focused primarily on these three antibiotics, because they serve as selective markers in transformation studies of actinomycets (Kok et al., 1984). These antibiotics have also been listed as antibiotics which are authorized for veterinary medicine in Europe for the treatment of food- producing animals, including avian, bovine, prscine and porcine species (Charteris et al., 1998). The tested *S. longsporesflavns* exhibited varying degree of resistance to chloramphenicol. The MIC ranges from 50 >100 µg/ml for chloramphenicol. Antibiotic resistance has been frequently found in *Streptomyces* species isolated from a variety of sources, chloramphenicol and erythromycin resistance have been found in *Streptomyces flavns* isolated from poultry (Robert et al., 1972; Varghese et al., 2015), whereas multiple-antibiotic resistance to cefoxitin,aztreonam, amikacin, gentamicin, kanamycin, streptomycin, sulphamethoxazole, trimethoprim, co-trimoxazole, metronidazole, polymyxin B and colistin sulphate has been observed in *Streptomyces* strains isolated from both the human GI tract and dairy prod-ucts (Schwarz et al., 2001). The antibiotic resistance

profile of microorganism depends largely on previous exposure histories of the micro-organism, for example, to the type of antibiotics, period of exposure, and contact with other resistant microorganisms (Danielsen, 2002). Thus, antibiotic resistance profiles differ between microorganisms isolated from different sources. In the present study, *Streptomyces* strains were found to be sensitive highly to chloramphenicol (100 µg/ml) isolated from dairy industries. Antibiotic resistance can be attributed to enzymatic inactivation, decreased intracellular drug accumulation, a lack of target sites for antibiotic drugs, or the presence of genes that confer antibiotic resistance (Castaman et al., 1993; Fons et al., 1997), or within the bacterial genome.

Most actinomycets species, regardless of their source (plants, meat, silage, sourdough or GI tract), harbour at least one indigenous plasmid (Adwan et al*.,* 1998). The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, amino acid metabolism, and bacteriocin production. Although antibiotic resistance is frequently linked to plasmids, exceptional cases have also been reported. In the present study, the elimination of chloramphenicol resistance in *S. longsporesflavns* was associated with any plasmid loss. Nwosu et al*.* (1999) suggested that more than one antibiotic resistance mechanism might be present, due to their observation that not all antibiotic- resistant strains harbored plasmids. This hypothesis is in agreement with the results of Vescovo et al. [1982], Roberts et al. (2014), in which the loss of one plasmids from *S. longsporesflavns* via the curing process was found to be related to antibiotic resistance. Wegener et al. (1993) also determined that resistances to penicillin in 44 strains, and kanamycin in 15 strains of *Staphylococcus hyicus* were not related to plasmids. By way of contrast, the chloramphenicol resistance of *S . longsporesflavns* in the present study may have been conferred by one plasmid (20.3 kb) that was lost in the cured derivative. However, this will remain un-confirmable pending further analysis with regard to the characteristics of the plasmid. Most antibiotic resistance in *Streptomyces* strains has ultimately been determined to be plasmid mediated. Danielsen (2002) has located the gene conferring tetracycline resistance *on L. plantarum* 5057 in one of the four plasmids that are present in the strain. The erythromycin-resistant gene in *L. reuteri* and *L. fermentum,* and the chloramphenicol-resistant gene in *L. reuteri*, are both also associated with plasmids (Cebeci and Gürakan, 2003.

The plasmid under study was found to be refractory to various physical and chemical plasmid curing agents. One of the reasons could be that it may be carrying unknown functions vital for cell viability. This is more likely for bulky plasmids. For example, none of the megaplasmids of Rhizobium or Agrobacterium that exceed 250 MDa have yet been cured (Charteris et al., 1998). Interestingly enough, we found some metabolic activities in this bacterial strain, such as degradation of salicylic acid or n-alkanes (C6±C18) which are so far reported to be carried on one or more well characterized plasmids such as the SAL and OCT plasmid respectively (Aghaiypour et al., 2001). Such metabolic markers with lower molecular weight have not previously been reported to be present in Bacteriocin production (6.2 kbp) and chloramphenical resistant (20.3) by *S. longsporesflavns*. From these data it may be concluded that the phenotypic expression of chloramphenical resistant genes is quite stable. Hence this strain may produce broad spectrum bacteriocin and it may be used as probiotic strain processes to work satisfactorily without spontaneous loss of bacteriocin production and chloramphenical resistant degrading ability; the fate is often predicted for plasmid encoded markers. Hence, it can be concluded that novobiocin could be a very useful curing agent for the elimination of highly stable plasmids from *S. longsporesflavns* and thus can help us in the construction of new strains with desirable characteristics through their genetic manipulation (Rong et al., 2012).

S. longsporesflavns and bacteriocin mutant

The present investigation highlighted the isolation, partial characterization and activity of bacteriocin produced by a *S. longsporesflavns* strain. Dairy industries seem to be a good source of actinomycets. Among ten strains tested, the most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it was *S. longsporesflavns.*

It was tested against 10 different bacterial pathogens which are usually present in food and can cause food borne illnesses in human beings. The bacteria selected were *L. bulgaricus, Salmonella typhimurium, Bacillus subtilis, Staphylococcus aureus, Salmonella paratyphi 'B', Escherichia coli, Klebsiella pneumoniae, Serratia marcescence, Pseudomonas aeruginosa and Vibrio cholerae.* The result indicated that the present strain seemed to have antagonistic activity against nine pathogens in the order of *L. bulgaricus, S. typhimurium, B. subtilis, S. aureus, S. paratyphi 'B', E. coli, K. pneumoniae, S. marsacence and P. aeruginosa*. The study had proved the possibility of using this strain as a probiotic.

Bacteriacins are antimicrobial agents produced by bacteria which are active against closely related bacteria (Balcao et al., 2001]. They have been proved active against many other bacteria including pathogens (Danielsen, 2002). Hence they may be used as probiotics or as bio-preservatives especially in the acid fermentation of foods. In the present study an attempt was made to study the bacteriocin produced by *S. longsporesflavns* and the bacteriocin production was further studied in detail.

Bacteriocin production was strongly dependent on pH, nutrient source and incubation temperature (Schwarz

et al., 2001). Various physicochemical factors seemed to affect bacteriocin production as well as its activity. In the present study maximum activity was noted at pH 4, temperature 50°C and at 0.9% NaCl. The results obtained proved that it can be used in acidic foods like pickle, yogurt etc., as the optimum pH for activity was found to be pH 4.0.

The bacteriocin seemed to be a pure protein of about 2.5 KDa and it was found to be active at pH 4. Hence it deserves further study especially the molecular aspects. As maximum inhibitory activity was found in the culture medium at the stationary phase, it might be a secondary metabolite. MRS broth seemed to be a more suitable medium compared to GP broth for the bacteriocin production.

The use of bacteriocin-producing starter cultures for raw sausage fermentation may contribute to more uniform and safer products. However, the bacteriocin activity levels in a meat matrix are less than the expected activity levels. This is due to the specific conditions in the food environment. For this reason it is necessary to select strains that adapt to the meat environment. *L. sakei* CTC 494, an isolate obtained from Spanish fermented sausage and an organism that produces the anti listerial bacteriocin sakacin (Robert et al., 1972), was found to be able to exhibit maximum bacteriocin activity levels in the temperature and pH ranges which are typical for the fermentation stage of raw fermented sausages (Robert et al., 1968). However, how other factors, such as the presence of salt and curing agents, influence bacteriocin titers was unclear.

Previously, the effect of salt on production and/or activity of bacteriocins produced by lactic acid bacteria has been reported to be beneficial (Ali et al., 1994; Orsini., 2013) or harmful. In this study, using a mathematical model, we investigated how sodium chloride and acidic pH interfere with the kinetics of bacteriocin production by S*. Longsporesflavns* during *in vitro* fermentation. Due to its water binding and ionic characteristics, salt affects the metabolism of a starter culture. The growth of lactic acid bacteria is sometimes enhanced in the presence of low concentrations of sodium chloride (0.1 to 0.3 M), but growth is clearly inhibited in the presence of NaCl concentrations greater than 0.3M (wt/vol) (Lin et al., 1996; Endrz et al., 1999). Homofermentative LABs are more resistant to sodium chloride than heterofermentative LAB are, and strains resembling *S. longsporesflavns* have been shown to be more resistant than strains resembling *L. curvatus* (Sieo et al., 2005) or *L. pentosus* (Whitehead et al*.,* 2001). Increases in the salt concentration decrease the growth rate of *S. longsporesflavns*. Indeed, the growth rate often decreases linearly at water activity below the optimum level (Arima, 1964). Moreover, sodium chloride negatively affects the production of bactericin by *S. longsporesflavns*. Production of bacteriocin decreases because the amount of biomass formed decreases; bacteriocin production generally exhibits primary metabolic kinetics (Pouwels et al., 1993; Derst et al., 1994) and because specific bacteriocin production decreases. It has been suggested that the decrease in bacteriocin production in the presence of salt is due to interference of sodium chloride molecules with binding of the induction factor, which is essential for bacteriocin production, to its receptor (Distasio, 1976). In the case of *S. longsporesflavns*, however, it appears that the water binding effect of salt molecules is the major factor responsible for the decrease in specific bacteriocin production since using glycerol as an agent to decrease water activity instead of salt has a comparable effect. Hence, because salt decreases water activity, the presence of relatively high salt concentrations in sausage batter may be one of the predominant factors. Combined inhibitory effects of sodium chloride and acidic pH on bactericin production reduce the efficacy of bacteriocin-producing starter cultures or co-cultures. During the fermentation stage, a salt concentration of 0.4 to 0.6M in the water phase of the sausage batter does indeed decrease bacteriocin production considerably. However, the activity probably is sufficient to have a significant anti- bacterial effect on the sausage environment, as demonstrated by Jones et al. (1985), Kak et al. (1984) and Blin et al. (2013). Moreover, the results of *in situ* experiments suggest that nitrite and pepper have a synergistic effect on the anti-listerial activity of *S. longsporesflavns* in sausage (Kekessyd et al., 1970).

pH is known mainly for its antimicrobial activity against sporeformers; it has a limited effect on the growth of lactic acid bacteria at acidic pH less than 2 (Datar, 1986), but at 4 inhibition is more pronounced (Castaman et al., 1993; Fons et al., 1997; Karthikeyan et al., 2009). It has been shown that biomass formation and bacteriocin production by *S. longsporesflavns* decrease as the concentration of pH increases. pH has no effect on specific bacteriocin production but decreases the bacteriocin titer indirectly because of its effect on cell growth. Since bacteriocin production is growth related, formation of a small amount of biomass results in a low bacteriocin yield. It has been mentioned previously that nitrite might interfere with active transport mechanisms. This would explain the surprisingly low V_{max} which is obtained when pH 4 is used. In this paper, we present a model that describes the combined effects of sodium chloride and pH on growth and bacteriocin production in *S. longsporesflavns*. The model accounts for a broad range of sodium chloride and acidic pH (0 to 8 and 2 to4, respectively) in MRS broth at 25°C and 2% glucose conditions that are encountered during sausage fermentation. The predictive capacity of the model may be extended to other temperatures and pH values if the equations are combined with a previously described temperature- pH model (Ramesh et al., 1987; Sinha et al., 1991; Sieciechowicz et al., 1989). However, the accuracy of such a combined model approach needs to be evaluated. In this work, we examined the effects of

sodium chloride and acidic pH on bacteriocin production by *S. longsporesflavns*, a potential starter culture for sausage fermentation. Whereas temperature affected bacteriocin production only slightly because it decreased cell growth, salt had a more drastic effect because it decreased both cell growth and specific bacteriocin production. Addition of salt may be one of the major causes of the reduced efficacy of bacteriocin-producing starter cultures in food environments.

Bacteriocin is a bacterial substance, which is a biological protein moiety having bactericidal mode of action against the closely related species. Chemical analysis indicated that some bacteriocin, including that of Staphylococcus, Clostridium and Lactobacillus spp are quite complex molecules with lipid and carbohydrate components in addition to protein with lipid (Klaenhammer et al., 1996; Michael et al., 1981; Nwosu et al., 1999). Bacteriocins of actinomycets are particularly important because of their essential role in the bacterial inhibition of majority of fermented foods. The present study showed that the bacteriocin is a simple protein moiety and the molecular weight of the bacteriocin was determined as 2.5 KDa.

Nisin is the first bacteriocin used as a food preservative agent in 1931. Nisin has first received approval by FDA to be used in pasteurized and processed cheese in 1988 (Wegener et al., 1993; Vescova et al., 1982). Like nisin the bacteriocin produced by *S. longsporesflavns* also has the potential to develop as a probiotic and can be used as a bio-preservative.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Suppression of duck hepatitis A virus Type 1 replication by lentivirus-mediated RNA-dependent RNA polymerase (RdRp) gene-specific siRNA

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To explore the application of RNAi technology for the control of duck hepatitis A virus type 1 (DHAV-1). The RNA-dependent RNA polymerase (RdRp) gene was amplified by PCR. pEGFP-RdRp was constructed for fusion expression of EGFP-RdRp protein. According to the sequence of RdRp, three gene-specific siRNAs were designed and the corresponding shRNA was inserted into pmiRZipΔ to construct pRdRp-shRNA. pRdRp-shRNA and pEGFP-RdRp were co-transfected into HEK293T cells for effective siRNA screening using fluorescent microscopy, flow cytometry, and quantitative fluorescence PCR. More effective siRNA was selected for further study using lentivirus vector pmiRZip. After demonstration of successful preparation of recombinant lentivirus, the suppressing effect was determined by calculating the 50% tissue culture infection dose ($TCID_{50}$) and RdRp gene expression of DHAV-1 in the duck embryo fibroblast (DEF) cells infected with recombinant lentivirus followed by DHAV-1 infection. The results indicated that all of the three siRNAs could suppress the RdRp gene expression, and the shRNA2 containing GDD motif had the highest efficiency. The recombinant lentivirus corresponding to shRNA2 reduced the $TCID_{50}$ of DHAV-1 by 6.2 l g and the RdRp gene expression by 89.6%, with the suppressing effect continued for at least 120 h. This work provides a new idea for the clinical control of duck virus hepatitis.

Key words: duck virus hepatitis, GDD motif, RNA-dependent RNA polymerase (RdRp), RNAi, siRNA.

INTRODUCTION

Duck hepatitis A virus type 1 (DHAV-1) is currently the primary pathogen causing duck virus hepatitis and can cause the acute hepatitis in ducklings, characterized by rapid propagation and the mortality up to 100%. Hence, duck virus hepatitis is classified as class B animal epidemics by the World Organization for Animal Health

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(OIE). The high incidence of virus hepatitis in ducklings inoculated with DHAV-1 vaccine in recent years indicates the variation of DHAV strains or reversion of virulence of traditional attenuated vaccine strains (Gao et al., 2012; Lambeth et al., 2006). The complete genome sequence of DHAV-1 was first reported in 2006 (Kim et al., 2006). Its genome is a single-stranded positive-sense RNA, encoding 2,249 amino acids and the protein 5′-UTR-VP0-VP3-VP1-2A1-2A2-2A3-2B-2C-3A-3B-3C-3D-3′ (Tseng et al., 2007; Li et al., 2013). 3D protein is an RNA-dependent RNA polymerase (RdRp), and plays a critical role in RNA replication. It can specifically recognize viral RNA, participates in the selection of RNA template and initiation site for RNA synthesis, maintains the extension of RNA synthesis, coordinates RNA synthesis procedure and regulates virus variation (Snijder et al., 2006). As an ancient and conservative defense mechanism, RNA interference (RNAi) has many unique advantages with wide applications in the control of animal virus diseases. Gene-specific siRNA can completely or partially interfere with the replication of several viruses, including chicken influenza virus (Ge et al., 2003), immunodeficiency virus (Boden et al., 2004), papillomavirus (Jiang and Milne, 2002), foot-and-mouth disease virus (Luo et al., 2011; Xu et al., 2012), bovine viral diarrhea virus (Lambeth et al., 2006; Ni et al., 2012), porcine reproductive and respiratory syndrome virus (Bao et al., 2010; Huang et al., 2006), porcine circovirus (Wang et al., 2008), and infectious bursal disease virus (Gao et al., 2008). However, there is still no application of RNAi in duck virus hepatitis. This study screened the siRNAs that can effectively silence the RdRp gene of DHAV-1, and used lentivirus vector to express siRNA to suppress DHAV-1 replication, which is of great significance to control duck virus hepatitis.

MATERIALS AND METHODS

Reagents and instruments

Restriction enzyme, T4 DNA ligase, RevertAid M-MuLV reverse transcriptase, Taq DNA polymerase, 1 kb DNA Ladder, RNase inhibitor and Trizol were purchased from Thermo Fisher Scientific; Dulbecco's modified Eagle's medium (DMEM) was purchased from *Gibco* Company); PEI (Sigma); Wizard DNA Clean-up System was purchased from Promega Corporation; RNAeasy Kit was bought from *QIAGEN* (Suzhou) Translational Medicine Co. Ltd.; Inverted fluorescence microscope belongs to Leica Microsystems. Conventional biochemical reagents were all analytical reagents made in China.

Biological materials

pEGFP-N1 vector was purchased from Clontech Company; lentivirus vector pmiRZip, modified green fluorescence-free lentivirus vector pmiRZipΔ and HEK293T cells were provided by professor Sun Huaichang of Yangzhou University; Ready-to-use lentivirus packaging plasmids pMD2.G and psPAX2 were purchased from FitGene BioTechnology Co. Ltd.; Duck embryo fibroblast (DEF) was purchased from Beijing ZhongYuan Ltd. (ATCC CCL141); DHAV-1 SH strain was provided by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences; *DH5α* competent cells and pVL-3CD-P1 plasmids were provided by Jiangsu Key Laboratory for High-Tech Research of Veterinary Bio-pharmacy.

RdRp gene amplification and construction of fusion expression vector

The primer (5' to 3') was designed according to the RdRp gene sequence of DHAV-1 (SH strain, HQ265433). RdRp-F: TTCGAATTCATGGGGAAAGTAGTGAGTAAGC (*Eco*RI); RdRp-R: GGTGGATCCTTGATCATCATGCAAGCTGTGTATG (*Bam*HI). The primer synthesis was carried out by Invitrogen Corporation (Shanghai). The pVL-3CD-P1 plasmid used as template was amplified to acquire the terminator-deleted RdRp gene. Common agarose gel electrophoresis was performed on the PCR product of RdRp gene, and the target gene band was recovered according to the specification of Wizard DNA Clean-up System. Then the recovered product was connected with the RdRp digested by *Eco*RI and *Bam*HI enzymes, as well as pEGFP-N1. Conventional methods were adopted for the transformation and screening of recombinant plasmid. Sequencing was conducted by Invitrogen Corporation (Shanghai).

Detection of RdRp protein expression

HEK293T cells were transfected by pEGFP-RdRp and pEGFP-N1 respectively, with three replicates for each plasmid. After incubation for additional 24 h, cells were examined directly under fluorescence microscope for EGFP expression and total RNA was extracted for RT-PCR amplification of the RdRp-EGFP transcript under the same conditions for RdRp gene amplification.

Sequence design and vector construction

Based on sequencing result of pEGFP-RdRp, a prediction of RdRp-specific siRNAs was carried out utilizing genscript software (http://www.genscript.com). Three were selected from the potential sequences, and shRNA primer was designed according to the requirements of lentivirus vector pmiRZipΔ (Table 1, the matched sequences of siRNA and RdRp were underlined). Primer synthesis was performed by Invitrogen Corporation (Shanghai).

Three pairs of shRNA primer sequences were annealed and cloned into the lentivirus vector pmiRZipΔ digested by *Bam*HI and *Eco*RI enzymes, respectively. These recombinants were sent to Invitrogen Corporation (Shanghai) for sequencing, and the names pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 were used for corresponding recombinants with correct sequencing result.

Detection of suppression efficiency of plasmid-delivered siRNA

HEK293T cells were cultured in 24-well plate by 10^5 cells per well. After culturing for 24 h, the purified pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 were separately transfected with pEGFP-RdRp according to the combination modes in Table 2 and the method of literature (Durcher et al., 2002). Each combination had 6 replicates.

Fluorescence microscopy

After transfection for 36 h, the culture plates were observed and

Table 1. Design of sequence with specific interference to RdRp.

Name of shRNA Primer	shRNA sequence (5' to 3')
RdRp-shRNA1–F(18-38)	GATCGCAATATGCAGGTAAGATTCTTCAAGAGAGAATCTTACCTGCATATTGCttttt
RdRp-shRNA1-R	AATTAAAAAGCAATATGCAGGTAAGATTCTCTCTTGAAGAATCTTACCTGCATATTGC
RdRp-shRNA2–F(952-972)	GATCGGGGATGACTGTGTTCTGTCATCAAGAGTGACAGAACACAGTCATCCCCttttt
RdRp-shRNA2 -R	AATTAAAAAGGGGATGACTGTGTTCTGTCACTCTTGATGACAGAACACAGTCATCCCC
RdRp-shRNA3–F(1194-1214)	GATCGGAAGCATTCAAACAGCAACTTCAAGAGAGTTGCTGTTTGAATGCTTCCttttt
RdRp-shRNA3-R	AATTAAAAAGGAAGCATTCAAACAGCAACTCTCTTGAAGTTGCTGTTTGAATGCTTCC

Table 2. Design scheme of transfection.

Group	Vector added	Proportion of vector
1	pRdRp-shRNA1+ pEGFP-RdRp	0.5 μg:0.5 μg
2	pRdRp-shRNA2+ pEGFP-RdRp	0.5 μg:0.5 μg
3	pRdRp-shRNA3+ pEGFP- RdRp	0.5 μg:0.5 μg
4	pmiRZipΔ+ pEGFP-RdRp	0.5 μg:0.5 μg
5	pEGFP-RdRp	1 μg
6	blank	1 μg

photographed under fluorescence microscope to record the fluorescent brightness and quantity of enhanced green fluorescent protein (EGFP) of cells in each well. Then the culture solution was discarded, and the cells were suspended with 100 μL PBS.

Flow cytometry

The cells in each well were collected for all transfection groups, and PBS was added to dilute the cell concentration to 10^9 cells/L. Then 3×10^4 cells in each transfection group were detected with flow cytometer to calculate the average fluorescence intensity of GFP. The extent of inhibition on RdRp protein (n=3) can be calculated by the following formula: Suppression efficiency = (Average fluorescent stregth of GFP in control group- Average fluorescent strength of GFP in experimental group)/ average fluorescent strength of GFP in control group×100%.

Quantitative fluorescence PCR

From each group, the cells in each well were collected. Total RNA was extracted by referring to the specification for Trizol. Reverse transcription was performed for 1 μg of total RNA, followed by quantitative fluorescence PCR, with GAPDH as reference gene. The primers for amplifying RdRp gene in quantitative fluorescence PCR were as follows: RdRp-Qpcr-F: 5'-TTATGGAGCAACTACAGA-3', RdRp-Qpcr-R: 5'-AAGTTACAAGCCTCAATG-3'. Reaction system included 10 μL of Premix Ex Taq TM (2×), 0.4 μL of forward primer and reverse primer (10 μM each), 0.8 μLof 10 μM probe and 1 μL of cDNA, and H_2O was supplemented to reach a 20 μL system. The reaction condition was as follows: 94°C/3 min; 95°C/10 s→55°C/10 s→72°C/15 s, 40 cycles; 72°C/7 min. Dissolution curve was used to analyze the specificity of amplification products. Rotor-Gene3000 Classic (Bio-Rad) was used to collect the Ct values after reaction. Data analysis was performed with $2^{-\Delta\Delta}$Ct method (n=3) (Livak and Schmittgen, 2001).

Preparation of recombinant lentivirus

The shRNA corresponding to siRNA with high suppression efficiency was cloned into the pmiRZip vector using conventional recombinant DNA technology. After sequencing, the qualified recombinant was called pmiRZip-shRNA.

HEK293T cells with good growth status were harvested at 70 to 80% confluence in advance. According to literature (Durcher et al., 2002), 20 μL packaging plasmid MIX and 4 μg siRNA expression vector with strong suppression screened were used for transfection. After 48 and 72 h, supernatant was collected respectively, and centrifuged at 3000 rpm and 4°C for 10 min. Then the supernatant was stored after filtering by 0.45 μm millipore filter. Meanwhile, pmiRZip was also transfected prepare negative recombinant lentivirus.

Purification of recombinant lentivirus

The lentivirus supernatant was fully mixed with 5×PEG8000 concentrating solution at the ratio of 4:1. After placed at 4°C overnight, the mixture was centrifuged at 4000 rpm for 20 min. The supernatant was discarded, and PBS was added to dissolve the lentivirus precipitate. Gradient dilution method (Yin and Liu, 1997) was employed for the titer determination of recombinant lentivirus. After cultured at 37°C and 5% CO_2 for 72 h, the original medium was replaced by 100 μL of fresh one to observe fluorescence expression. The number of fluorescent cells in the last well showing fluorescence expression was counted. The titer of original virus solution = the number of fluorescent cells/virus volume after dilution.

Detection of DHAV-1 $TCID_{50}$ on DEF cells

DEF cells were infected with DHAV-1 SH strains by routine method. 50% tissue culture infection dose ($TCID_{50}$) of the virus was calculated by Karber's method (Yin and Liu, 1997).

Detection of suppression efficiency of recombinant lentivirus - delivered siRNA

Detection of DHAV-1 $TCID_{50}$

DEF cells were inoculated into 24-well plate at the ratio of 1×10^5 cells/well. When the cell density reached about 80%, the cells were inoculated with recombinant lentivirus at a dose of 10 TU/cell, that is, 500 µL for each well. About 48 h later, fluorescence-positive cells appeared. By referring to the dose of 0.001 $TCID_{50}$/TU and method in literature (Zhou et al., 2011), DHAV-1 SH strain was used for infection (n=3). After infection for 24, 48 h, 72, 96 and 120 h, 100 µL of cell supernatant was collected for $TCID_{50}$ determination, respectively. The $TCID_{50}$ of the supernatant of cells infected with DHAV-1 through the transduction of negative recombinant lentivirus was taken as a control, to quantify the suppression of replication of DHAV-1 SH strains by the recombinant lentivirus-mediated siRNA.

Detection of RdRp gene expression

After infection by DHAV-1 SH strains for 120 h, the cells of each well were collected for the extraction of cellular and viral nucleic acid. According to the quantitative fluorescence PCR method above, the expression of RdRp gene was determined with GAPDH as reference gene, so as to judge the suppressing effect of siRNA on the RdRp gene expression in DHAV-1.

RESULTS

RdRp gene amplification and expression

The terminator-deleted RdRp gene was obtained through the PCR with pVL-3CD-P1 plasmid as template. It was proved by the appearance of an expected band of about 1.4×10^3 bp on electrophoresis (Figure 1). The fusion expression vector pEGFP-RdRp was acquired by recombinant DNA technology and was identified through enzyme digestion (Figure 2). The sequencing result indicated that the cloned RdRp gene sequence had 100% homology with the published one of DHAV-1 SH strains in a complete reading frame with GFP gene.

pEGFP-RdRp and pEGFP-N1 were transfected into HEK293T cells, respectively. After 24 h, typical EGFP-positive cells were observed by fluorescence microscopy and an expected 1.4×10^3 bp amplicon was detected by RT-PCR using RdRp-specific primers (Figure 3), suggesting the successful expression of RdRp proteins by the pEGFP-RdRp recombinant.

Construction of lentivirus vector for siRNA expression

Three pairs of shRNA primers were designed according to the requirement of pmiRZipΔ on siRNA expression. By using recombinant DNA technology, the corresponding recombinant plasmids were generated and called pRdRp-shRNA1, pRdRp-shRNA2 and pRdRp-shRNA3 respectively after identification by sequencing.

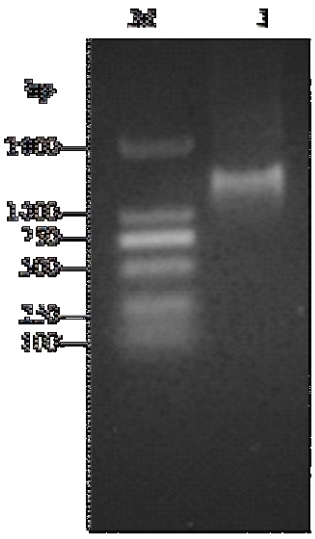

Figure 1. PCR product of DHV-Ⅰ RdRp gene. M: DL2000 DNA Marker; 1: PCR product of RdRp.

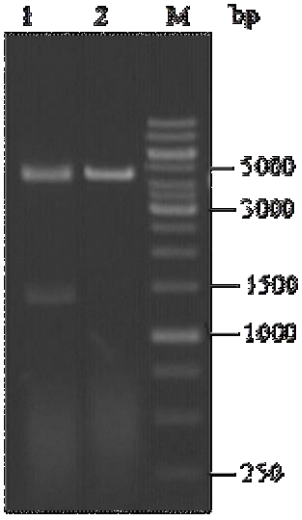

Figure 2. Identification of pEGFP-RdRp by restriction enzyme digestion 1: Double digestion of pEGFP-RdRp by *Eco*RI and *Bam*HI; 2: Double digestion of pEGFP-N1 by *Eco*RI and *Bam*HI M: 1kb DNA Marker.

Detection of suppression efficiency of plasmid-delivered siRNA

The fluorescent brightness and quantity of EGFP of cells in each well were observed under fluorescence microscope 36 h after transfection (Figure 4). The

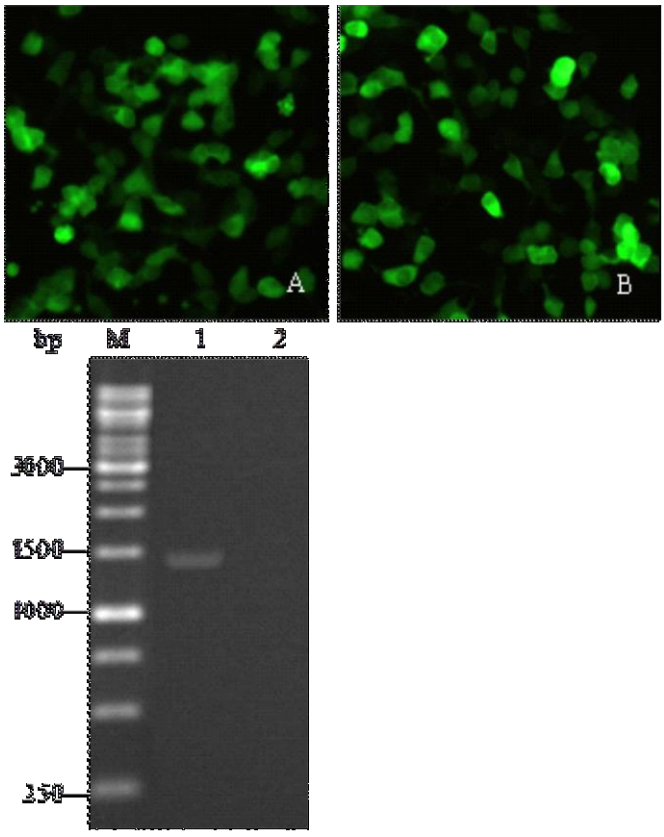

Figure 3. Detection of RdRp-EGFP expression in HEK293T cells (10×10) A: fluorescence of pEGFP-RdRp transfeced cells; B: fluorescence of pEGFP-N1 transfected cells; M: 1kb DNA Marker; 1: RT-PCR amplicon of pEGFP-RdRp transfeced cells; 2: RT-PCR amplicon of pEGFP-N1 transfected cells.

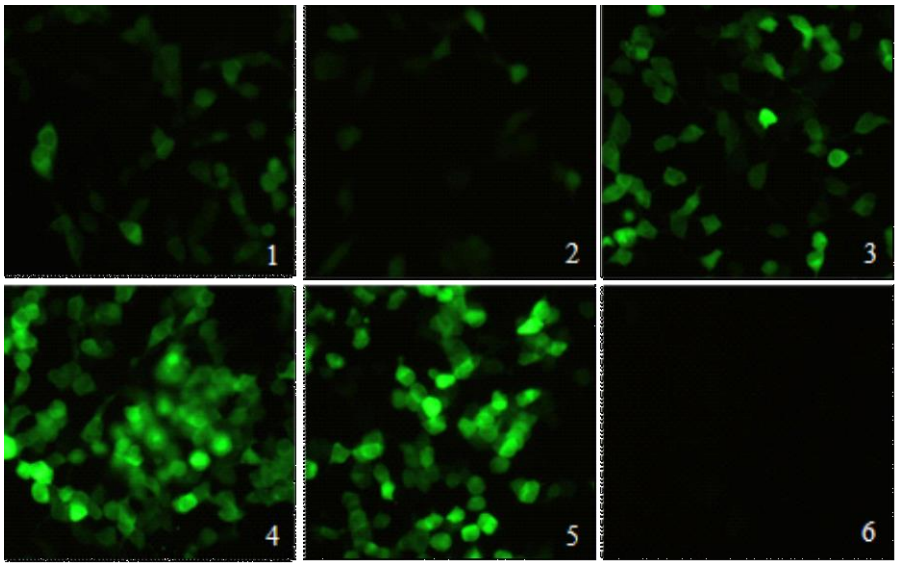

Figure 4. GFP-RdRp expression by fluorescence microscopy (10×10). 1. pRdRp-shRNA1+pEGFP-RdRp transfection group; 2. pRdRp-shRNA2+pEGFP-RdRp transfection group; 3. pRdRp-shRNA3+pEGFP-RdRp transfection group; 4. pmiRZipΔ+pEGFP-RdRp transfection group; 5. pEGFP-RdRp transfection group; 6. Control group.

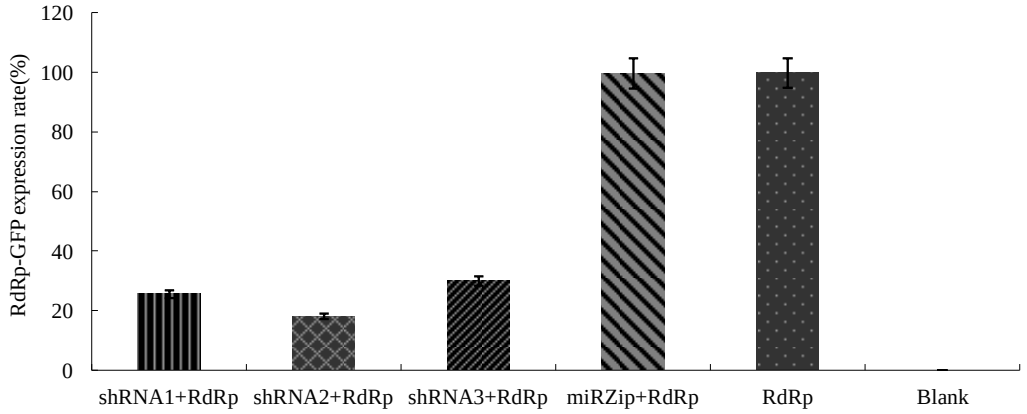

Figure 5. Expression of fusion protein RdRp-GFP by flow cytometry (n=3).

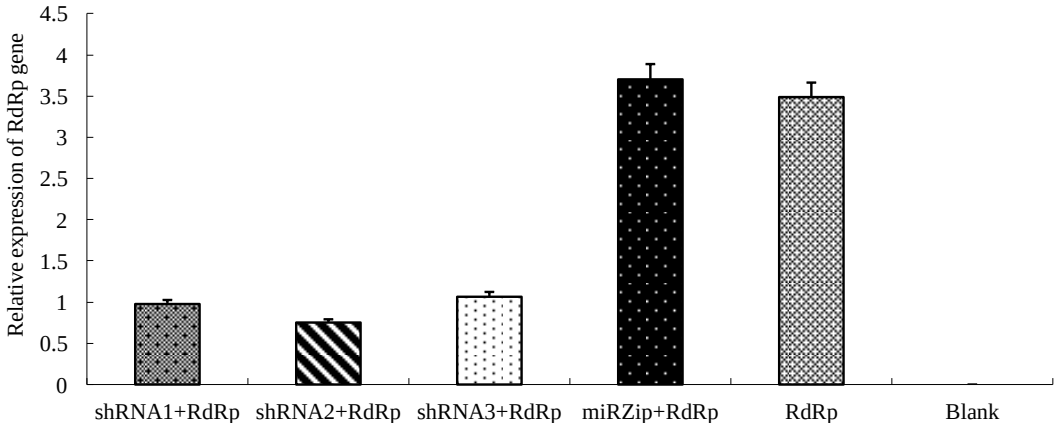

Figure 6. Relative expression of RdRp gene in transfected cells by quantitative fluorescence PCR (n=3).

fluorescent brightness in shRNA transfection attenuated obviously. The expression of fusion protein RdRp-GFP detected by flow cytometry was shown in Figure 5, and it was found that the reduction of protein expression ranged from 69.9 to 81.9%. In the meantime, the cells transfected with different combination of pRdRp-shRNA1, pRdRp-shRNA2, pRdRp-shRNA3 and pEGFP-RdRp were collected for the quantification of RdRp gene. Specific quantitative fluorescent PCR indicated the decrease of RdRp gene expression by approximately 80%, as seen in Figure 6. Among these combinations, pRdRp-shRNA2+pEGFP-RdRp group had the lowest RdRp gene expression, which indicated the suppressing effect of shRNA2 was the strongest.

Preparation of recombinant lentivirus

The recombinant lentivirus corresponding to pmiRZip-shRNA and pmiRZip was packaged in HEK293T cells. After concentrated by PEG8000, the recombinant lentivirus was used to infect HEK293T cells (Figure 7). By gradient dilution method, the titer of original virus solution was determined as 1.3×10^8 TU/mL.

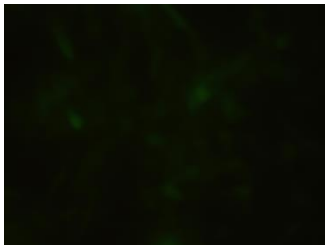

Figure 7. Infection of recombinant lentivirus into HEK293T cells (10×10).

Detection of suppression efficiency of recombinant lentivirus-delivered siRNA

Detection of DHAV-1 $TCID_{50}$

The recombinant lentivirus corresponding to pmiRZip-shRNA and pmiRZip was inoculated into DEF cells at the dose of 10 TU/cell. After 48 h, these DEF cells were

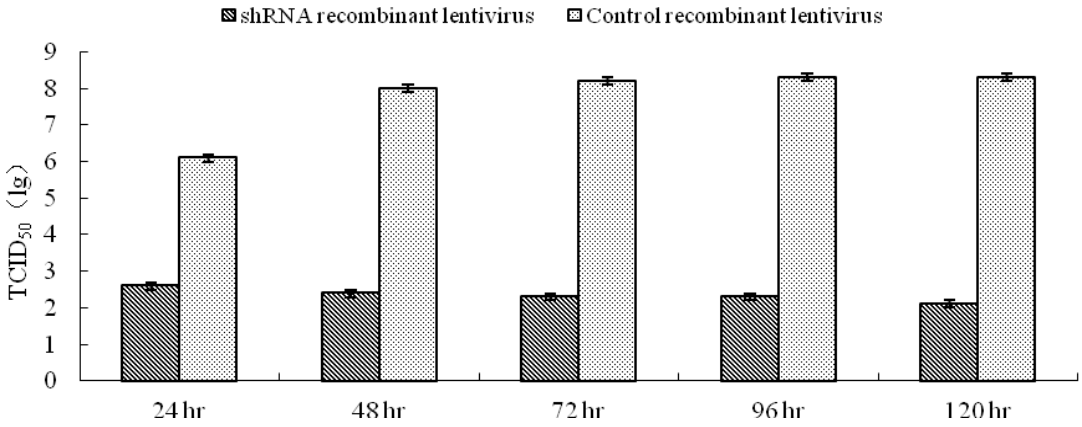

Figure 8. Suppressing effect of shRNA recombinant lentivirus on DHAV-1 determined by $TCID_{50}$ (n=3).

infected with DHAV-1 SH strains, respectively (n=3). After infection for 24, 48, 72, 96 and 120 h, $TCID_{50}$ of the cell supernatant was determined as virus titer. As seen in Figure 8, the suppressing effect on virus had emerged at 24 h, and it could continue to 120 h at least. At 120 h, the $TCID_{50}$ of DHAV-I in the control group was 8.3 lg, while that in the lentivirus infection group with siRNA was only 2.1 lg.

Detection of RdRp gene expression

Total RNA was extracted from the cells after infection by DHAV-1 120 h. The expression of RdRp gene was determined by quantitative fluorescence PCR. Compared with the control group, the RdRp gene expression decreased 89.6% in the lentivirus group carrying siRNA.

DISCUSSION

All known RNA viruses encode a RNA-dependent RNA polymerase, which combines with the protein of hosts (viral protein also needed sometimes) to catalyze RNA polymerization and to modify RNA. Hence, such polymerase is critical to the replication of RNA viruses. Previous studies indicated that the RdRp gene-specific shRNA had stronger viral suppression efficiency than the shRNA specific to the gene of viral structural protein (Gao et al., 2012; Wang et al., 2008). So we selected the RdRp gene of DHAV-1 as target gene to design three shRNAs. Our experimental result showed that under the mediation by plasmid vector, the highest efficiency in silencing RdRp gene expression reached up to 81.9% for the three specific shRNAs. The corresponding recombinant lentivirus reduced the $TCID_{50}$ of DHAV-1 by 6.2 lg and the RdRp gene expression by 89.6%, which agreed well with our expectation. A new idea is found for the clinical control of duck virus hepatitis, which will contribute to the alleviation of huge losses brought by DHAV-1 to breeding industry.

There are several conserved regions (domain or motif) in RdRp protein of various viruses. It is believed that RdRp contains 8 conserved motifs, which can be found in RNA viruses, whether double-strand, positive-strand or negative-strand. These motifs are crucial for RNA replication. The typical GDD motif marks RdRp, and it has been reported that the glycine in GDD motif has important functions (Beerens et al., 2007; Sánchez and Juan, 2005). However, GDD motif is substituted by SDD motif in porcine reproductive and respiratory syndrome virus (PRRSV). When the serine in SDD motif mutated into glycine through artificial induction, PRRSV could be rescued (Zhou et al., 2011). Therefore, we speculate that GDD motif plays an important role in the viral virulence. We designed a specific siRNA containing GDD sequence, that is, shRNA2. The experimental results showed that shRNA2 had higher suppression efficiency on RdRp gene compared with other shRNA, and the corresponding recombinant lentivirus had a strong, long-running suppression on DHAV-I. This implies the potential influence of GDD deletion on viral virulence. Hence, a new control strategy is provided for the control of duck virus hepatitis and other diseases induced by RNA viruses.

In order to achieve the qualitative screening and quantitative judgments of effective shRNA, and exclude the influence from the differences in fluorescence intensity of GFP expressed by various vectors, the lentivirus vectors with or without GFP report gene were both selected for tests. Although an additional procedure of vector modification was introduced, GFP as an indicator could reflect transfection or infection efficiency intuitively, saving a lot of time and labour.

The efficiency of viral gene suppression was generally above 70%. But even at the optimal condition, both gene transfection efficiency of cells and transfection efficiency of recombinant lentivirus could not reach this level. This

suggests that siRNA expressed by infected/transfected cells may have the ability of transcellular transmission (Rausch, 2006). The reason of why the suppression efficiency failed to reach 100% is still unclear, possibly indicating a connection with the RNAi vector expression system, siRNA design software, and cell transfection and infection efficiency. More efforts are needed to clarify the underlying mechanisms.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Nodular bacterial endophyte diversity associated with native *Acacia* spp. in desert region of Algeria

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Five species of *Acacia (Acacia ehrenbergiana* Hayne, *A. nilotica* (L.) Delile, *A. seyal* Delile, *A. tortilis* (Forssk.) Hayne and *A. laeta* Delile) indigenous to Tamanrasset (Algeria) were investigated for their nodulation status and nodular endophytic diversity. *A. ehrenbergiana* showed the highest nodulation ability across the different sites in this region, indicating the widespread occurrence of compatible rhizobia in the soils. Altogether 81 strains were purified. Among this endophytic strain collection, only four bacterial endophytes nodulated their respective host plants. On the basis of partial 16S rDNA sequencing, they were affiliated to *Ensifer* sp., *Ensifer teranga*, *Mesorhizobium* sp. and *Rhizobium* sp. Among the 79 non- symbiotic endophytes, 24 representative strains on the basis of PCR-RFLP profile obtained with MSPI enzyme digestion were characterized. They belonged to nine genera, namely: *Paenibacillus, Ochrobactrum, Stenotrophomonas, Pseudomonas, Microbacterium, Rhizobium, Agrobacterium, Brevibacillus* and *Advenella.* The isolated nodular endophytes in this study revealed a strong tolerance profile to salinity and high temperature. Principal component analysis confirmed that no correlation was found between bacterial tolerance to a maximum temperature of growth and soil depth of sampling. This tolerance profile was distributed over the three levels of soil depth sampling: 20, 40 and 60 cm. On the other hand, there was no relationship between *in vitro* tolerances of rhizobial strains to NaCl and high temperature and corresponding edaphoclimatic characteristics of the sampling sites. This study is a contribution to nodular bacterial diversity knowledge of desert African *Acacia* species growing in preserved ecosystems.

Key words: *Acacia* spp., bacterial endophytic diversity, desert, nodules, salinity tolerance, temperature tolerance.

INTRODUCTION

Endophytes can be defined as non-phytopathogenic organisms, which colonize plant tissues at least part of their lifetime (Wilson, 1995). They could accede to all vegetal compartments passively or actively (Hardoim et

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al., 2008). This is true for legumes, which are characterized by their ability to establish symbiotic relationships with Bacteria Nodulating Legumes (BNL). This plant-microorganism interaction gives rise to a neo-organ: the nodule where atmospheric nitrogen fixation takes place. It may host in addition to "nodulating endophytes" (BNL) a great variety of endophytic bacteria, including some rhizobial non-nodulating strains together with non rhizobial strains. Scientific attention focused on diversity of nodular endophytic bacteria, especially for cultivated species of legumes (Sturz et al., 1997; Bai et al., 2002; Li et al., 2008; Stajković et al., 2009; Ibanez et al., 2009). These efforts converged on improving crop yield both in quality and quantity. In contrast, little attention has been paid to the root-nodule bacterial endophyte of truly wild legumes; those whose ecology is not directly affected by human activities (Muresu et al., 2008) especially the trees. Endophytes have become in the last decades of a great interest due to their biotechnological applications. Their beneficial effects had been reviewed intensively as they could act as Plant Growth Promoters (PGP), enhance plant resistance to stress and disease and are producers of active secondary metabolites (Compant et al., 2010).

Tamanrasset is a vast desert region with an annual rainfall of 48 mm and mean temperature that ranges from -1 to 48°C following Algerian National Organization of Meteorology –ONM (2006). It is located in the South-east of Algeria. It harbors an interesting flora and is the only Algerian region where five indigenous species of *Acacia* are localized (Sahki et al., 2004; Ozenda, 1983): *Acacia ehrenbergiana* Hayne, *A. nilotica* (L.) Delile, *A. seyal* Delile, *A. tortilis* (Forssk.) Hayne and *A. laeta* Delile. These rustic leguminous trees survive under extremely harsh conditions of drought in dry oueds (dry rivers), are widely used for animal and human medicines preparation, and are source of fuel and animal feeding. In their natural distribution area, most of the African *Acacia* spp. were associated with *Mesorhizobium* (de Lajudie et al., 1998; Haukka et al., 1998; McInroy et al., 1999; Ba et al., 2002; Odee et al., 2002), *Ensifer* (syn. *Sinorhizobium*) (de Lajudie et al., 1994; Haukka et al., 1998; Khbaya et al., 1998; Ba et al., 2002) and, to a lesser extent, *Rhizobium* (McInroy et al., 1999; Nick et al., 1999) and *Bradyrhizobium* (Dupuy et al.,1994; McInroy et al., 1999; Odee et al., 2002). Some studies revealed leguminous tree non-symbiotic nodular endophytes as *Conzattia multixora* grown in Mexico (Wang et al., 2006), woody and shrub legumes growing in Ethiopia: *Crotalaria* spp., *Indigofera* spp., and *Erythrin abrucei* (Aserse et al., 2013). Many scientists working on African *Acacia* species symbionts did encounter bacteria non-nodulating endophytes; for example: *Ensifer meliloti, Rizobium tropici* and *Agrobacterium* –like strains associated with *A. tortilis* in Tunisia (Ben Romdhane et al., 2005); *Agrobacterium* associated with *Acacia gummifera* in Morroco (Khbaya et al., 1998) and *A. cyclops* and *A. mollissima* in Senegal (de Lajudie et al., 1999). Only one study (Hoque et al., 2011) did reveal non-symbiotic endophytic bacteria diversity associated with two species of *Acacia*, namely *Acacia salicina* and *Acacia stenophylla* in arid and semi-arid regions of Australia. The aim of this work is to reveal nodulation status of *Acacia* species indigenous to Tamanrasset and the diversity of endophytic bacteria isolated from their nodule; it also aims to establish a possible relationship between bacterial affiliation/plant host and bacterial tolerance profile/ depths of soil sampling (20, 40 and 60 cm surrounding native trees).

MATERIALS AND METHODS

Soil sampling and site description

Soil samplings were collected from three depths (20, 40 and 60 cm) in a maximum diameter of 1meter around the trunks of vigorous trees. Each soil sample was kept in sealed bag for further use. Prospect soils were neutral to slightly alkaline and non-saline except Oued Idekel with CW of 0.637 which is considered as slightly saline according to Durand scale (1983) (Table 1). All soils showed rocky, sandy and loamy texture, but Djnan Biga which was loamy, clayey and sandy. *Acacia* species were encountered in or on the sides of dried rivers (oueds). Tree species were not distributed homogeneously in the eight prospected sites. There is generally a numerical predominance of *A. ehrenbergiana* and *A. tortilis* when present in the studied sites.

Nodules sampling

Sampling was conducted in November (Rainy season is generally situated between June and September). Tree root nodules were prospected at the three depths cited above. No nodules were found *in natura*. Seeds were harvested by the trees of every sampling site when they were present; for *Acacia laeta*, they were supplied gracefully by Forestry Direction of Tamanrasset. Trapping assay was performed using the collected soil with seeds of associated *Acacia* species: seeds of native species *Acacia nilotica*, *A. ehrenbergiana*, *A. seyal*, *A. tortilis*, and *A. laeta* were scarified and surface sterilized by treating with concentrated (95%) H_2SO_4 for 120, 30, 30, 30, and 20 min, respectively. They were then rinsed thoroughly with sterile distilled water and germinated on 0.8% (w/v) water agar at 28°C for two to five days. The germinated seeds were then transferred in aseptic conditions into Gibson tubes (Gibson, 1980) containing sterile Jensen nitrogen-free medium (Vincent, 1970). Plants were grown in an incubation chamber as described by Diouf et al. (2007). After 1 week of growth, 1 mL of stirred soil suspension was added to each tube. Soil suspension was obtained from 10 g of each soil sample added to 90 mL of sterile buffered saline, pH 7 (NaCl 0.15 M, KH_2PO_4 0.002 M, Na_2HPO_4 0.004 M) and shaken for 1 h. Four replicates were tested for each soil sample. Un-inoculated plants served as controls. 208 tubes were prepared. Plants were observed for nodule formation 6 weeks after germination, and fresh nodules were collected and conserved under desiccated condition in tubes containing $CaCl_2$ crystals.

Bacterial strains isolation and culture conditions

The root nodules were washed by immersion in 70% (v/v) ethanol for 30 s, rinsed with sterile distilled water, then rehydrated for 1 h.

Table 1. Sampled soil characterization and localization and *Acacia* spp. nodulation status.

Sampling sites localization in Tamanrasset	*Acacia* species encountered	Number of nodulated plants/4Gibson tubes inoculated by 10^{-1} of soil suspension	Depth of soil sampling cm	Soil conductivity (mS/cm^2)	Soil pH
Djnen Biga 22°33.683 N 005°23.754 E ALT 3735FT	*A. ehrenbergiana*	3	20	ND	ND
Oued In Deladg 22°56.004 N 005°52.851 E ALT 4554FT	*A. albida*	1	20	0.105	7.70
		2	40		
		2	60		
	A. ehrenbergiana	4	20		
		2	40		
		3	60		
	A. nilotica	0	20		
		2	40		
		2	60		
Oued Tassena 35°35.240 N 000°48.527O ALT 358FT	*A. albida*	0	20	0.078	8.17
		0	40		
		0	60		
	A. tortilis (predominant)	2	20		
		4	40		
		4	60		
	A. leata	3	20		
		3	40		
		2	60		
	A. ehrenbergiana	2	20		
		4	40		
		3	60		
Oued Tamezzegine 22°35.218 N 005°23.815 E ALT 3740FT	*A. ehrenbergiana*	3	20	0.149	7.8
		4	40		
		4	60		
	A. nilotica	2	20		
		0	40		
		1	60		
	A. tortilis	3	20		
		4	40		
		3	60		
Oued Idekel 22°33.783 N 005°24.020E ALT3732 FT	*A. nilotica*	0	20	0.637*	7.8
		0	40		
		1	60		
Oued Taghemout 22°58.640 N 005°40.892 E ALT 5461FT	*A. seyal* (predominant)	0	20	0.148	7.7
		2	40		
		0	60		
	A. seyal	0	20		
		0	40		
		1	60		
	A. tortilis	0	20		
		0	40		
		0	60		

Table 1. Contd.

Oued In Tounin (Tasekra) 22°56.004 N 005°52.851 E ALT 4554FT	*A. tortilis* (predominant) *A. ehrenbergiana*	0 1 1 2 3 3	20 40 60 20 40 60	0.326	7.69
Oued Tan-Assennane 22°44.543 N 005°38.291 E ALT 4112FT	*A. leata*	0 2 1	20 40 60	0.082	7.04

*****Soil slightly saline.

They were individually surface-sterilized by immersion in 35% H_2O_2 for 15 s to 2 min according to their size, and then rinsed in successive baths of sterile distilled water. After crushing, the nodule homogenates were spread onto yeast extract-mannitol agar (YMA) plates (Vincent, 1970). One to three isolated colonies per nodule homogenate were obtained from successive streaking on YMA plates. Their purity and membrane nature were checked by phase-contrast microscopy and Gram coloration respectively. Colony shape and color were determined using a magnifying glass. Pure cultures were then cryogenically preserved (-80°C) in yeast extract-mannitol medium (YEM) adjusted to 30% (v/v) glycerol.

Tolerance of bacterial isolates to salinity and temperature

All tests were carried out in triplicate. Inoculation isolates were grown in YEM liquid medium to log phase (corresponding to approximately 10^9 colony-forming units mL^{-1}). In test plates, inoculation was performed with 10 µl of these cultures. The results were scored after 7-day incubation. Positive results were reported when bacterial colonies were observed under a binocular microscope at 950 magnification. Tolerance to salinity was tested on YEM agar plates containing NaCl concentrations of 170, 340, 510, 680, 860, and 1034 mM incubated at 28°C. For temperature tolerance, YMA plates were inoculated and incubated at four different temperatures: 35, 40, 45, and 50°C.

Nodulation test

The nodulation ability of the obtained bacterial isolates was checked by inoculating seedlings of the corresponding *Acacia* species grown in hydroponic conditions. Tubes were filled with Broughton and Dilworth N-free nutrient solution (Broughton and Dilworth, 1971); a tab paper for plant support was introduced, then the tubes were sealed with aluminum folder and a rubber band and were sterilized. After cooling, central hole was dug for seedling introduction. One week after germination, three plants per Acacia species were inoculated with approximately 2 mL of bacterial suspension at 10^9 cells mL^{-1} of each isolate. Uninoculated controls were included. Plants were transferred to a greenhouse and watered with a sterilized Broughton and Dilworth (1971) N-free nutrient solution (Broughton and Dilworth), when it was necessary. Five weeks after inoculation, plants were harvested to estimate the infectivity and effectivity of bacterial isolates based on the presence of nodules, nodule color, visual observation of plant vigor, and foliage color. The bacterial isolates were recorded as nodulating or non-nodulating.

16S PCR-RFLP

Selected and isolated colonies were homogenized into sterile 1.5-mL centrifuge tubes with 50 µl of sterile Milli-Q water; suspensions were centrifuged at 6000 g for 5 min and washed twice. Pellets were re-suspended and then stored at 20°C until PCR use. The universal eubacterial 16SrRNA gene primers FGPS 6 (5′-GGA GAG TTA GATCTT GGC TCA G-3′) and FGPS 1509 (5′-AAG GAGGGG ATC CAG CCG CA-3′) (Normand et al., 1992) were used, and PCR was performed as follows: An initial cycle of denaturation at 94°C for 5 min, 36 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR products were checked by 0.8% (w/v) agarose gel electrophoresis in Tris/acetate/ EDTA (TAE) buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.3). Bands were excised and DNA was purified using a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). The final 16S ADN product was digested by restriction enzyme MPS I (Promega) as follows: 8 µ of PCR product, 2 µL buffer 10X, restriction enzyme (10U/µL) 0.5 µL and qsp 20 µL PCR water. The restriction digests were incubated during 1 h 30 min at 37°C and checked on agarose-gel (3%) electrophoresis to ensure they had gone to completion in TBE buffer (89 mmol Tris base, 89 mmol boric acid, 2 mmol EDTA, pH 8.0). The gels were run at 40V:15 min then at 80V: 1 h 45 min. Gels were stained by addition of ethydium bromide (1mg mL^{-1}), and photographed by RAISER camera (Germany) with Perfect Image software, V-5.3 (Clara vision). The gel profiles of RFLP pattern were done with phoretix 1D software (Totallab, 2013). The patterns were converted to matrix of zero and one depending on the presence or absence of bands for different strains. Dice's similarity coefficients were then calculated between pairs of bands. Strains were grouped by using the unweighted pair group method with arithmetic averages (UPGMA), dendrogram construction utility (DendroUPGMA) at S. Garcia-Vallve, Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain [http://genomes.urv.cat/UPGMA/] and tree dyn application (Dereeper et al., 2010).

Partial 16S rRNA gene sequencing

The final 16S ADN product (following the methodology cited previously) was sequenced using primers FGPS6, FGPS1509, and 16S-1080r (5′-GGG ACT TAA CCC AAC ATC T-3′) (Sy et al., 2001). Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA automated sequencer using a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems). Sequences were corrected using CHROMASPRO 5

(Technelysium Pty Ltd., Tewantin, Australia) before alignment and analysis of the 16S rRNA gene sequences using CLUSTAL X software (Thompson et al., 1997). Phylogenetic analysis was inferred using the neighbor-joining method (Saitou and Nei, 1987) calculated by the Kimura method (Kimura, 1980). The 16S rRNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank/EMBL database and used for cladistic analysis. A bootstrap analysis using 1000 replications was performed. The 16S rRNA gene sequences of selected Acacia non-nodulating strains were deposited in the GenBank database under accession numbers (Table 2) and were included in the phylogenetic tree.

Statistical analyses

Principal component analysis (PCA) was performed to examine the relationships between depth of sampling and *in vitro* bacterial tolerance to salinity and maximum temperature of growth, and a Factorial correspondence analysis (FCA) was performed to visualize the relationships between rhizobial taxa, as defined from the partial 16S rRNA gene-based phylogeny, and Acacia host species. Computations and graphical display for both methodologies were made using the XLSTATTM software package (version 2010.5.04, Addinsoft, Paris, France, http://www.xlstat.com)

RESULTS AND DISCUSSION

Nodulation status of Tamanrasset native *Acacia* species

Tamanrasset is considered as a desert region with an annual rainfall less than 100 mm. Sampling was conducted in November, two months after the rainy season to optimize the chances to find nodules, but none was encountered *in natura*. Host trapping assay showed the highest number of nodulated plants inoculated with soil suspension obtained from 40 and 60 cm instead of 20 cm of sampling depth. This could be due to relative soil humidity which allows rhizobial surviving; in stressful environments compatible rhizobial populations may be transiently insufficient or non-existent in the surface soil (Wolde-meskel et al., 2004).

There was no nodulation on root of *A. albida* with Oued Tassena soil suspension contrary to that of Oued in Daladg. The same goes for *A. laeta* which was poorly nodulated in association with Oued Tan-Assennane soil compared to those of Oued Tassena. Whereas, *A. nilotica* was poorly nodulated whatever the soil origin. This could be due to the low level of compatible rhizobial population. The highest nodulation pattern was observed on roots of *A. ehrenbergiana* followed by *A. tortilis.* Both species were dominant numerically when they were present at the prospected sites, so we could assume that rhizobial population correlates to tree's density as reported by Thrall et al. (2007).

The obtained nodules had a determinate and indeterminate shape whatever host species and prospected site; while observed Acacia's nodules in literature were indeterminate and woody (Sprent, 1989).

Nodular endophytic bacteria phentotypic and symbiotic characterization

81 isolates were obtained from nodules of the five *Acacia* spp. All purified strains showed fast growth (2 to 3 days). The majority of colonies was translucent or white watery; some were pointy in form and others were "marbled" (milky curdled type). All were gram negative coccobacillus with the occurrence of some rods.

Only four endophytic bacteria were true symbiont by their nodulation ability of host trap species (Table 2). This paucity of nodulation for endophytic nodular bacteria had been reported for true wild legumes by Muresu et al. (2008). These authors had given some hypotheses to explain this phenomenon: sterilization techniques did not allow rhizobial recovery; moreover salinity or acidity of isolation culture media could interfere with surviving of rhizobia. Finally the predominant endophytic non rhizobial bacteria may produce antagonistic compound affecting symbiont growth.

There were no marked difference between uninoculated plants and inoculated ones, so we could not suggest a plant growth promotion effect of these non nodulating endphytes.

Nodular endophytic bacteria genetic diversity

The blast results from nearly full-length 16S rRNA gene sequences (> 1300 nt) for symbiotic nodulating bacteria classified them as *Ensifer teranga*, *Ensifer* sp., *Mesorhizobium* sp. and *Rhizobium* sp. (Table 2). These results are in agreement with studies on African symbiont diversity (de Lajudie et al., 1994, 1998; Nick et al., 1999). Among the 79 non nodulating endophytes, 24 representative strains were genetically characterized. Bacteria were selected on the basis of their macroscopic aspects and PCR-RFLP profile after digestion with MSPI enzyme. In our study, it is highly discriminating. This discrimination power has also been reported by Fall et al. (2008). Selected strains belonged to nine genera (Table 2) namely: *Paenibacillus, Ochrobactrum, Stenotrophomonas, Pseudomonas, Microbacterium, Rhizobium, Agrobacterium, Brevibacillus, Advenella*. On the basis of phylogenetic study, five major groups were distinguished (Figure 1). The first group included nodulating reference strains and an *Agrobacterium* sub-group affiliated with *Agrobacterium tumefaciens* ; it included two strains blasted as *Rhizobium* sp. (N141a, L120T) and *Agrobacterium tumefaciens* (N70a) ; the second sub-group included *Ochrobactrum* genus: E85b close to *O. intermedium* ; and L30b, S180b, E46b close to *O. anthropi*. The second group was constituted by *Pseudomonas aeruginosa* sub-cluster close to N141B, E136a; an *Advenella kashmirensis* branch associated with T21b and *Stenotrophomonas maltophila* close to N97b3, E136c, N97B. For the two other clusters, the first is affiliated to *Microbacterium oxydans* including N142b2,

Table 2. Molecular and tolerance profile characterization of *Acacia* species nodular endophytes.

Strains (Accession number)	Closest partial 16S rRNA Gene sequence *	Maximum strain temperature tolerance (°C)	Maximum strain salinity tolerance (mM)	Associated host plant	Site of sampling localization	Depth of sampling (cm)
Renodulating						
E60 (HQ836175)	*Ensifer teranga*	50	340	*A. ehrenbergiana*	Oued Tin Amezzegin	60
T82 (HQ836175)	*Ensifer* sp.	40	1034	*A. tortilis*	OuedTassena	60
A121 (HQ836160)	*Mesorhizobium* sp.	40	680	*F. albida*	Oued In Daladg	60
N145 (HQ836162)	*Rhizobium* sp.	40	680	*A. nilotica*	Oued In Daladg	60
Non nodulating						
E85b (KM894187)	*Ochrobactrum intermedium*	40	1034	*A. ehrenbergiana*	Djnen Biga 22°33.683 N 005°23.754 E ALT 3735FT	20
E136c (KM894178)	*Stenotrophomonasmaltophilia*	50	1034	*A. ehrenbergiana*		60
E136a (KM894176)	*Pseudomonas aeruginosa*	45	1034	*A. ehrenbergiana*		60
N142B (KM894185)	*Microbacterium oxydans*	40	1034	*A. nilotica*	Oued In Deladg 22°56.004 N	40
N142b2 (KM894196)	*Microbacterium oxydans*	40	1034	*A. nilotica*	005°52.851 E ALT 4554FT	40
N141B (KM894177)	*Pseudomonas aeruginosa*	45	1034	*A. nilotica*		40
N141a (KM894198)	*Rhizobium* sp.	50	1034	*A. nilotica*		40
L120T (KM894194)	*Rhizobium* sp.	40	680	*A. laeta*		40
E46b (KM894190)	*Ochrobactrum anthropi*	40	1034	*A. ehrenbergiana*		60
L30b (KM894179)	*Ochrobactrum anthropi*	40	1034	*A. laeta*	Oued Tassena 35°35.240 N	40
L29b (KM894183)	*Peanibacillus*sp.	50	510	*A. laeta*	000°48.527O ALT 358FT	40
T21b (KM894192)	*Advenella kashmirensis*	40	1034	*A. tortilis*		60
T20C (KM894195)	*Paenibacillus humicus*	40	510	*A. tortilis*		40
N70a (KM894180)	*Agrobacterium tumefaciens*	40	1034	*A. nilotica*	Oued Tamezzegine 22°35.218 N 005°23.815 E ALT 3740FT	60
T76c (KM894181)	*Paenibacillus glycanilyticus*	40	510	*A. tortilis*		20
N97b3 (KM894189)	*Stenotrophomonasmaltophilia*	40	1034	*A. nilotica*	Oued Idekel 22°33.783 N 005°24.020EALT3732 FT	60
N97B (KM894184)	*Stenotrophomonasmaltophilia*	45	1034	*A. nilotica*		60
S178a (KM894182)	*Paenibacillus glycanilyticus*	50	1034	*A. seyal*	Oued Taghemout	40
S186a (KM894193)	*Paenibacillus humicus*	50	1034	*A. seyal*	22°58.640 N 005°40.892 E	20
S186B (KM894191)	*Brevibacillus nitrificans*	50	1034	*A. seyal*	ALT 5461FT	20

Table 2. Contd.

S180b (KM894186)	*Ochrobactrum anthropi*	45	1034	*A. seyal*		40
T160b (KM894199)	*Pseudomonas* sp.	45	680	*A. tortilis*	Oued In Tounin (Tasekra)	60
T159b (KM894197)	*Peanibacillus* sp.	40	1034	*A. tortilis*	22°56.004 N 005°52.851 E ALT 4554FT	60
L105b (KM894188)	*Peanibacillus* sp.	45	1034	*A. laeta*	Oued Tan-Assennane 22°44.543 N 005°38.291 E ALT 4F96FT	40

***** Similarity of 99%.

N142B and the second to *Brevibacillus nitrificans* associated with S186B. The last group included all the *Paenibacillus* genera: *Paenibacillus glycanilyticus* close to T76c, S178a and P*aenibacillus humicus* affiliated with S186a, T20C. We recorded a strong sub-group blasted as *Paenibacillus* sp. not affiliated to reference strains which included L105b, L29b and T159b.

N70a and N141a are at the same phylogenetic position; the first one is affiliated to *A. tumefaciens* while the second to *Rhizobium* sp. Moreover, in the phylogenetic tree (Figure 1), strain L120T has the same phylogenetic position as *A. tumefaciens* but is affiliated to *Rhizobium* sp.. These results highlighted the monophyletic status of *Rhizobium* and *Agrobacterium* (Young et al., 2001; Sawada et al., 2003; Farrand et al., 2003); even partial sequencing of 16S R DNA did not give an exact taxonomic position.

Generally, *Paenibacillus* is the second most abundant genera from nodule endophyte isolates (Velasquez et al., 2013). In our study, it was predominant. A study conducted by Hoque et al. (2011) on nodular endosymbiotic bacteria isolated from *Acacia stenophylla* and *Acacia salicina,* native species in Australia reported an unexpected diversity of about 19 genera. Eight of them are similar with our findings but *Advenella kashmirensis* was not reported as presenting a status of endophyte in literature. There are other studies on native legumes in arid and semi-arid regions in Tunisia (Benhizia et al., 2004, Zakhia et al., 2006); non-inoculated legumes in the Mediterranean basin (Muresu et al., 2008) and perennial *Lespedesa* in South Korea (Palaniappan et al., 2010) revealed the same or different affiliations as cited above, but always a remarkable diversity.

Tolerance profile to salinity and high temperatures of bacterial strains

Nearly 80% of characterized strains tolerated 1034 mM of salinity and five grew at 50°C (Table 2). A Principal Component Analysis (PCA) was used to explore variation of *in vitro* bacterial tolerance to salinity and a maximum temperature of growth according to depth of sampling. The three principal components accounted for 41.43, 36.06 and 22.5%, respectively. There was no relationship between these parameters. As clearly confirmed by PCA (Figure 1), no correlation was found between bacterial tolerance to a maximum temperature of bacterial growth and depth of sampling (r= -0.196); even a slight correlation was revealed between bacterial tolerance to salinity and depth of sampling with r= 0.201.

In a previous work, we found out (Boukhatem et al., 2012) that there were no relationship between *in vitro* tolerances of rhizobial strains to NaCl and high temperature and corresponding edaphoclimatic characteristics of the sampling sites. These findings were in agreement with this present study; maximum *in vitro* strain salinity tolerance was reported for bacterial endophytes originated from slightly saline soil (Oued Idekel) than those from non-saline sites (Table 1 and Figure 2). The isolated nodular endophytes in this study revealed a strong tolerance profile which made them to survive in soils suffering from harsh conditions. It was proven previously that salt-tolerant and temperature-resistant characteristics made the bacteria adapt to extreme environments (Tripathi et al., 2002; Tehei and Zaccai, 2005). This resistance profile was distributed over the three levels of soil sampling: 20, 40 and 60 cm (Table 2). Whilst, endophyte colonization may originate from other source other than rhizosphere, such as phyllosphere, anthosphere, or spermosphere (Hallmann et al., 1997). In this study, soil suspension trapping methodology was used. The main source of endophytes was soil and to a lesser degree endophytes were acquired by horizontal transfer (they were present into seeds). We suppose that in natural conditions, even bacteria presented a high tolerance profile. Environmental temperature could affect the fluctuation of bacterial endophytes (Mocali et al., 2003) and stressful

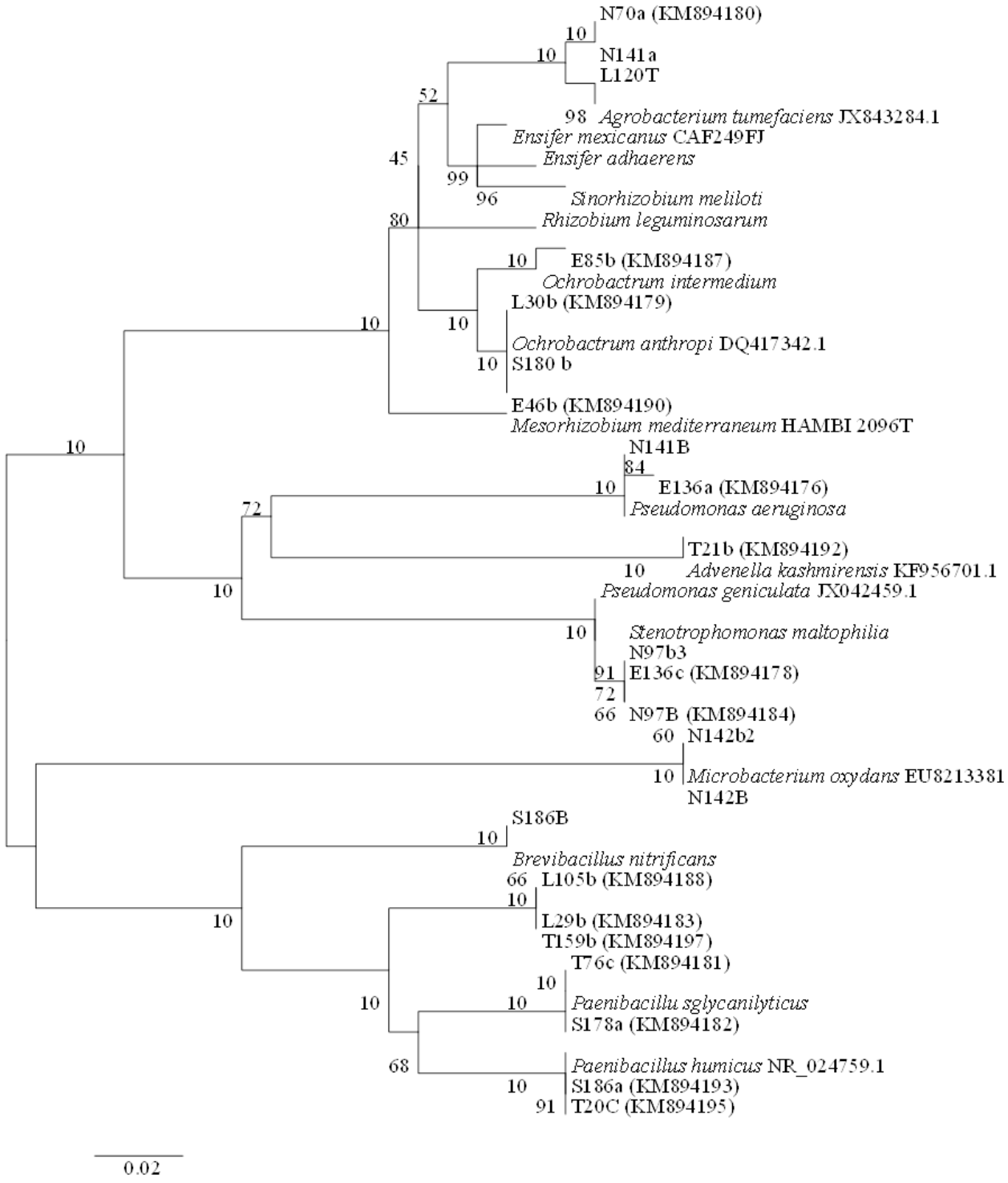

Figure 1. Phylogenetic tree based on nearly full-length 16S rRNA gene sequence (> 1300 nt) analysis of nodule endophytic bacteria of native *Acacia* sp. in Tamanrasset and reference strains (renodulating and endophytic). Neighbor-Joining method integrating Kimura 2 distance was used. Data are bootstrap values issued from 1000 repetitions. One strain listed in Table 1 was not included in the phylogenetic tree since its nucleotide length was <1300 nt.

conditions could drive the selection toward higher frequencies of infection as it has been proven for grasses associated with fungal endophytes (Jensen and Roulund, 2004). On the other hand, when legume plants are exposed to complex communities they selectively regulate access and accommodation of bacteria

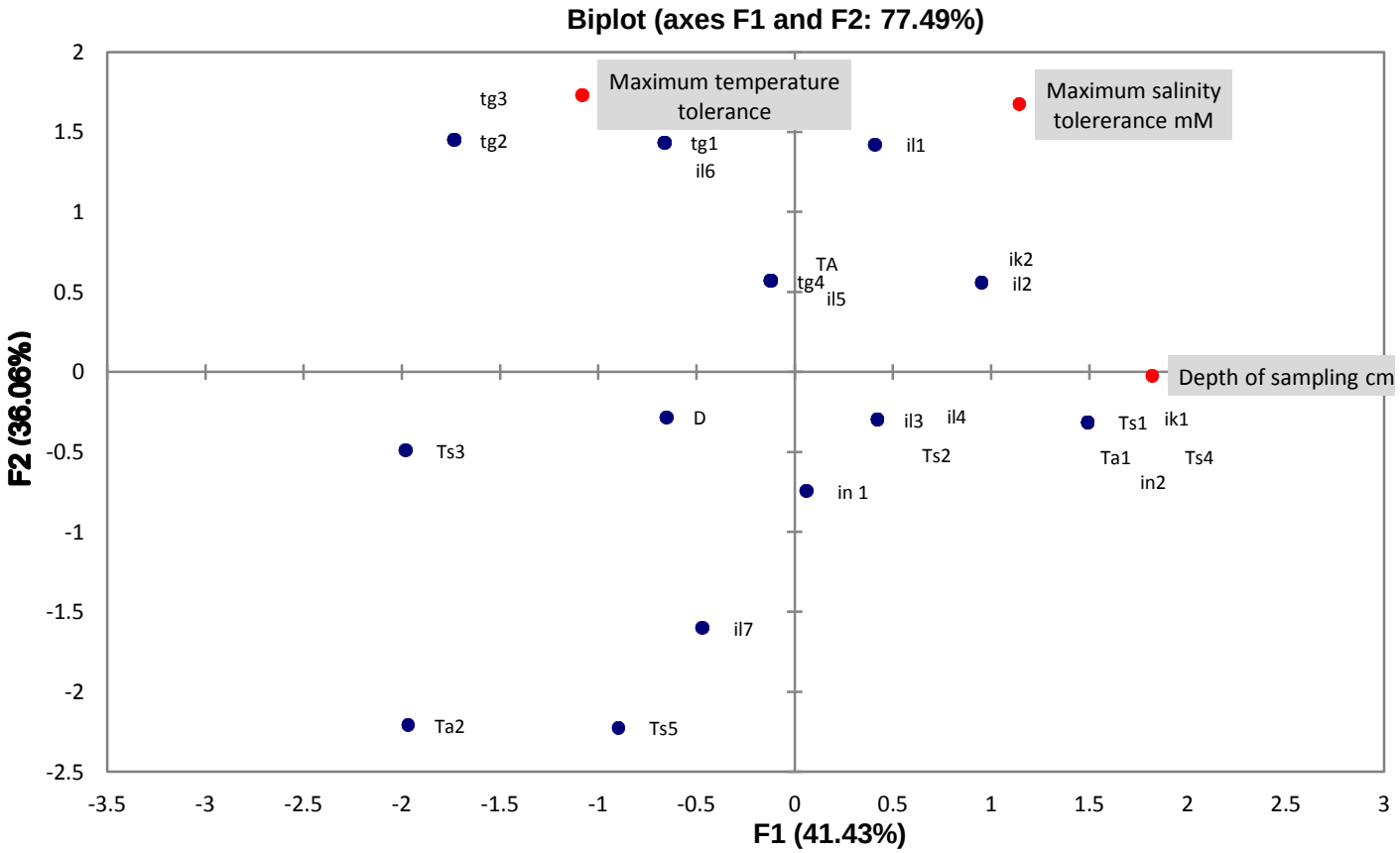

Figure 2. PCA representing the relationships between *in vitro* tolerance of nodular endophytic isolates to NaCl and high temperature and depth of sampling. Each dot corresponds to a single bacterial isolate from its site of sampling and is indicated by a symbol as follows: **D**: Djnen Biga; **il**: Oued In Deladj; **Ts**: Oued Tassena; **Ta**: Oued Tin Amezzejin; **ik**: Oued Idekel; **tg**: Oued Taghemout; **TA**: Oued Tan-Assennane, **in**: Oued In Tounin (Tasekra).

occupying the root nodule which is considered as a specialized environmental niche (Zgadzaj et al., 2015).

Plant host specificity

Acacia nilotica presented the largest panel of endophytic diversity due to the highest number of isolates (8). It did not harbor the dominant genera *Paenibacillus* or *Ochrobactrum*. Only *A. ehrenbergia* and *A. nilotica* share more than one genus (*Stenotrophomonas* and *Pseudomonas*) (Figure 3). The hypothesis that differentplant species can be colonized by a different spectrum of endophytic bacteria was given by McInroy and Kloepper (1995) in experiments conducted on sweet corn and cotton. Another study (Izumi et al., 2004) performed on coniferous and deciduous tree species by comparing the most abundant cultivable bacteria in the rhizosphere and root samples suggested that root's endophytic bacteria may be in residence through a process of selection or by active colonization rather than by passive diffusion from the rhizosphere. There are influencing factors prior colonization including plant-species-specific factors: root architecture, surface structure and root's exudates and non-plant factors like wounding or mycorhization. On the other hand after the root colonization, size of the intercellular space, nutrient composition within the apoplastic fluid and the plant's response to endophytic colonization are probably the main factors determining bacterial selectivity and thus the bacterial spectrum found inside the roots (Hallmann and Berg, 2006).

Conclusion

Tamanrasset hosts a great biodiversity of native *Acacia* spp. These trees are highly resistant to drought and survive under extremely harsh conditions. It is interesting to reveal bacterial diversity associated with their nodules, symbiotic and non-symbiotic, especially in this ecologically preserved and fragile ecosystem. The weak recovery of non nodulating bacteria gives rise to some questions about specific interactions between bacterial

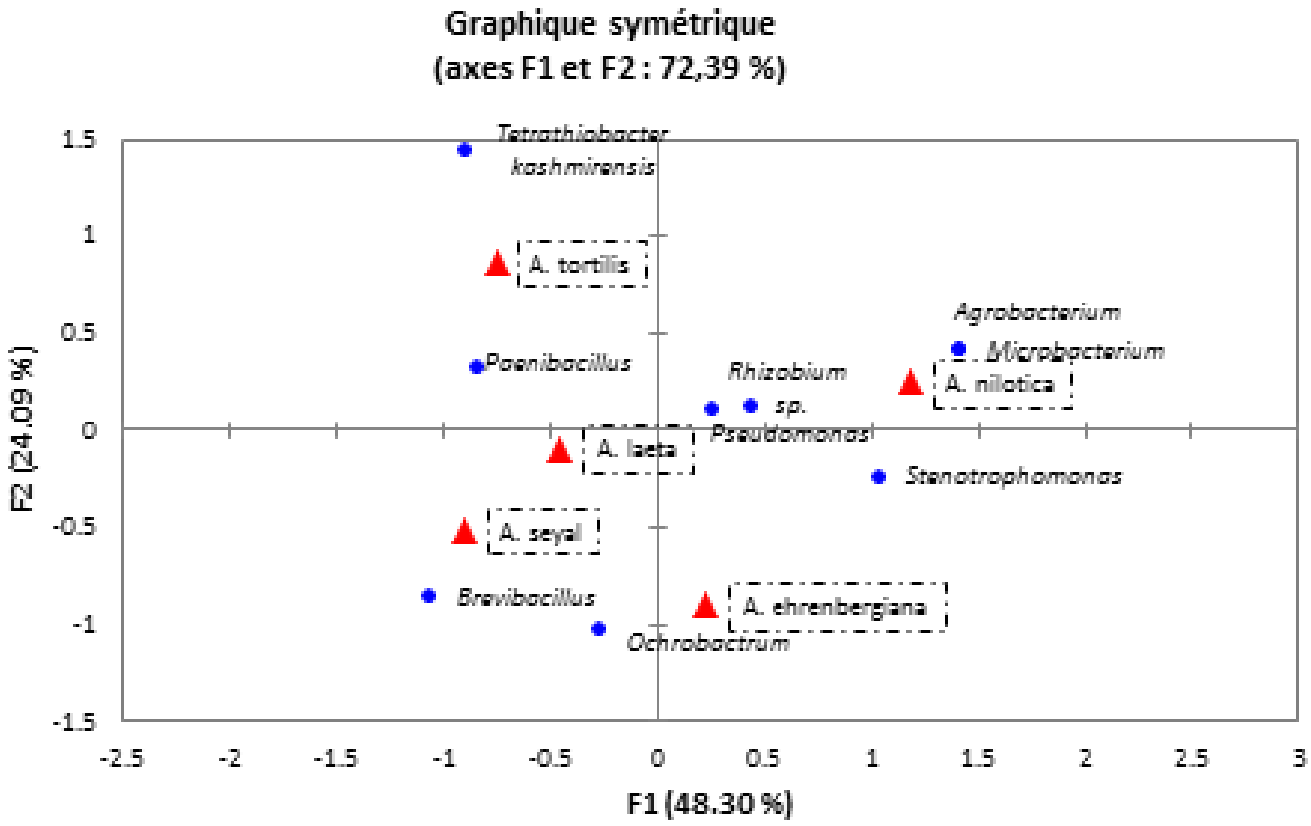

Figure 3. First factorial plane projection of FCA between endophytic bacterial taxa (circles), as defined from the 16S rRNA gene-based phylogeny, and five *Acacia* species (triangles).

endophytes and symbionts which initiate nodules. We wonder if endophytic population size or metabolite production inhibits rhizobial growth. Even these non-symbiotic endophytes did not show a PGP effects; other beneficial actions have to be investigated as their effect on seedling emergence, bio-control ability and production of metabolites with biotechnological uses.

Conflict of Interests

The authors have not declared any conflict of interests.

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