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Origanum syriacum ssp. *sinaicum* associated Growth Promoting Bacteria

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Abstract: *Plant growth promoting bacteria have several applications in agriculture as they could be used as bio-fertilizers and/or bio-control agents. Isolation, characterization and identification of Origanum syriacum ssp. sinaicum associated growth promoting bacteria was the aim of this study as Origanum is one of the most important wild endemic medicinal plants in Egypt. Out of 268 bacterial isolates whether rhizospheric or endophytic obtained firstly, the initial test for the production of gibberellic acid led to a reduction of this number to be 25. The nitrogenase activity was the second potential test by which 4 isolates were finally selected and tested for further plant growth promoting characteristics. 16S rRNA analysis identified two of them to be Serratia sp. (Serratia SK3 and Serratia SK1a) and the other two to be Bacillus sp. (Bacillus SK1 and Bacillus SK2). The current investigation represents four local bacterial strains that could be used to enhance the growth of the commercially grown and widely used Origanum syriacum ssp. sinaicum in Egypt.*

Keywords: *Bacillus, Gibberellic acid, Origanum syriacum ssp. sinaicum, phosphate solubilization and Serratia*

I. Introduction

Soil is often considered as a black box as it represents a suitable environment for a diverse and complicated community of bacteria, fungi, actinomycetes, protozoa, and algae. These microorganisms are always in direct or indirect contact with plant and the outcome might be in one of three forms; neutral, harmful, or beneficial relationship. The interaction between plant and these microorganisms may occur at the phyllosphere (above-ground portions of plant), rhizosphere (soil region directly influenced by root exudations) or endosphere (the internal transport system of the plant). Plant growth promoting bacteria might be termed as rhizospheric (PGPR), if they are inhabiting the root plane or it might be termed as plant growth promoting endophytic bacteria (PGPE) when they are present inside the plant tissues. The treatment of plants with plant growth promoting bacteria (either rhizospheric or endophytic bacteria) has been reported to increase the growth parameters such as germination rates, root growth, leaf area, yield, chlorophyll content, and shoot and root weights [1]. Plant growth promoting bacteria have several applications in agriculture as they might be used as bio-fertilizers and/or bio-control agents [2]. In agricultural applications, the advantage of inoculating seeds with endophytic PGPB is that, they will establish populations which will have a competitive advantage over organisms invading the rhizosphere [3]. Generally, plant growth promoting bacteria may stimulate the growth either directly by supplying the plant with fixed nitrogen, soluble phosphate, phytohormones, and the1-aminocyclopropane-1-carboxylate (ACC) deaminase activity [4-6] and/or indirectly by preventing phytopathogens from attacking the plant [7, 8]. One of the most important wild endemic medicinal plants in Egypt is *Origanum syriacum* subsp. *sinaicum* (Boiss.) Greuter&Burdet [9] which is a rare species **[10]** endemic to Sinai Peninsula in Egypt [11]. The over exploitation in addition to habitat loss, deforestation coupled with over harvesting all together have resulted in dwindling population of important medicinal plants around the world and *Origanum* different species were not an exception [12]. Therefore, Finding solutions for problems facing such rare plants is a must. Each medicinal plant harbors a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms [13]. Up to our knowledge, there is no report on *Origanum syriacum* spp. *sinaicum* associated bacteria, therefore this study aims to isolate and characterize wild and cultivated oregano associated bacteria to assess their PGP traits. The outcome of this study will form the basis for the selection of a suitable PGP bacteria that would be used later as a bio-fertilizer to facilitate oregano domestication and to improve its growth and metabolism.

II. Materials and Methods

Collection of the plant materials and isolation of plant growth promoting bacteria from different niches:

Origanum syriacum spp. *sinaicum* samples were collected from Saint Katherine Protectorate, the wild environment of this plant in Egypt. From Wadi Garagnia, four plant samples were carefully removed with roots from different land forms and at different heights up to 2000 meters above the sea level. Nine additional aerial parts were obtained from Wadi Garagnia and Wadi Meserdy. Additionally, three other cultivated plants were collected from Medicinal Plants Conservation Project in Saint Katherine. All samples were collected in sterile polythene bags and brought to the laboratory for the isolation of endophytic and rhizospheric bacteria within 24 h after collection.

- **Isolation of rhizospheric and endophytic bacteria:**

To isolate rhizospheric bacteria, wild oregano samples were uprooted along with soil adhering to the root. About 5 g of the rhizospheric soil, which collected by shaking in sterilized bags,were transferred to 50 ml sterilized tap water and shaken for 15 min. at room temperature. Serial dilutions were prepared as described previously [14]. For streaking, a loop of each dilution was streaked on Luria Broth (LB) plates. The plates were incubated at 30ºC for 24 h . Colonies with different morphology were picked up and purified on LB plates by repeated streaking.

To analyze endophytic bacteria, the collected oregano samples were separated into root and aerial parts then the following procedures were done separately. The obtained root samples were washed with sterilized dist. water then, the root parts were subjected to surface sterilization by 0.2 M $HgCl_2$ in 50% ethanol for 4 minutes and finally additional washing by sterilized dist. water was performed. Under aseptic conditions, the sterilized roots were cut into small segments, dried over sterilized filter paper, and finally spread over nutrient agar plates and incubated at 30ºC for 24 h in order to confirm the success of surface sterilization process [15-17].

The surface sterilization of the aerial parts was performed by 70% ethanol for 1 minute and then 3% sodium hypochloride for 6 minutes [3]. The success of the surface sterilization was also confirmed as mentioned previously.

In order to isolate the endophytic bacteria, the aerial and root parts were macerated separately in a sterilized 0.8 % KCl solution by using a sterilized mortar and pestle under aseptic conditions. Then the serial dilution method was performed and the samples were streaked on LB plates and incubated at 30ºC for 24 h. The colonies with different morphology were further purified and kept at glycerol stocks for further experiments.

Selection of the best plant growth promoting (PGP) isolates:

Whether rhizospheric or endophytic, the obtained isolates were further subjected to a set of experiments in order to select the PGP bacteria among them. These experiments aimed to test the production of gibberellic acid and nitrogen fixation activity [4-6].

- **Gibberellic acid production test:**

25 ml of LB media was inoculated with each bacterial isolate and the cultures were incubated at 30°C for 3 days in dark. After centrifugation, 5 ml of the culture supernatant was adjusted to pH 2.8 by 0.1N HCl and then extracted 3 times by equal volume of ethyl acetate in a separating funnel and the resulting ethyl acetate fractions were collected. The ethyl acetate fraction was then passed through sodium sulphate anhydrous. Then 1ml Folin reagent was added to 1 ml of the treated ethyl acetate fraction in addition to 1ml conc. HCl and 3ml of distilled water in a boiling water bath for 5 min. and the absorbance was measured at 750 nm [18]. The isolates produced the highest amounts of GA were passed to the next selection step; the nitrogenase activity test.

- **Nitrogenase activity assay:**

Nitrogenase (EC 1.18.6.1) activity was assayed for the selected bacterial isolates according to acetylene reduction method described by [19] and modified by [20]. At first, one colony of each bacterial isolate was inoculated in Watanabi broth, a specific medium for the selection of nitrogen-fixing bacteria [21]. Subsequently, 20 ml of fresh Watanabi semi-solid medium was inoculated with 1ml of the culture of the tested isolates. The incubation was carried out at 30°C for 24 h and sterilized sob seal was used to prevent gas leakage. 10% of the bottle`s air was then replaced by acetylene gas and then after four hours, the gas phase of the bottles was mixed thoroughly and 0.5ml gas sample was taken for ethylene measurement using Gas Chromatography (model HP6890 GC). Serial dilutions of pure ethylene were used in constructing the standard curve. Whether endophytic or rhizospheric, the isolates that gave the highest nitrogenase activity were nominated as potential plant growth promoting bacteria.

Morphological characteristic and molecular identification of the selected isolates:

The selected isolates were described morphologically on nutrient agar plates. Furthermore, Gram staining, motility, pigment production and catalase activity were also determined [22].

The Bacterial genomic DNA of each strain was extracted and used as a template for PCR amplification of the 16S rRNA gene using forward primer 5´ACTCCTACGGGAGGCAGCAG, and a reverse primer 5´ATTACCGCGGCTGCTGG. The PCR product was purified and sequenced. The generated sequences were analyzed by Finch TV software and the phylogenetic tree was generated via Seaview software using the closest published type strains sequences.

Testing for further plant growth promoting activities:

In order to confirm the potentiality of the finally selected isolates as plant growth promoting bacteria, the isolates were subjected to the following tests;

- **Production of phytohormones:**

The selected bacterial isolates were inoculated in 25 ml Watanabi media supplied with 5 mM tryptophan and incubated for 2 days in dark conditions at 30°C. After centrifugation, the pH of the culture supernatant was shifted to 2.8 then the samples were extracted with ethyl acetate [23-25].

The phytohormones in the ethyl acetate fractions were separated by thin layer chromatography using a solvent system of isopropanol/ammonia/distilled water (10:1:1 w). IAA, GA_3, and ABA bands were detected under UV according to the standards of all of them. Bands detected were scraped and dissolved in methanol and analyzed by High performance liquid chromatography using a reverse phase Supelcosil LC-18 column (25 cm × 4,6 mm) by Cecil 1100 Series Liquid Chromatograph. Samples were analyzed under isocratic conditions with 35% methanol (in 1% acetic acid) for 1AA, 30% methanol (adjusted to PH 3.0 with 0.1 M H_3PO_4) for GA_3 and 55% methanol (in 0.1 M acetic acid) for ABA. Wave lengths in the UV detector were 280, 208 and 265 nm for IAA, GA3 and ABA respectively. The total run time for separations was approximately 15 min at a flow rate of 1 ml/min.

- **Ammonia production:**

For testing ammonia production, the isolates were raised in 10 ml of peptone water medium (containing:10 g peptone, 5 g NaCl in one liter and then was adjusted to pH 7.2 and incubated at 30°C for 4 days. One ml of culture was added to a test tube containing one ml of Nesseler's reagent. Development of the yellow to brown color indicates the production of ammonia [26].

- **Phosphate solubilization:**

Phosphate solubilization was assayed by using Pikovaskaya's medium [27]. The plates were inoculated with 10 µl of the fresh isolate cultures spotted on the four quarters of the plate and incubated at 30°C for 7 days. The plates routinely checked for formation of transparent haloes around the bacterial growth.

- **Hydrogen cyanide (HCN) production:**

The fresh cultures of the selected isolates were streaked on King's B medium supplied with 4.4 g/l glycine. Whatman no.1 filter paper discs soaked in 0.5% picric acid (in 2% sodium carbonate) were placed in the lid of the Petri dishes in order to detect the production of HCN. The plates were tightly sealed with parafilm and incubated at 30°C for 4 days till the development of deep orange colour [28].

- **1-Aminocyclopropane-1-carboxylate deaminase assay:**

The activity of 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminse) was determined according to the previously described method [29]. The selected isolates were inoculated in 100 ml of Dobereiner medium (nitrogen free based medium) [30]. The cultures were incubated at 30°C for 2 days to get rid of any nitrogen traces. 3 ml of DF salt minimal medium was supplemented with ACC solution (as the sole nitrogen source) to 3 mM final concentration [31]. This DF-ACC medium was inoculated with 200 µl of Dobereiner culture and incubated for 24 h at 30°C. After centrifugation, 1 ml of the supernatant was mixed with ninhydrine reagent. The tubes were closed tightly then put in a boiling water bath. The absorbance of samples color was measured at 570 nm.

III. Results

- **Isolation and selection of *Origanum syriacum* ssp. *sinaicum* associated growth promoting bacteria:**

Several rhizospheric as well as endophytic isolates were obtained from both wild and cultivated plants. To discriminate PGPB from others, all the isolates were tested for the production of gibberellic acid colourimetrically. The bacterial isolates that gave the highest amount of GA, 25 isolates, were passed for the next selection step; the nitrogenase activity which led to the final selection of 4 bacterial isolates. Among them,

the isolates B2 and B3 were originally isolated as endophytes from cultivated (root) and wild (shoot) of oregano respectively. The other two B4 and B5 were obtained from cultivated and wild oregano rhizosphere respectively. Beside these isolates, an active reference stain B1 (*Bacillus polymyxa*), known for its PGP characteristics, has been used as a positive control for the proceeding experiments [32]. The morphological characteristics of these isolates , in addition to the reference strain, were observed on nutrient agar media as indicated in table 1. The 16S rRNA sequence analysis showed a very high sequence similarity between B2 and B5 isolates and the members of genus *Serratia* (figure 1) while B3 and B4 showed the identity to *Bacillus* sp. (figure 2). The isolates B2 and B5 were further given the names *Serratia* SK3 and *Serratia* SK1a respectively and B3 and B4 were given the names *Bacillus* SK1 and *Bacillus* SK2 respectively. The partial sequence obtained for 16s rRNA genes of all the identified organisms were submitted to the gene bank under the accession numbers KU379654, KU379656, KU379655, KU379657 for *Bacillus* sp. SK1, *Bacillus* sp. SK2, *Serratia* sp. SK1a and *Serratia* sp. SK3 respectively.

- **Further plant growth promotion characteristics**

The HPLC analysis performed to detect the production of phytohormones by the isolates under investigation showed that, all of them did not produce a detectable level of IAA and ABA, however, all the isolates produce GA_3. The highest producer was **B5** *Serratia* SK1a, while the lowest production was detected by **B4** *Bacillus* SK2 compared to the reference strain **B1** *Bacillus polymxa* (table 2).

All the selected isolates show the ability to produce ammonia. Ammonia production was significantly higher for **B2** *Serratia* SK3 and **B4** *Bacillus* SK2 than other isolates and the reference strain (table 2).

Phosphate solubilization has been indicated for all the selected isolates as indicated in table 2. The isolates **B2** *Serratia* SK3 and **B5** *Serratia* SK1a showed the highest phosphate Solubilization capacity compared to the reference strain as indicated by the wider area of the clear zone.

Hydrogen cyanide (HCN) production was indicated for **B2** *Serratia* SK3 ,however, the other isolates, including the reference strain, did not produce HCN under the same experimental conditions.

All the tested isolates including the reference strain showed ACC-deaminase activity as shown in table 2. However, the higher activity was recorded to the isolates **B3** *Bacillus* SK1, and **B4** *Bacillus* SK2 compared to the reference strain.

IV. Discussion

Due to the significance of *Origanum syriacum* spp. *sinaicum* as a medicinal plant, the decision was taken to perform this study aiming to isolate its naturally associated bacteria and to evaluate their growth promoting criteria, a step that might enable their use as bio-inoculants to improve the plant growth and metabolism. Among 268 isolates originally obtained, it became experimentally evident that, the finally selected isolates; **B3** *Bacillus* SK1 ,**B4** *Bacillus* SK2 ,**B2** *Serratia* SK3 and **B5** *Serratia* SK1a showed the highest capacity to produce GA3 and the highest nitrogenase activity.

Although the identified bacteria were not previously recorded as an associate of *Origanum syriacum* ssp. *sinaicum* whether as rhizospheric or endophytic bacteria, several studies showed the association of *Serratia* sp. [33, 34] and *Bacillus* [35], [36, 37] both as rhizosphere and/or endophyte with other plants.

The ability to produce GA_3 might be a key tool that assists the obtained *Serratia* and *Bacillus* to invade the plant tissue and rhizospere region as valuable endophytes. This explanation has been indicated previously by various authors [38, 39]. The production of GA_3 has been reported for several species such as *Bacillus licheniformis* and *Bacillus pumilus* [40]. Moreover several *Serratia* species has been figured as gibberellic acid producer [41].

An additional essential role that could be played by the PGPB is the ability to fix atmospheric nitrogen [42]. The isolated strains in this study have the ability to fix nitrogen as indicated by the results of nitrogenase assay and supported by measuring ammonia production (table 2). Earlier studies reported the ability of *Serratia sp.* isolated from the rice to fix nitrogen [43] and reported the same ability to an isolated *Bacillus sp.* [44]. In addition to its direct influence on plant growth and metabolism, ammonia production might indirectly influence the plant growth [45] by shifting the pH to alkaline condition leading to growth suppression of certain fungi and nitrobacteria [46].

Phosphate solubilization is another help that could be provided by PGPB to the plant to support its growth and metabolism. Phosphate-solubilizing bacteria are able to solubilize phosphate inorganic compounds such as tricalcium phosphate by the production of organic acids [47]. In agreement with previous studies, the obtained bacterial isolates showed the ability to solubilize phosphate. High phosphate solubilization activity by *Serratia* sp. was reported by previous studies [48-50] that supported observation that gave *Serratia* isolates higher grade of phosphate solubilization compared with *Bacillus* isolates. The isolation of **B2** *Serratia* SK3 as endophyte from cultivated root and **B5** *Serratia* SK1a from the rhizospere of the wild oregano supports that it might be associated with the plant to enhance phosphate uptake.

The ability of **B2** *Serattia* SK3 to produce HCN has been reported previously for other *Serattia* species [51] and it add to us another benefit for its association with oregano as the production of HCN might protect plant from various biotic stresses [52].

The ability to metabolize 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene, by ACC-deaminase activity is another potential role of the obtained isolates that might be used to reduce the production of ethylene [53, 54]. Both *Bacillus* [35] and *Serratia* [55] different species showed ACC-deaminase activity in other previous studies.

In conclusion, this study spot the light on the plant growth promoting bacteria associated with *Origanum syriacum* ssp. *sinaicum* and their abilities in various metabolic processes. The desired characters founded in these isolates open the door for their use later as bio-inoculants that would improve oregano growth and metabolism.

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Table 1: Morphological and biochemical characters of the selected PGPBs.

Isolate/Phenotypic of colony	**B1**	**B2**	**B3**	**B4**	**B5**
On Solid Agar					
Colony form	Circular	Circular	Circular	Circular	Irregular
elevation	Convex	Umbonate	Raised	Flat	Raised
margin	Entire	Entire	Dentate	Entire	Lobate
surface	Smooth	Smooth	Smooth	Smooth	Smooth
color	Off-white	Off-white	White	Off-white	White
size	Moderate	Moderate	Punctiform	Big	Punctiform
consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Optical Density	Translucent	Translucent	Opaque	Translucent	Opaque
On nutrient broth					
Amount of growth	Abundant	Abundant	Moderate	Moderate	Abundant
Surface Growth	-ve	-ve	-ve	-ve	-ve
Sub-surface growth (Dob.semiliquid)	+++ve	++ve	+ve	-ve	-ve
Sediment growth	-ve	-ve	-ve	+ve	+ve
On nutrient agar –slant					
Amount of growth	Abundant	Abundant	Abundant	Abundant	Abundant
Form	Distributed	Distributed	Branched	Beady	Distributed
Pigmentation	-ve	-ve	-ve	-ve	-ve
Colour	Off-White	Off-White	Off-White	Off-White	Off-White
Opacity	Translucent	Translucent	Opaque	Opaque	Translucent
Organism identified	***Bacillus* sp.**	***Serratia* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Serratia* sp.**

B1: *Bacillus polymxa*, **B2:** *Serratia* SK3, **B3***: Bacillus* SK1, **B4:** *Bacillus* SK2 ,**B5** *Serratia* SK1a.+ve=positive,-ve=negative.

Table 2: Plant growth promoting traits of (B1,B2, B3, B4 and B5).

Traits	**HCN**	**Phosphate Solubilization**	**Ammonia Production**	**ACC deaminase**	**N_2ase (nmoles C_2H_4/ml /h)**	**IAA**	**GA_3**	**ABA**
B1	-	+	+	+	8.9 $\pm 1.2^{b}$	n.a.	+++	n.a.
B2	++	+++	++	+	17.87 $\pm 5.6^{a}$	n.a.	++	n.a.
B3	-	+	+	++	9.5 $\pm 3.4^{b}$	n.a.	++	n.a.
B4	-	++	++	++	12.6 $\pm 1.7^{ab}$	n.a.	+	n.a.
B5	-	+++	+	+	11.75 $\pm 2.2^{ab}$	n.a.	++++	n.a.

Numerical values are mean ± SD. Treatments with identical letters are not significant at $P \leq 0.05$. HCN= hydrogen cyanide; P= phosphorous; IAA= indole acetic acid; GA3= gibberellic acid; ABA= abscisic acid ;n.a=not attained;+ trait is present; + low; ++ moderate ;+++high,++++very high **B1:** *Bacillus polymxa***, B2***: Serratia* SK3, **B3:** *Bacillus* SK1,**B4***: Bacillus* SK2, **B5:** *Serratia* SK1a,+ve=positive,-ve=negative.

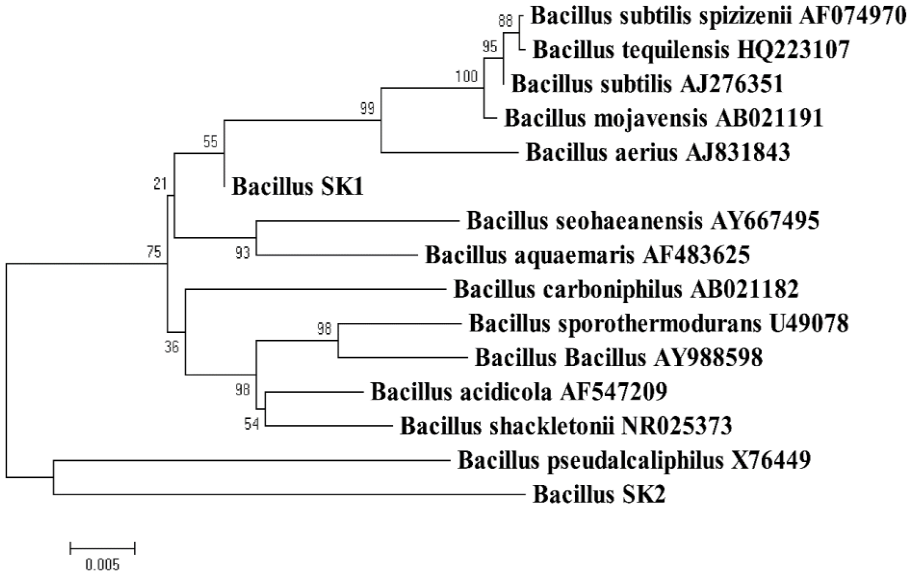

Figure 1: The phylogenetic tree that indicates the position of the *Bacillus* isolates among other *Bacillus* type strains.

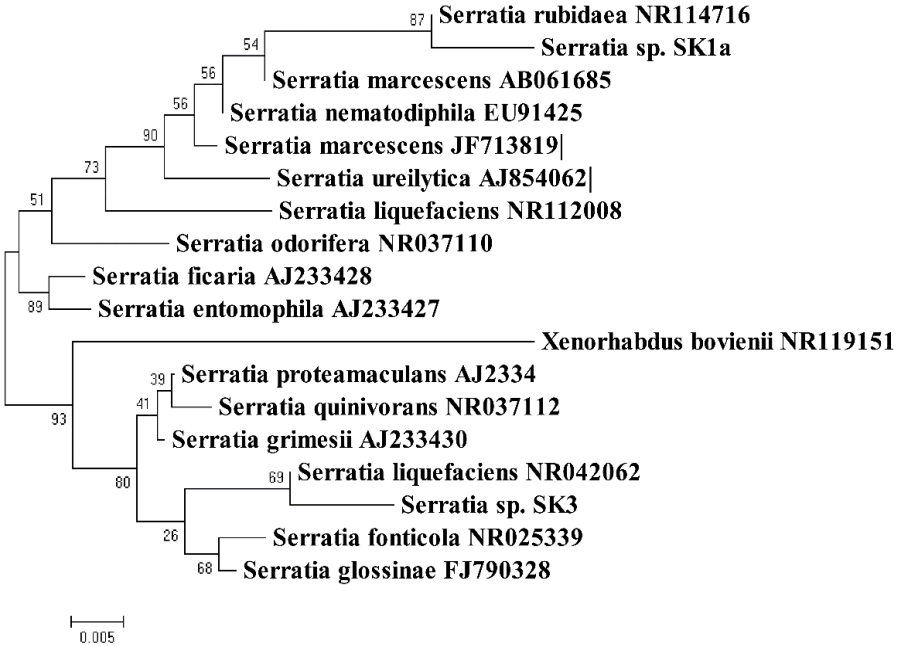

Figure 2: The phylogenetic tree that indicates the position of the *Serratia* isolates among other *Serratia* type strains.