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Research Article

New reports on dixenic associations between the symbionts of entomopathogenic nematodes, *Photorhabdus* and *Xenorhabdus*, and non-symbiotic bacteria

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Abstract: By conducting three different methods, we report on the isolation of five novel strains of non-symbiotic bacteria from crushed infective juveniles (IJs) of four species of entomopathogenic nematodes (EPN) including *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *Steinernema feltiae*, and *Steinernema glaseri* and five bacterial species from hemolymph of insect larvae infected with EPNs. Samples of hemolymph of infected *Galleria mellonella* L. larvae by EPNs and crushed surface sterilized IJs were bulk streaked onto both MacConkey and NBTA agar. To further ensure diagnoses, extracted DNA from IJs bulk was subjected to PCR by 16S-rRNA bacterial universal primers. Bacteria were identified using biochemical and phylogenetic analysis. Based on 16S-rRNA gene sequence, maximum parsimony, maximum likelihood and neighbour joining phylogenetic analyses were conducted, as well as comparisons of predicted RNA secondary structures. Four species of bacteria were identified including: *Stenotrophomonas maltophilia* strain IR11 from *S. feltiae*; *S. pavanii* strain IR20 from *S. glaseri*; *Acinetobacter junii* strain IR8 from *S. carpocapsae*; and *Alcaligenes faecalis* strains IR1 & IR15 from *S. feltiae* and *H. bacteriophora* respectively as non-symbiotic bacteria from IJs and five species probably originated from *G. mellonella* intestine including *Citrobacter gillenii* isolate S3, *Enterobacter asburiae* isolate S4, *Klebsiella oxytoca* isolate S5, *Morganella morganii* isolate S6 and *Serratia marcescens* isolate S6.

Keywords: *Stenotrophomonas maltophilia, S. pavanii, Acinetobacter junii, Alcaligenes faecalis, Citrobacter gillenii, Enterobacter asburiae Klebsiella oxytoca, Morganella morganii, Serratia marcescens*

Introduction

Entomopathogenic nematodes (EPN) from the Heterorhabditidae (Poinar, 1976) and Steinernematidae (Travassos, 1927) families are obligate insect parasites, which can infect and kill a broad range of insect hosts (Kaya and Gaugler, 1993). These nematode families are symbiotically associated respectively with the entomopathogenic bacteria *Photorhabdus* sp. (Boemare *et al.*, 1993) and *Xenorhabdus* sp. (Thomas and Poinar, 1979). The bacterial symbionts are carried monoxenically in a special receptacle in the infective stage (IJ) of members of the Steinernematidae and throughout the whole intestine of IJs of the Heterorhabditidae. The bacterial symbionts help to establish and

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maintain suitable conditions for nematode reproduction (Boemare*et al.*, 1993) by producing secondary metabolites that overcome the host insect's immune system (Akhurst and Dunphy, 1993), kill the insect, and inhibit the growth of various fungal and bacterial competitors (Akhurst, 1982; Chen *et al.*, 1996).

However, in addition to these symbiotic bacteria, some non-symbiotic bacterial species have been identified, which are able to co-inhabit or colonize the insect cadaver. Several such bacterial species have been identified from both the hemolymph of insect cadavers infected with EPNs and from IJs. Surface sterilised IJs of *Steinernema carpocapsae* were found to contain non-symbiotic bacteria from different genera including *Alcaligenes* sp., *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Serratia liquefaciens* and *Acinetobacter* sp. (Gouge and Snyder, 2006; Lysenko and Weiser, 1974). Similarly, *Ochrobactrum anthropi*, *Paracoccus denitrificans* and *Pseudomonas maltophilia* have been found to be associated with *Steinernema scapterisci* (Aguillera, 1993; Aguillera and Smart, 1993), *Heterorhabditis indica* and *Heterorhabditis bacteriophora* (Babic *et al.*, 2000). More recently, bacteria including *Flavobacterium* sp., *Providencia vermicola*, *P. rettgeri*, *Citrobacter freundii*, *Staphylococcus succinus* and *Alcaligenes faecalis* were isolated from *S. abbasi*, *S. feltiae* and *Rhabditis blumi* (Eivazian Kary and Alizadeh, 2016; Park *et al.*, 2011; Sharifi and Eivazian Kary, 2016; Somvanshi *et al.*, 2006). Several non-symbiotic bacteria have been reported recently from EPN infected cadavers, for example, *Flavobacterium* sp. was isolated from sawfly larvae infected with *Steinernema kraussei*, *Ochrobacterum cytisi* and *Schineria larvae* were isolated from *Steinernema siamkayai* and *Ochrobacterum anthropic* isolated from *H. indica* (Razia *et al.*, 2011).

This study was conducted to identify IJs non-symbiotic bacteria by three methods to accurately differentiate IJs originated non-symbionts separated from galleria. Four species of EPNs that have been recently isolated from north-west Iran were used in the study (Agazadeh *et al.*, 2010; Eivazian Kary *et al.*, 2012). Following biochemical tests we conducted molecular phylogenetic analyses of the isolated bacteria by: 1) comparing their 16S-rDNA sequences; and 2) inferring the RNA secondary structures of aligned sequences of their 16S-rDNA, and comparing them with those of related species.

Materials and Methods

Isolation and identification of EPN species

During a survey of EPNs throughout the north-west Iran in 2014, several isolates of EPNs were recovered from soil samples and identified based upon their morphological and morphometric characters, cross breeding tests, and molecular data. EPN were recovered from soil samples using an insect baiting method, described by Bedding and Akhurst (1975). In brief, ten final instar *G. mellonella* larvae were placed in a 300 ml jar containing moistened soil obtained from one of the samples and stored at room temperature (25 ± 2 °C) for 2 weeks. After five days the–traps were checked every two days for 2 weeks. Dead insect larvae from each container were placed in White traps (White, 1929) to collect emerging IJs. To verify the pathogenicity of collected nematodes and to establish new cultures, emerging nematodes from each sample were pooled and used to infect fresh *G. mellonella* larvae.

Morphological identification was made using well established taxonomic criteria (Hominick *et al.*, 1997; Stock and Kaya, 1996). For molecular studies DNA extraction and PCR were made following the methods of Eivazian Kary et al. (2009). In brief, DNA was extracted from a single IJ by crushing it in 10μl 1 × PCR Buffer and then incubating it in a sterilized 0.2 ml tube containing 10μl of the same buffer at −70 ºC for 15 min and thawing at 60 ºC. The process was followed by inoculation with 2μl of 60μgml−1 proteinase K at 65 ºC for 2 h, and then at 95 ºC for 15 min. The supernatant containing nematode DNA was collected and subjected to PCR. The internal transcribed spacer was amplified by PCR in a 50μl reaction containing: 15μl of worm lysis mix, 5μl of 10 × PCR Buffer, 2μl of dNTP mix, 1.2

unit of *Taq* DNA polymerase, 1μl of each primer and double distilled water to final volume. TW81 and AB28 were used as forward and reverse primers (Joyce *et al.*, 1994) respectively. Phylogenetic relationships of studied isolates within the genus were obtained by neighbor joining, equally weighted maximum parsimony (MP) and maximum likelihood (ML) methods using MEGA 6.

Isolation of non-symbiotic bacteria from EPNs infected insect hemolymph

Nematodes were initially surface sterilized; IJs were suspended in 2 ml of sterile water in a 3 ml Eppendorf tube. The solution was then spun at 13,000 rpm for 10 s at room temperature (ca. 24 °C) to obtain a concentrated nematode pellet. The supernatant was discarded and 2 ml of freshly prepared 1% bleach solution was added to the nematode pellet. The IJs were resuspended and mixed well with the bleach then centrifuged again and the nematode pellet was washed in 1 ml of sterile distilled water to remove the bleach residue. This washing step was repeated five times (Yadav *et al.*, 2015). Surface sterilized IJs were used to infect final instar *G. mellonella* larvae which had been already immersed in 70% alcohol to remove putative bacterial contaminations. In a sealed sterile Petri dish 10 larvae were exposed to 500 IJs for 24 h at room temperature (26 ± 3 °C) then transferred to a new sterile Petri dish for another 24 h. Hemolymph from infected cadavers with typical signs of EPNs infection were chosen for bacterial isolation. Hemolymph was extracted by dissecting larvae ventrally between their 5^{th} and 6^{th} abdominal segments and was collected with a sterile loop and streaked on both MacConkey and NBTA agar. In addition, hemolymph of uninfected and surface-sterilized final instar *G. mellonella* larvae was tested to act as a control. The plates were incubated at 30 °C for 48 h. Bergeys manual was followed for biochemical characterization of the isolates (Krieg and Holt, 1984).

Isolation of non-symbiotic bacteria from crushed IJs

Mentioned method in former section was used to prepare surface sterilized IJs in 1.5 ml eppendorf tube. The surface-sterilized nematodes were suspended in nutrient broth and crushed manually by sterile tissue grinder then a sterile loop was used to streak suspension on both MacConkey and NBTA agar. In addition, IJs-free nutrient agar was tested to act as a control.

Monitoring non-symbiotic bacteria by PCR amplification of 16S-rRNA gene and sequencing

The proposed method by Eivazian Kary et al. (2009) for IJs DNA extraction was used in part for IJs complete lysis to ensure the efficiency of bacterial DNA extraction processor. The nematode bulk was crushed in 20µl 1 × PCR Buffer and transferred to a precooled sterilised 1.5ml eppendorff tube containing 20µl of the same buffer. The tube was incubated at −70 ◦C for 20 min and thawed at 60 ºC then inoculated at 65 ºC for 2 h and heated at 95 ºC for 15 min. After vortexing, the suspension was subjected to genomic DNA purification using the DNeasy tissue kit (Qiagen) as per the manufacturer's protocol. DNA was eluted into 20 µl of TE buffer and stored at -20 °C. 16S-rRNA gene amplification was carried out by a standard PCR reaction mixture that included 10X Taq buffer, 1.25 mM of $MgCl_2$, 0.25 mM dNTPs, 1 mM of each primer and 1 µl of Taq polymerase using forward primer 5′-GAAGAGTTTGATC ATGGCTC and reverse primer 5′-AAGGAGGT GATCCAGCCGCA-3′. All amplifications were performed with an initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were purified using QIAquick PCR purification kit (Qiagen) in order to remove the salts, primers and unincorporated dNTPs and then subjected to direct sequencing. DNA sequences were analyzed and assembled using the SeqMan program of the DNAstar Lasergene software. Sequences data generated for 16S-rDNA (accession numbers KT261415 to KT261419 and KR024664 to KR024668) have been deposited in GenBank.

Phylogenetic analysis: Maximum Parsimony, Maximum Likelihood and Neighbor Joining trees

DNA sequences were edited with Chromas 2.01 and aligned using Clustal X 1.64 (Thompson *et al.*, 1997) with the homologous sequences of other species of the genus obtained from NCBI databases linked in LPSN (List of Prokaryotic names with Standing in Nomenclature) (http://www.bacterio.net). The evolutionary history was inferred using the Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods. MEGA6 with the following settings was used for evolutionary analysis. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Figures 1, 3 and 5). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences with ten replicates. For ML analysis, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. In the case of NJ, the evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

Secondary structure and minimum free energy calculations

The GeneBee-Net program (Brodsky *et al.*, 1995; Brodsky *et al.*, 1992) using greedy algorithm was used for RNA secondary structure prediction by minimum free energy calculations of aligned sequences. The parameters were set as: energy threshold = -4.0; cluster factor = 2; conserved factor = 2; compensated factor = 4 and conservativity = 0.8.

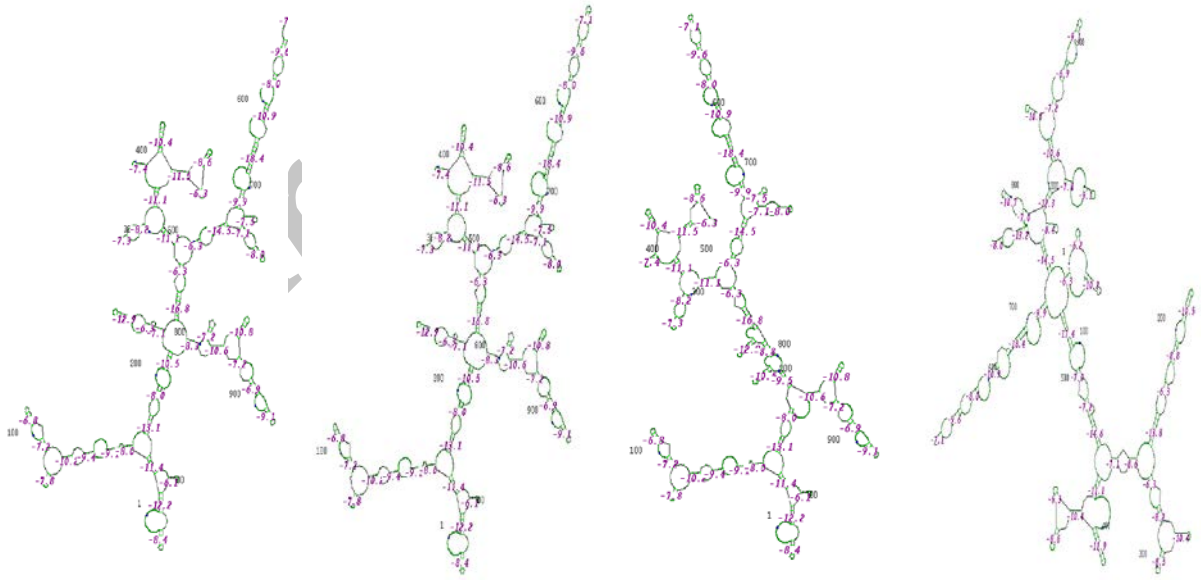

Figure 1 Hypothesis of 16s-rRNA gene based phylogenetic relationships of isolates *A. faecalis* IR1 & IR15 to members of the genus *Alcaligenes*. The tree was constructed by the maximum likelihood method. Bootstrap values are shown at the nodes.

Results

Isolated bacteria from EPNs infected galleria hemolymph

Ten strains of non-symbiotic bacteria including nine species were isolated from the infected cadavers of *G. mellonella*. Utilising biochemical tests (Table 1), these strains were tentatively identified as *Stenotrophomonas maltophilia* strain IR11, *Alcaligenes faecalis* strain IR1 and *Citrobacter gillenii* strain S3 (non-symbiont bacteria isolated from *S. feltiae* infected hemolymph of galleria); *Stenotrophomonas pavanii* strain IR20, *Enterobacter asburiae* strain S4 and *Klebsiella oxytoca* strain S5 (non-symbiont bacteria isolated from *S. glaseri* infected hemolymph of galleria); *Acinetobacter junii* strain IR8, *Morganella morganii* strain S8 and *Serratia* marcescens strain S6 (non-symbiont bacteria isolated from *S. carpocapsae* infected hemolymph of galleria) and *Alcaligenes faecalis* strain IR15 S6 (non-symbiont bacteria isolated from *H. bacteriophora* infected hemolymph of galleria).

Isolated bacteria from crushed IJs

Five strains of non-symbiotic bacteria including four species were isolated from the crushed IJs. Utilising biochemical tests (Table 1), these strains were tentatively identified as *Stenotrophomonas maltophilia* strain IR11 (non-symbiont of *S. feltiae*), *Stenotrophomonas pavanii* strain IR20 (non-symbiont of *S. glaseri*), *Acinetobacter junii* strain IR8 (non-symbiont of *S. carpocapsae*), *Alcaligenes faecalis* strain IR1 (non-symbiont of *S. feltiae*) and *Alcaligenes faecalis* strain IR15 (non-symbiont of *H. bacteriophora*). No bacterial species were identified from the control tests (Nutrient broth).

Monitored bacteria using PCR amplification of 16s-rRNA gene

Three species of non-symbiotic bacteria were isolated from the crushed IJs. Phylogenetically these strains were identified as *Stenotrophomonas maltophilia* strain IR11, *Stenotrophomonas pavanii* strain IR20 and *Acinetobacter junii* strain IR8. No bacterial species were identified from the control tests

Phylogenetic analysis

All isolates, regardless of origins, were subjected to phylogenetic analysis based on 16s-rRNA sequence for species identification but here we present those results for isolates with crushed IJs origin.

Genus *Alcaligenes*

MP, ML and NJ analyses of the 16S-rDNA sequence for *Alcaligenes* spp yielded fully dichotomous trees with no conflict among them. Bootstrap ML analysis of the 16S-rDNA dataset revealed high support for all 4 clades (Clade I: *A. faecalis* IR1, *A. faecalis* IR15, *A. faecalis* type strain, *A. aquatilis* type strain and *A. defragrans*; Clade II: *A. piechaudii*, *A. xylosoxidans*, *A. ruhlandii* and *A. denitrificans*; Clade III: *A. latus* and *A. paradoxus* and Clade VI: *A. venusta* and *A. cupidus*). The two symbionts isolated from *S. feltiae* and *H. bacteriophera*, *A. faecalis* IR1 and *A. faecalis* IR15, grouped together in Clade I as closest relatives to the *A. faecalis* type strain. *Alcaligenes aquatilis* appeared as a sister group to this monophyletic group (Figure 1).

Minimum free-energy analysis of rRNA secondary structure showed that, in the monophyletic group in Clade I, 16S-rDNA sequences of strains IR1 and IR15 and type strain possessed similar secondary structures in comparison to *A. aquatilis*. The free energy of the structure in IR1, IR15 and type strains was -219.3 kcal/mol, -221.2 kcal/mol and -217.8 kcal/mol respectively, in comparison to -205.3 kcal/mol in *A. aquatilis* type strain. This indicates that IR1 and IR15 strains are more closely related to *A. faecalis* type strain compared to their sister group, *A. aquatilis* (Figure 2).

Table 1 Biochemical characteristics of isolated bacteria from entomopathogenic nematodes infected hemolymph of galleria larva and crushed nematodes.

Characteristic	*Ea*	*Ko*	*Cg*	*Mm*	*Sm*	*Af*	*Aj*	*Sm*	*Sp*
Gram	-	-	-	-	-	-	-	-	-
Metabolism	An	An	An	An	An	Ae	Ae	Ae	Ae
Pigments	-	-	-	-	+	-	+	+	+
Motility	-	-	+	+	+	+	-	+	-
Catalase	+	+	*	+	+	+	+	+	+
Lipase	-	-	-	-	+	+	+	*	-
Oxidase	-	-	-	-	-	+	-	-	-
Lactose	+	+	+	*	-	-	+	+	-
Maltose	+	+	+	-	+	*	-	+	-
L-rhamnose	-	*	+	-	*	*	+	*	-
Sucrose	+	+	-	-	+	-	+	+	-
Adonitol	-	+	-	-	-	*	*	-	+
Arabinose	+	+	+	-	-	*	*	-	-
Glucose	*	*	*	*	*	-	+	+	-
Manitol	+	*	+	-	+	-	+	+	-
D-glucosamine	*	*	*	*	*	*	+	*	+
N-Acetylglucosamine	*	*	+	*	*	-	-	*	-
Voges-Proskauer	-	+	-	-	+	-	-	-	*
Methyl red	+	-	+	+	-	-	-	-	*
Gelatin	-	-	-	-	+	-	-	+	+
Starch	*	*	*	*	*	*	*	-	-
Nitrate-red	+	+	+	-	+	-	-	+	+
Phenylalanine Deaminase	-	-	-	+	-	*	*	-	-
Acetate	+	+	*	-	+	+	+	*	*
Citrate	+	+	+	-	+	+	+	+	+
Succinate	*	*	*	*	*	+	+	*	*
D-galactose	+	*	*	+	*	-	+	+	-
D-glucose	+	+	+	+	+	-	+	*	-
D-Mannose	+	+	+	+	+	*	-	+	-
Salicin	+	+	-	-	+	*	*	-	-
Sorbitol	+	*	+	-	*	*	*	-	-
Raffinose	+	+	-	-	-	*	+	*	-
Beta-glucuronidase	+	*	*	*	*	*	-	+	+
Phosphatase	*	*	*	*	*	*	+	*	*
Urease	+	+	-	+	-	-	-	+	-
Indol	-	+	-	+	-	-	-	-	-
D-Fructose	*	*	+	*	*	-	+	+	-
Glycerin	*	*	*	*	*	-	*	*	*

Ea: *Enterobacter asburiae* S4; Ko: *Klebsiella oxytoca* S5; Cg: *Citrobacter gillenii* S3; Mm: *Morganella morganii* S8; Sm: *Serratia marcescens* S6; Af: *Alcaligenes faecalis* IR1 & IR15; Aj: *Acinetobacter junii* IR8; Sm: *Stenotrophomonas maltophilia*; Sp: *S. pavanii;* An: Anaerobic; Ae: Aerobic.
+: positive; -: Negative; *: No data.

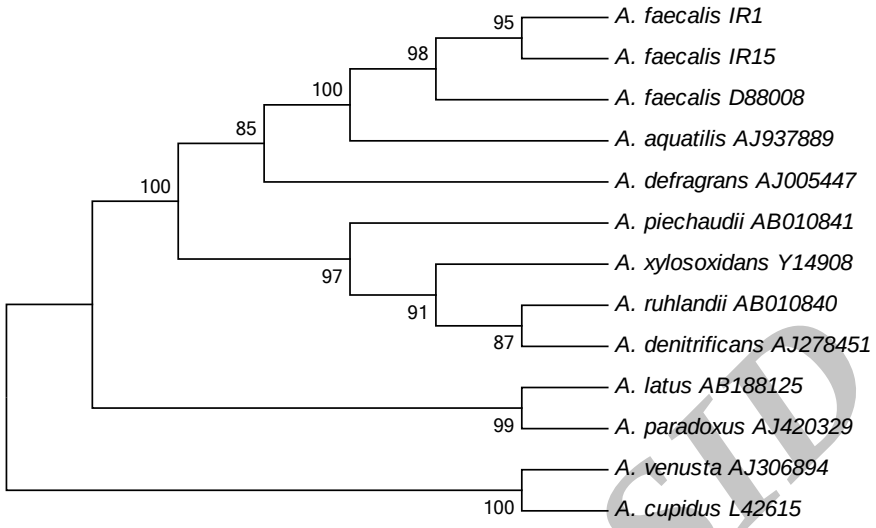

Figure 2 Graphical depiction of the predicted minimum free energy secondary structure for the 16S-rDNA sequences of *Alcaligenes faecalis* strains (A) IR1; (B) IR15 and (C) type strain, and of (D) *A. aquatilis*.

Genus Acinetobacter

In the MP, ML and NJ trees *A. junii* IR8 and *A. junii* type strain were grouped together with a high bootstrap value (> 90%) to form a highly supported monophyletic group (Figure 3). However, the three methods indicated different relationships with sister groups. In the ML and NJ trees *A. baumannii* appears to be the most related species to the *A. junii* clade, but in the MP tree *A. gerneri* replaced it. In all trees, terminal nodes received high bootstrap support. Although these groups were common between trees, all trees differed in depicting relationships within and between clades with low bootstrap values and did not have enough resolution to show genealogic relationships. Polytomies were observed in deeper nodes.

The results of the 16S rRNA secondary structure analysis of the monophyletic group including *A. junii* IR8, *A. junii* type strain and *A. baumannii*, as a sister group, showed that the 16S–rDNA sequences of strain IR8 and the type strain generated similar structures compared to *A. baumannii*, despite having minor differences in loop structures. The free energy of the 16S–rDNA structures in the IR8 and type strain were -307.3 kcal/mol, -298.9 kcal/mol respectively. In comparison the free energy for the *A. baumannii* type strain was -286.7 kcal/mol, indicating a greater relatedness between strain IR8 and *A. junii* type strain (Figure 4).

Genus Stenotrophomonas

MP analysis of the 16S-rDNA dataset yielded 2 fully dichotomous trees with minimal conflict, as revealed by the consensus tree. Bootstrap MP analysis revealed moderate to high support for most clades recovered in the strict consensus of most parsimonious trees (Figure 5A). The ML tree (Figure 5B) was identical in topology to the MP tree but the NJ tree (Figure 5C) differed from them, depicting relationships within 1 clade differently (*S. chelatiphage*, (*S. africae*, ((*S. maltophila* AB4553, *S. maltophila* IR11), (*S. pavanii* FJ748683, *S. pavanii* IR20)). In all constructed trees isolate IR11 grouped with *S. maltophila* type strain and isolate IR20 formed another monophyletic group with *S. pavanii*.

The minimum free energy of structure for the *S. pavanii* type strain and *S. pavanii* IR20 was -312.3 kcal/mol and -310.2 kcal/mol respectively and for the *S. maltophilia* type strain and *S. maltophilia* IR20 was -303.5 kcal/mol and -300.9 kcal/mol respectively (Figure 6).

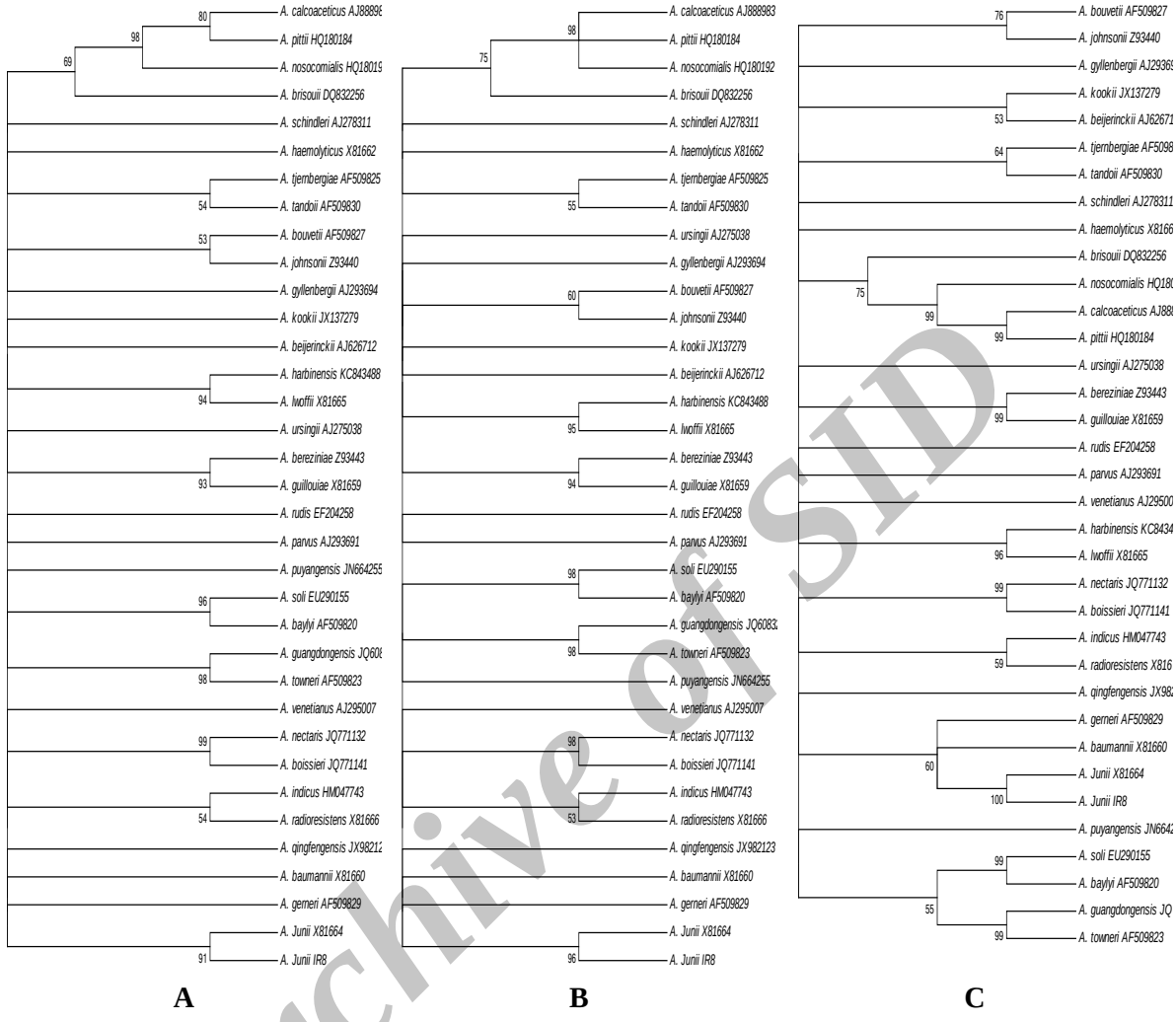

Figure 3 Hypothesis of 16s-rRNA gene based phylogenetic relationships of *A. junii* IR8 to members of the genus *Acinetobacter* constructed by (A) maximum likelihood (B) maximum parsimony and (C) neighbor-joining methods. Bootstrap values are shown at the nodes.

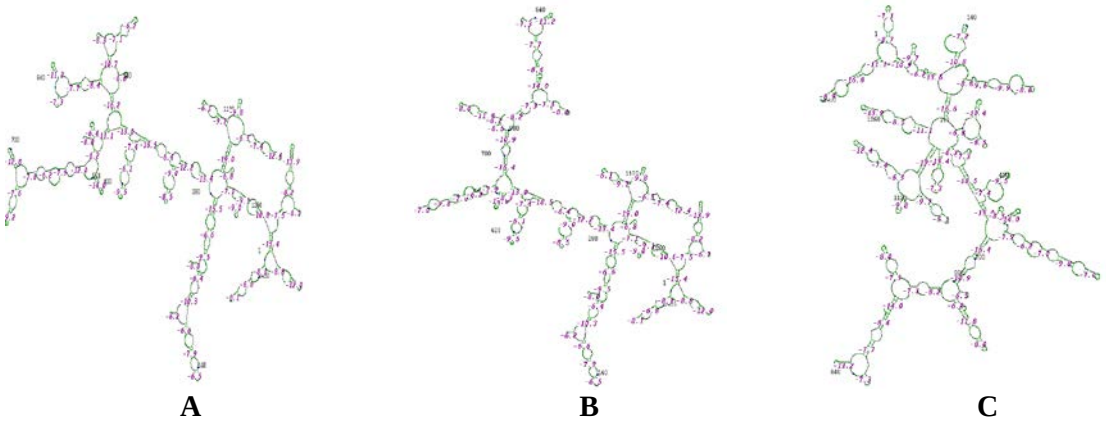

Figure 4 Graphical depiction of the predicted minimum free energy secondary structure for the 16S-rRNA gene sequences of (A*) Acinetobacter junii* strain IR8; (B) *A. junii* type strain and (C) *A. baumanii* type strain.

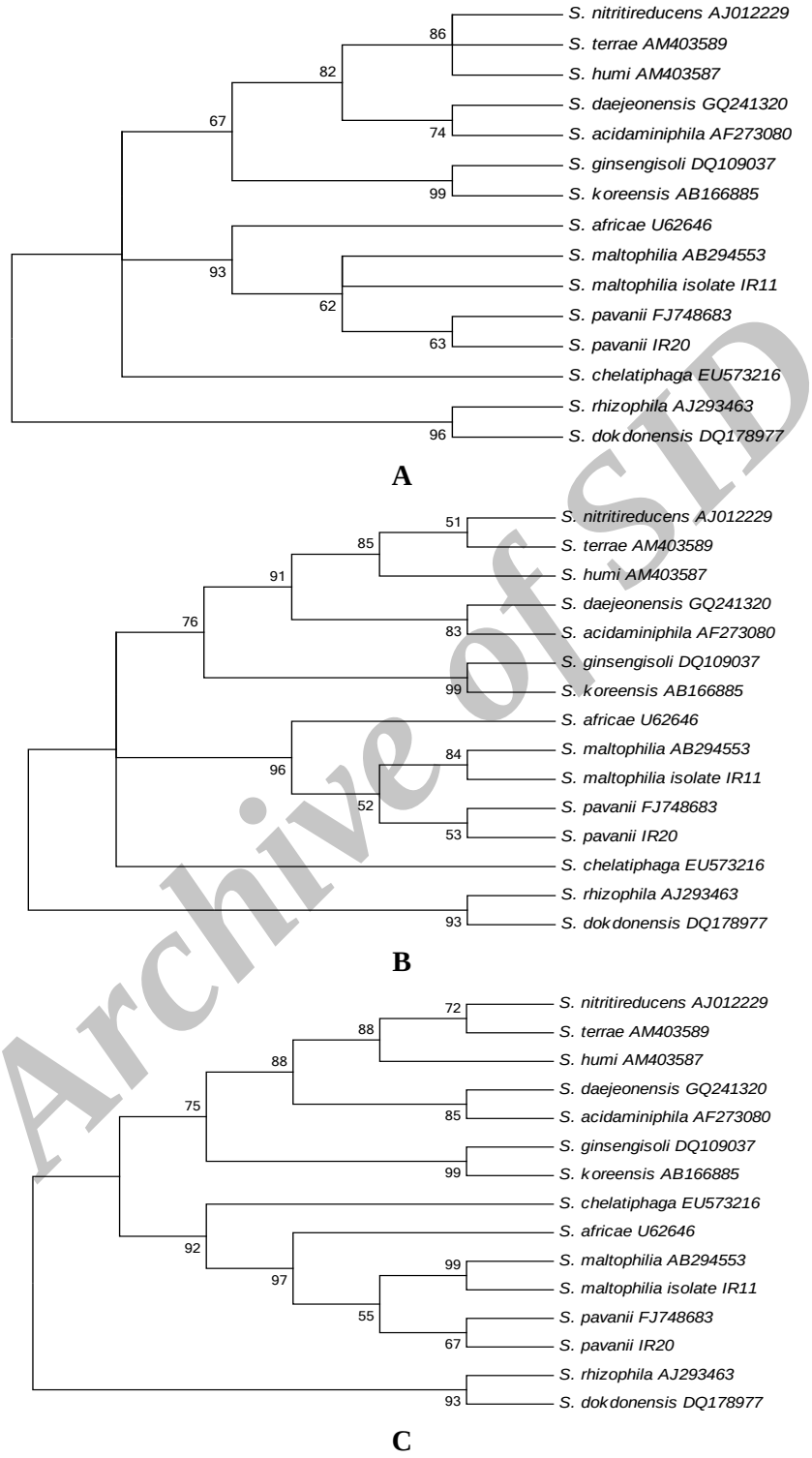

Figure 5 Hypothesis of 16s-rRNA gene based phylogenetic relationships of *S. maltophila* IR11 *and S. pavanii* IR20 to members of the genus *Stenotrophomonas* constructed by (A) maximum parsimony (B) maximum likelihood and (C) neighbor-joining methods. Bootstrap values are shown at the nodes.

Figure 6 Graphical depiction of the predicted minimum free energy secondary structure for the 16S-rRNA gene sequences of (A*) Stenotrophomonas maltophila* IR11, (B) *S. maltophila* type strain, (C) *S. pavanii* IR20 and (D) *S. pavanii* type strain.

Discussion

We isolated five novel non-symbiotic bacteria from the crushed IJs of four species of EPNs indigenous to Iran. Previous studies have demonstrated that the symbiotic bacteria carried by EPN produce broad-spectrum antibiotics that inhibit the co-existence of other bacterial species (Maxwell *et al.*, 1994). However, our study provides evidence for the existence of non-symbiotic bacteria occurring alongside the EPNs symbiotic bacteria in four different nematode species. Earlier gnotobiological experiments have also shown naturally occurring combinations of symbiotic with non-symbiotic bacteria, for example Babic *et al* (2000) reported the occurrence of dixenic associations between the bacterial symbiont *P. luminescens* and bacteria related to *Ochrobactrum* sp. in 33% of tropical entomopathogenic *Heterorhabditis* species, however, it is possible that the identified bacterial strains were members of the insect gut microbiome that were translocated into the hemolymph due to EPN infection, which has been supported by a previously published study (Singh *et al.*, 2014). Alongside of isolated bacteria from crushed IJs, here we distinguished five different species from the hemolymph of EPNs infected larva including *Citrobacter gillenii* isolate S3, *Enterobacter asburiae* isolate S4, *Klebsiella oxytoca* isolate S5, *Morganella morganii* isolate S6 and *Serratia marcescens* isolate S6. It is possible that these bacteria are the members of galleria gut (or may be its respiratory system) that are translocated into the coelom by nematodes. Comparing the isolated bacteria from EPNs infected hemolymph with crushed IJs and subsequent PCR based monitoring method showed that IJs can efficiently cause penetration of bacteria from insect gut to haemocoel but interestingly in our study each EPN showed specificity for this transmission. Two reasons could be postulated for this non-symbiont-nematode specificity, it is possible that the presence of bacteria in insect haemocoel is not a simple penetration through the holes on the intestine wall caused by IJs. Nematode cuticle surface is covered by glycoprotein-rich negatively charged surface coat that has been associated with immune evasion in several parasitic nematode species (Blaxter *et al.*, 1992; Page *et al.*, 1992), this layer may function to aid locomotion and prevent bacteria adhesion to nematode (Cipollo *et al.*, 2004; Hoflich *et al.*, 2004; Politz and Philipp, 1992). It is possible that by having some kinds of evolutionary adaptation, some bacteria from intestinal flora (or may be respiratory tract) are

capable of involving in phoretic relationship with specific species of EPNs. On the other hand, this non-symbiont-EPN association could be attributed to the lower adverse effects of symbiosis on some non-symbiont bacteria compared to other members of insect flora that gives them opportunity for growth. In the case of *H. bacteriophora*, *A. faecalis* was the only non-symbiont that was isolated from both IJs infected hemolymph of galleria and crushed nematodes as well. Comparing to *Steinernema-Xenorhabdus*, *Heterorhabditis-Photorhabdus* complex is more specific and produces a wider variety of metabolites including lipases, proteases, antibiotics, lipopolysaccharides, and a number of other secondary metabolites that are active against a wide range of microbial pathogens of animals and plants, including bacteria and fungi (Forst and Clarke, 2002) then limited number of opportunistic bacteria can interface with this complex. Regardless of bacterial association with IJs or insect, the results clearly imply that these isolates are capable to resist *Xenorhabdus* and *Photorhabdus*-derived antibiotics. In our studies we isolated non-symbiotic bacteria from infected larvae 48 h post-infection. Isaacson and Webster (2002) reported that antimicrobial activity of *X. cabanillasii* increases from 72 h post-exposure and reaches its highest level at 144 h post-exposure. Therefore, it remains a possibility that that the Gram-negative bacteria isolated in this study may have been detectable at 48 h post-infection because antimicrobial substances produced by the symbionts may not have reached high enough levels to eradicate these non-symbiont species. However, it has been shown that a number of Gram-negative non-symbiotic bacteria do continue to increase during such increases in antimicrobial activity of symbiotic bacteria (Isaacson and Webster, 2002).

We isolated two strains of *A. faecalis* from Iranian EPNs, IR1 from *S. feltiae* and IR15 from *H. bacteriophera*. *Alcaligenes faecalis* is an obligate aerobe that is commonly found in the environment. A recent study has shown that *A. faecalis* strain MOR02, isolated in Mexico, is lethal to *G. mellonella* within 24 h of infection when directly injected into the haemocoel of larvae. *A. faecalis* MOR02 was shown to inhabit the digestive tract of *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora*, whereas control bacteria (*Escherichia coli*) were observed to be transmitted externally on the cuticles of the nematodes, which demonstrated the specificity of the *A. faecalis*-nematode interaction (Quiroz-Castaneda*et al.*, 2015). That we have independently isolated alternative strains of *A. faecalis* from *S. feltiae* and *H. bacteriophora* in a different continent suggests the possibility that interactions between these bacteria and nematode species may be more closely linked than previously considered.

We are not aware of a previous association of *S. maltophila* with any EPN species, however it has been shown to have strong nematotoxic activity against a free-living nematode and a plant parasitic nematode (Huang *et al.*, 2009). Similarly, we are not aware of any previous reports of associations between EPNs infected cadavers and *S. pavanii*, however this species has been previously isolated from rhizosphere and endosphere of cucumber in Iran (Rafiei and Khodakaramian, 2015) and therefore may occur as a soil dwelling organism in this region. The presence of *S. pavanii* as the most related species to *S. maltophila* in our study provides support for the theory that this monophyletic group has ability to colonize EPNs infected cadavers as a synapomorphic character of the group. To our knowledge this is also the first study to indicate the occurrence of *Acinetobacter junii* in any EPN species infected cadaver, although strains of *A. junii* have previously been isolated from a number of other locations including from the mid-gut of healthy *Culex quinquefasciatus* mosquitos (Pidiyar *et al.*, 2004).

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گزارش جدید از ارتباط باکتری‌های همزیست نماتدهای بیمارگر حشرات، *Photorhabdus* و *Xenorhabdus*، با باکتری‌های غیر همزیست

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چکیده: با استفاده از سه روش مختلف، پنج جدایه جدید از باکتری‌های غیرهمزیست از لاروهای بیمارگر له شده چهار گونه از نماتدهای بیمارگر حشرات شامل *Heterorhabditis bacteriophora*، *Steinernema carpocapsae*، *S. feltiae* و *S. glaseri* شناسایی شد. پنج گونه باکتری نیز از همولنف لارو سن آخر پروانه موم‌خوار بزرگ، *Galleria mellonella*، آلوده به نماتد بیمارگر حشرات جداسازی و شناسایی شدند. نمونه‌هایی از همولنف آلوده به نماتد لاروهای پروانه موم‌خوار بزرگ و توده له شده از لاروهای آلوده‌کننده نماتدهای بیمارگر حشرات که قبلاً استریل سطحی شده بودند در محیط کشت‌های MacConkey و NBTA agar کشت شدند. برای اطمینان بیش‌تر، DNA استخراج شده از توده لاروهای بیمارگر با استفاده از پرایمرهای عمومی ناحیه 16S-rRNA باکتری‌ها تحت واکنش زنجیره‌ای پلیمرازی قرار گرفت. بعد از ترادف‌یابی، تجزیه‌وتحلیل نسب شناختی با استفاده از سه روش Maximum parsimony، Maximum likelihood و Neighbour joining انجام و پیش‌بینی ساختار ثانویه RNA جدایه‌های مورد بررسی جهت تفکیک بهتر گونه‌ها در گروه‌های مونوفایلتیک انجام شد. در نهایت چهار گونه باکتری غیرهمزیست شامل: *Stenotrophomonas maltophilia* strain IR11 از لاروهای آلوده‌کننده نماتد بیمارگر *S. feltiae*، *S. pavanii* strain IR20 از *S. glaseri*، *Acinetobacter junii* strain IR8 از *S. carpocapsae* و *Alcaligenes faecalis* strains IR1 & IR15 به‌ترتیب از *S. feltiae* و *H. bacteriophora* جداسازی و شناسایی شدند. پنج گونه باکتری که احتمالاً از دستگاه گوارش لاروهای پروانه موم‌خوار بزرگ منشأ گرفته بودند نیز به‌عنوان گونه‌های *Citrobacter gillenii* isolate S3، *Enterobacter asburiae* isolate S4، *Klebsiella oxytoca* isolate S5، *Morganella morganii* isolate S6 و *Serratia marcescens* شناسایی شدند.

واژگان کلیدی: *Acinetobacter junii*، *Alcaligenes faecalis*، *Citrobacter gillenii*، *Enterobacter asburiae*، *Klebsiella oxytoca*، *Morganella morganii*، *Serratia marcescens*، *Stenotrophomonas maltophilia*، *S. pavanii*