JOURNAL OF ENTOMOLOGICAL SOCIETY OF IRAN
2016, 36(2): 111-119

Non-symbiotic association of *Citrobacter freundii* and *Staphylococcus succinus* with the entomopathogenic nematode *Steinernema feltiae*

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Abstract

Soil-dwelling nematodes from Steinernematidae family are obligate parasites of insects and usually referred to as entomopathogenic nematodes (EPNs). These nematodes are symbiotically associated with entomopathogenic bacteria *Xenorhabdus* spp. The bacterial symbionts are carried monoxenically in a special vesicle in the infective juveniles (IJs). In the present study we report the isolation of two species of non-symbiotic bacteria from infected insect cadavers by the EPN, *Steinernema feltiae*. *Galleria mellonella* L. larvae were exposed to surface sterilized infective juveniles of *S. feltiae* and transferred to sterile Petri dishes for a further 24 hours. Hemolymph was collected and streaked onto both MacConkey and NBTA agar. Bacteria were identified using biochemical and phylogenetic analysis. 16S-rRNA gene sequence based maximum parsimony, maximum likelihood and neighbour joining phylogenetic analyses were conducted. Two non-symbiotic species including *Citrobacter freundii* and *Staphylococcus succinus* were identified and reported to be associated with *S. feltiae*. Our results provide further evidence for the existence of non-symbiotic bacteria associated with EPNs infection.
Keywords: Entomopathogenic nematode; *Steinernema feltiae*; *Citrobacter freundii*; *Staphylococcus succinus*

چکیده

رابطه غیر همزیستی Citrobacter freundii و Staphylococcus succinus با نماتود بیمارگر حشرات Steinernema feltiae

ناصر عیوضیان‌کاری و ژیلا علیزاده

نماتودهای خاکزی متعلق به خانواده Steinernematidae (Nematoda: Rhabditida) پارازیت های اجباری و کشنده حشرات بوده که معمولا تحت عنوان نماتودهای بیمارگر حشرات EPNs (Entomopathogenic nematodes)، از آن‌ها نام برده می شود. این گروه از نماتودها به طور طبیعی با باکتری های جنس Xenorhabdus رابطه همزیستی داشته و نماتود مرحله سوم آلوده کننده یا Infective Juvenile، این باکتری ها را در یک وزیکول اختصاصی در روده‌ی خود حمل می کنند. در این مطالعه وجود دو گونه از باکتری های غیر همزیست از لاشه حشرات آلوده به نماتود بیمارگر حشرات Steinernema feltiae گزارش می گردد. شناسایی باکتری‌های مذکور بر اساس آزمون های بیوشیمیایی و تجزیه و تحلیل نسب شناختی ترادف ژن ۱۶s-rRNA انجام گرفت و در نهایت دو گونه باکتری به عنوان Citrobacter freundii و Staphylococcus succinus شناسایی شدند. مطالعه اخیر تاییدی بیشتر بر وجود ارتباط بین باکتری های غیر همزیست با نماتودهای بیمارگر حشرات و توانایی نماتودها در انتقال آن‌ها می باشد.

واژگان کلیدی: نماتودهای بیمارگر حشرات، Entomopathogenic nematode*; Steinernema feltiae*; *Citrobacter freundii*; *Staphylococcus succinus*

Introduction

Soil is the natural habitat of EPNs from the families Steinernematidae and Heterorhabditidae (Koppenhoffer *et al*., 2004). In both genera *Steinernema* and *Heterorhabditis* there is a single free-living stage, the infective juvenile (IJ), that carries in its gut bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Kaya & Gaugler, 1993). On encountering a suitable insect, the IJ enters through the mouth, anus or spiracles and makes its way to the haemocoel (Eidt & Thurston, 1995). In the haemocoel, the IJ releases cells of its bacterial symbiont. Bacteria multiply rapidly in haemolymph and produce toxins, which contribute to the weakening of the host's defense mechanism. The host attacked by EPN usually dies because of poisoning or failure of certain organs in 24 to 72 h. after the infection (Forst & Clarke, 2002). The bacterial symbionts also contribute to the symbiotic relationship by establishing and maintaining suitable conditions for nematode reproduction, providing nutrients and antimicrobial substances that inhibit the growth of a wide range of microorganisms (Boemare, Akhurst, & Mourant, 1993). Although the nematode-bacteria interaction is unique, several non-symbiotic bacteria species have been identified from the hemolymph of insect cadavers infected with EPNs even after attempting to surface sterilize infective juveniles, including: *Flavobacterium* sp. (Mracek, 1977), *Ochrobacterum cytisi*, *Schineria larvae*, *O. anthropic* (Razia *et al*., 2011), *Alcaligenes* sp., *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Serratia liquefaciens* and *Acinetobacter* sp. (Gouge & Snyder, 2006; Lysenko & Weiser, 1974), *Ochrobactrum anthropi*, *Paracoccus denitrificans* and *Pseudomonas maltophilia* (Aguillera, 1993; Aguillera & Smart,

1993). Recently, the bacteria *Flavobacterium* sp., *Providencia vermicola*, and *Alcaligenes faecalis* were isolated from the nematode *Rhabditis blumi* (Park *et al*., 2011). These reports clearly show that non-symbiotic bacterial associations with EPNs are common and relevant phenomena. Such associations could be one of the major reasons for EPNs liquid mass production failure. On the other hand such associations could be postulated as a primary steps in symbiosis evolution providing simple frame for evaluating involved mechanisms (Gaugler & Han, 2002). Accurate identification and understanding the span of this phenomenon between EPNs and bacteria are the first and important step for uncovering underlying evolutionary frameworks of parasitism and symbiosis in details and understand the molecular basis of these phenomena. Such non-symbiotic bacteria with different levels of relatedness with EPNs will enable us to complete the puzzles of parasitism and symbiosis evolution.

Materials and methods

EPN species

During a survey of EPNs throughout north-west of Iran in 2014, several isolates of EPNs were recovered from soil samples and identified based on morphology and morphometric characters, cross breeding test, as well as molecular data. In total, 200 soil samples were collected randomly from different cultivated and non-cultivated areas of north-west Iran. Each soil sample was a composite of 10-20 random sub-samples taken in the same location, but at least 10 m away from each other and to a depth of 30 cm, using a small shovel. The soil was thoroughly mixed on a plastic sheet and half of each sample was used for extraction of EPNs. EPNs were recovered from soil samples using an insect baiting method, described by (Bedding & Akhurst, 1975). Ten last instar *Galleria mellonella* larvae were placed in a 300 ml jar containing moistened soil and stored at room temperature (25±2°C) for 2 weeks. The–traps were checked every two days for possible infected great wax moth larvae. Dead larvae from each container were placed in White traps to collect emerging IJ and were replaced by healthy larvae. To verify the pathogenicity of the collected nematodes and to establish new cultures, emerging nematodes were pooled for each sample and used to infect healthy *G. mellonella* larvae. Morphological identification was made using taxonomic criteria suggested by Hominick *et al*. (1997). For molecular studies DNA extraction and PCR were made using suggested methods by Eivazian Kary *et al*. (2009). 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 bacterial non-symbionts from insect hemolymph

To surface sterilize the nematode, infective juvenile (IJs) of *S. feltiae* were suspended in 2 ml of sterile water in 3 ml Eppendorf tube. The solution was spun at 13,000 rpm for 10 s at room temperature 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 suspension was mixed well and the nematode pellet was washed in 1 ml of sterile distilled water to remove the bleach residue. The washing step was repeated five times (Yadav *et al*., 2015). Surface sterilized IJs were used to infect last instar great wax moth larvae which have been already immersed in 70% alcohol to remove putative bacterial contaminations. In each sealed Petri dishes 10 larvae were exposed to 500 IJs for 24 h. at room temperature (26±3°C) then transferred to sterile Petri dishes for another 24 h. Hemolymph from infected cadavers with typical signs of EPNs infection was chosen for bacterial isolation. Hemolymph was extracted by dissecting larvae ventrally between the 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. Bergey's manual (1984) was followed for biochemical characteristics of the isolates.

DNA extraction and PCR

Genomic DNA was purified from isolates in culture using the DNeasy tissue kit (QIAgen) as per the manufacturer's protocol. DNA was eluted from the column 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´-GAAGAGTTTGATCATGGCTC and

reverse primer 5′-AAGGAGGTGATCCAGCCGCA-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 then subjected to direct sequencing. DNA sequences were analyzed and assembled using the SeqMan program of the DNAstar Lasergene software. Sequence data generated for 16s-rDNA (accession numbers KT261414 and KT261420) 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 *Citrobacter* and *Staphylococcus* species 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, Maximum Likelihood and Neighbor-Joining 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. 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.

Results

Entomopathogenic nematodes were recovered from 12 sites; 5 sites were positive for the occurrence of heterorhabditids and seven sites for steinernematids. Based on morphological and molecular characterization one species of *Heterorhabditis* and *Steinernema* were found. All *Heterorhabditis* isolates were identified as *H. bacteriophora* and *Steinernema* species as *S. feltiae.*

Two species of non-symbiotic bacteria were isolated from the infected cadavers of *G. mellonella* with EPN, *S. feltiae*. Utilizing biochemical tests, these species were identified as *Citrobacter freundii* isolate S1 and *Staphylococcus succinus* isolate S2. To increase confidence in the biochemical identification, 16S-rDNA based phylogenetic analysis were conducted within genus with a complete set of well-defined species using MP, ML and NJ methods.

Citrobacter freundii isolate S1

C. freundii is a species of facultative anaerobic, gram-negative bacilli of the Enterobacteriaceae family with medium-sized translucent to opaque colonies (2-4 mm in diameter) with glossy surface. The bacteria are long rod-shaped with a typical length of 2–6 μm mostly surrounded by several flagella used for locomotion, but a few are not mobile (Table 1). It can be found in soil, water, sewage, food, and the intestinal tracts of animals and humans. As an opportunistic pathogen, *C. freundii* is responsible for a number of significant infections. It is known to be the cause of a number of nosocomial infections of the respiratory tract, urinary tract, blood, and many other normally sterile sites in patients. It represents about 29% of all opportunistic infections (Whalen *et al*., 2007).

MP, ML and NJ analysis of the 16S rDNA sequence of the genus *Citrobacter* yielded partial dichotomous trees without any confliction among them. All phylogenetic reconstruction methods yielded trees containing two major clades including: clade I: *C. amalonaticus*, *C. farmer*, *C. rodentium*, *C. sedlakii*, *C. koseri* and *C. diversus* and clade II: *C. werkmanii*, *C. youngae*, *C. fraudii* and *C. braaki* appeared in trees with similar topologies. Predicted phylogenectic MP tree were differed in having polytomy in clade II. All members of this clade were appeared as separate branches in tree. In ML and NJ trees, constructed genealogic relationships in this clade were not fully resolved and polytomy was observed in ancestral node of *C. youngae*, *C. fraudii* and *C. braaki* (Fig. 1).

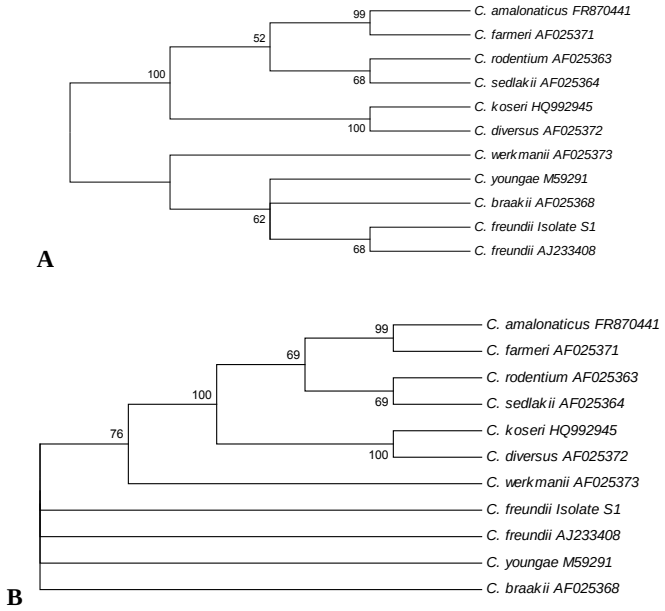


Fig. 1. Hypothesis of 16s-rRNA gene based phylogenetic relationships of isolate *C. freundii* S1 to members of the genus *Citrobacter*. The trees were constructed by A (Maximum Likelihood and B) Neighbor Joining methods.

Table 1. Biochemical characteristics of *Citrobacter freundii* isolate S1. Reactions are interpreted as the following: +, Positive and -, Negative

Characteristic	*C. freundii* S1
Gram	-
Metabolism	Facultatively anerobic
Catalase	+
Oxidase	-
Citrate	+
Methyl red	+
D-glucose	+
Lactose	+
Maltose	+
L-rhamnose	+
Sucrose	+
L-arabinose	+
D-mannitol	+
Salicin	-
Trehalose	+
Glycerol	+
D-sorbitol	+
Voges-Proskauer	-
Mannose	+

Staphylococcus succinus isolate S2

Gram-positive cocci, 0.6-2.0 µm in diameter, in rosettes including of 3-6 cells, colonies are white, opaque with elevated center. Biochemical characters are presented in table 2.

All phylogenetic analysis methods yielded seven monophyletic groups on trees with minor differences including, clade I: *S. pseudintermedius*, *S. intermedius*, *S. delphini*, *S. schleiferi*, *S. felis*, *S. chromogenes*, *S. agnetis*, *S. hyicus*, *S. muscae*, *S. rostri* and *S. microti*; clade II: *S. simulans*, *S. carnosus*, *S. piscifermentans* and *S. condiment*; clade III: *S. kloosii*, *S. nepalensis*, *S. cohnii*, *S. gallinarum*, *S. arlettae*, *S. xylosus*, *S. equorum*, *S. succinus S2* and *S. succinus*; clade IV:

S. hominis, *S. jettensis*, *S. devriesei*, *S. haemolyticus* and *S. lugdunensis*; clade V: *S. pasteuri* and *S. warneri*; clade VI: *S. aureus*, *S. argenteus*, *S. simiae*, *S. epidermidis*, *S. saccharolyticus*, *S. capitis* and *S. caprae* and clade VII: *S. sciuri*, *S. lentus*, *S. vitulinus*, *S. stepanovicii* and *S. fleurettii*. In all trees *S. succinus* isolate S2 appeared as a closest relative to *S. succinus* type species with 100% bootstrap value as a member of clade III. In all trees *S. xylosus*, *S. equorum* and *S. succinus* appeared as the most relative descendants with unresolved relationships and polytomy in ancestral node. Trees differed in depicting the position of *S. massiliensis*. In NJ and ML trees this species appeared as a separate branch without any resolved relationship with other clades but predicted hypothesis by MP method differed in putting *S. massiliensis* as a sister group of clade II and making a larger monophyletic clade. In all constructed phylogenetic trees, *S. auricularis* and *S. pettenkoferi* appeared as a single branch without resolved relationships with remaining taxa. Constructed ML tree showed the highest rate of polytomy in which members of clade IV appeared as individual branches (Fig. 2).

Discussion

We isolated two species of non-symbiotic bacteria from the hemolymph of *G. mellonella* infected by *S. feltiae*. Previous gnotobiological experiments have shown some combinations of symbiotic with non-symbiotic bacteria. Ehlers *et al*. (1990) reported that *E. coli*, allow development of the EPN and also that non-symbiotic bacteria are able to create provisional associations with these nematodes, however the naturally occurring symbiotic bacteria still enable the most efficient development. Furthermore, these non-symbiotic bacteria do not support long-term experimental associations (Akhurst & Boemare, 1990; Ehlers *et al*., 1990; Han *et al*., 1998). Bonifassi *et al*. (1999) proposed that the space between the cuticles of 2nd and 3rd juvenile stage could harbor non symbiotic bacteria for *S. scapterisci*. Similarly, Gouge and Synder (2006) showed that there was no significant differences between bacterial species identified from non-sterile or surface sterilized nematodes, suggesting that the bacteria identified originated from either inside the nematode or between the second and third stage juvenile cuticles. In the presence of symbiotic bacteria, non-symbiotic bacteria not only directly can compete with symbiotic bacteria for resources but also their activity can change cadaver in a detrimental manner for symbiotic bacteria activity (Rehfuss & Urban, 2005) and this may negatively affects symbiotic bacteria and hence EPNs growth and reproduction.

The evolutionary impact of this temporary tripartite association in Insect-Nematode-Bacteria triangle remains to be elucidated. Phylogenetically this scenarios could be postulated as a plesiomorphic character of EPNs ancestor which tried to establish a permanent symbiotic association with bacteria which is obvious in its extreme form in *Xenorhabdus* and *Photorhabdus* as an autapomorphic character. For example, compared to five genes in symbiotic bacteria, as a EPNs non-symbiotic bacterium, *A. faecalis* MOR02 genome consist of two genes encoding proteins with 66% and 71% similarity to GalU (glucose-1-phosphate uridyl transferase or UDP-glucose pyrophosphorylase) and GalE (UDP-glucose 4-epimerase), respectively (Quiroz-Castaneda *et al*., 2015). The activities of these proteins are important for the production of polysaccharides, an important factor for colonization that is considered as an important virulence factor in Gram-negative pathogens (Easom *et al*., 2010; Ramjeet *et al*., 2008). Here we report *Staphylococcus succinus* and *Citrobacter freundii* as opportunistic non-symbiotic bacteria of EPN *S. feltiae*, but their virulence and ability in killing host insect and providing appropriate condition for EPNs growth and reproduction remain to be completely elucidated.

Phylogenetic analysis of 16S-rRNA nucleotide sequences of genus *Citrobacter* clearly revealed that this monophyletic group consists of members with well-defined genealogic relationships, although the resulting NJ tree was differed from ML and MP in having unresolved relationships for clade II members, ML and MP trees are phylogenetically more robust. The NJ algorithm produces a single tree, without giving any means to compare it to other potential trees but ML and MP methods, investigates the space of all possible phylogenetic trees and consider all to identify the best ones. In the case of *S. succinus* in all constructed trees approximately seven clades were discriminable, considering ancestral node polytomy. All methods of phylogenetic inference have optimal ranges of data variation. If the variation between taxa (or sequences) is too low, there are an insufficient

number of changes to resolve divergences. This can happen if the number of informative sites is too small or due to presence of recombination or gene flow (Teyssier, Marchandin, Simeon De Buochberg, Ramuz, & Jumas-Bilak, 2003). Recent study clearly revealed that 16S rDNA sequence heterogeneity is relevant in bacteria (Harth, Romero, Torres, & Espejo, 2007), nonetheless for a number of reasons 16S-rRNA gene sequences are still used as a useful genetic marker to study bacterial phylogeny. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S-rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S-rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

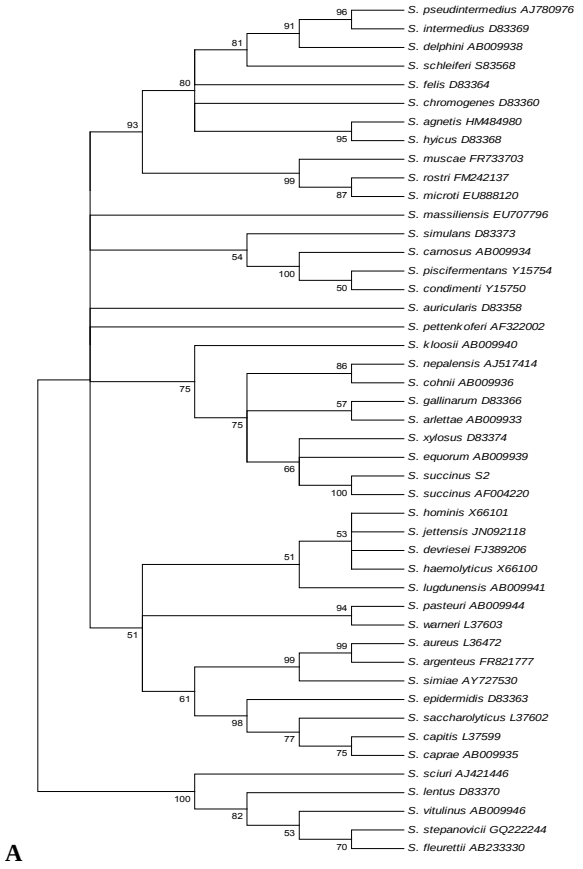
Table 2. Biochemical characteristics of *Staphylococcus succinus* isolate S2. Reactions are interpreted as the following: +, positive; -. Negative and w, weak positive reaction.

Characteristic	***S. succinus S2***
Gram	+
Catalase	+
Oxidase	-
Lactose	+
Maltose	+
L-rhamnose	w
Sucrose	+
L-arabinose	-
D-mannitol	+
Salicin	+
Trehalose	+
Voges-Proskauer	-
Mannose	-
Raffinose	-
Beta-glucuronidase	+
Beta-galactopyranosidase	+
Phosphatase	+
Urease	+
Fructose	+
Galactose	w
Melizitose	+
Turanose	+

Acknowledgements

The authors gratefully acknowledge financial support from the Azarbaijan Shahid Madani University.

The authors thank Dr. M.R. Morshedloo for his valuable suggestions and contribution to the developing manuscript.



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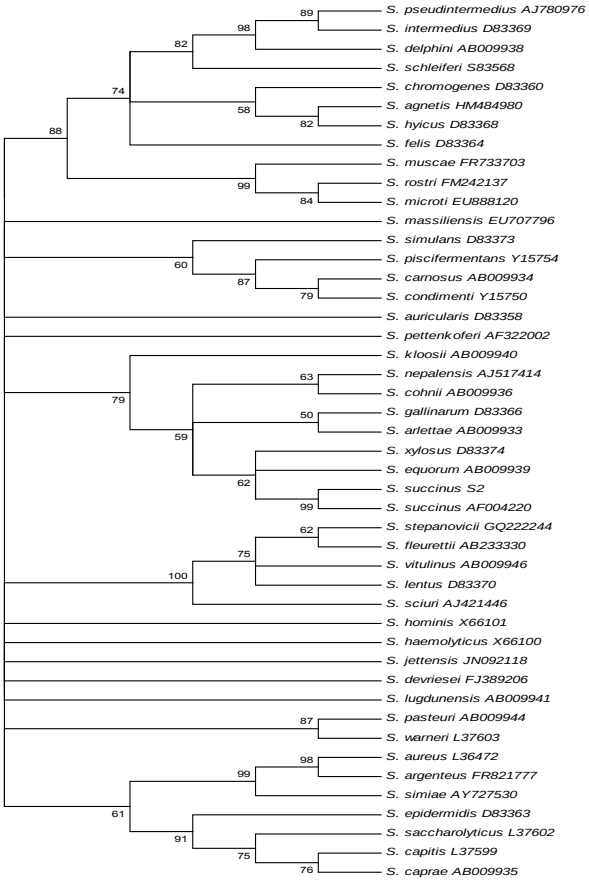

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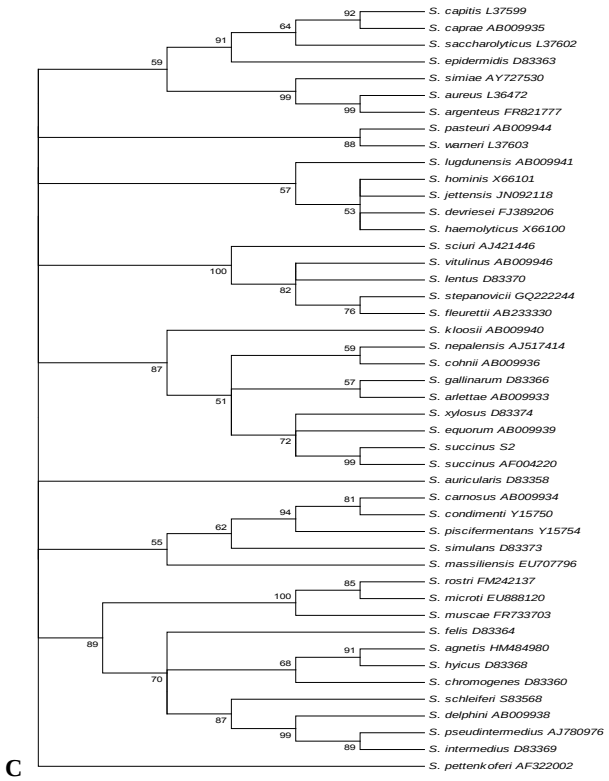

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Fig. 2. Hypothesis of 16s-rRNA gene based phylogenetic relationships of isolate *S. succinus* isolate S2 to members of the genus *Staphylococcus*. The trees were constructed by A) Maximum Likelihood, B) Maximum Parsimony and C) Neighbor Joining methods.

References

Aguillera, M. M. (1993) Bacterial symbionts of *Steinernema scapterisci*. *Journal of Invertebrate Pathology 62*, 68-72.

Aguillera, M. M., & Smart, G. C. (1993) Development, reproduction, and pathogenicity of *Steinernema scapterisci* in monoxenic culture with different species of bacteria. *Journal of Invertebrate Pathology* 62, 289-294.

Akhurst, R. J., & Boemare, N. E. (1990) Biology and taxonomy of *Xenorhabdus*. pp. 75–92 in R. Gaugler & H. K. Kaya (Eds.) *Entomopathogenic Nematodes in Biological Control* . Boca Raton, FL: CRC Press.

Bedding, R. A., & Akhurst, R. J. (1975) A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica 21*, 109-110.

Boemare, N. E., Akhurst, R. J., & Mourant, R. G. (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology 43*, 249-255.

Bonifassi, E., Fischer-Le Saux, M., Boemare, N., Lanois, A., Laumond, C., & Smart, G. (1999) Gnotobiological study of infective juveniles and symbionts of *Steinernema scapterisci*: A model to clarify the concept of the natural occurrence of monoxenic associations in entomopathogenic nematodes. *Journal of Invertebrate Pathology 74*(2), 164-172. doi: 10.1006/jipa.1999.4866

Easom, C. A., Joyce, S. A., & Clarke, D. J. (2010) Identification of genes involved in the mutualistic colonization of the nematode *Heterorhabditis bacteriophora* by the bacterium *Photorhabdus luminescens*. *BMC Microbiology 10*(45), 10. doi: http://dx.doi.org/10.1155/2015/570243

Ehlers, R.-U., Stoessel, S., & Wyss, U. (1990) The influence of phase variants of *Xenorhabdus* spp. and *Escherichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Revue de Nématologie 13*, 417-424.

Eidt, D. C., & Thurston, G. S. (1995) Physical deterrents to infection by entomopathogenic nematodes in wireworms (Coleoptera: Elateridae) and other soil insects. *Canadian Entomology 127*, 423-429.

Eivazian Kary, N., Niknam, G., Griffin, C. T., Mohammadi, S. A., & Moghaddam, M. (2009) A survey of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the north-west of Iran. *Nematology 11*(1), 107-116. doi: 10.1163/156854108X398453

Forst, S., & Clarke, D. (2002) Bacteria-Nematode Symbiosis. pp. 57-77 in R. Gaugler (Ed.) *Entomopathogenic Nematology*. Wallingford, UK: CABI Publishing.

Gaugler, R., & Han, R. (2002) Production technology. pp. 289-310 in R. Gaugler (Ed.), *Entomopathogenic nematology*. CABI International

Gouge, D. H., & Snyder, J. L. (2006) Temporal association of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) and bacteria. *Journal of Invertebrate Pathology* 91(3), 147-157. doi: 10.1016/j.jip.2005.12.003

Han, R. C., Wouts, W. M., & Li, L. (1998) Development of *Heterorhabditis* spp. strains as characteristics of possible *Xenorhabdus luminescens* subspecies. *Revue de Nématologie 13*(411-415).

Harth, E., Romero, J., Torres, R., & Espejo, R. T. (2007) Intragenomic heterogeneity and intergenomic recombination among *Vibrio parahaemolyticus* 16S rRNA genes. *Microbiology 153*(Pt 8), 2640-2647. doi: 10.1099/mic.0.2007/009175-0

Hominick, W. M., Briscoe, B. R., del Pino, F. G., Heng, J., Hunt, D. J., Kozodoy, E., Mracek, Z., Nguyen, K. B., Reid, A. P., Spiridonov, S. E., Stock, P., Sturhand, D., Waturu, C & Yoshida, M. (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *J Helminthol 71*(4), 271-298.

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology 38*, 181-206.

Koppenhoffer, A. M., Fuzy, E. M., Crocker, R., Gelernter, W., & Polavarapu, S. (2004) Pathogenicity of Steinernema scarabaei, Heterorhabditis bacteriophora and S. glaseri to twelve white grub species. *Biocontrol Science and Technology 14*, 87-92.

Krieg, N. R., & Holt, J. G. (1984) *Bergey's Manual of Systematic Bacteriology* (Vol. 1). Baltimore, USA: Williams and Wilkins.

Lysenko, O., & Weiser, J. (1974) Bacteria associated with the nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. *Journal of Invertebrate Pathology 24*, 332-336.

Mracek, Z. (1977) *Steinernema kraussei*, a parasite of the body cavity of the sawfly, *Cephaleia abietis*, in Czechoslovakia *Journal of Invertebrate Pathology*, 30, 87-94.

Park, H. W., Kim, Y. O., Ha, J. S., Youn, S. H., Kim, H. H., Bilgrami, A. L., & Shin, C. S. (2011) Effects of associated bacteria on the pathogenicity and reproduction of the insect-parasitic nematode *Rhabditis blumi* (Nematoda: Rhabditida). *Canadian Journal of Microbiology 57*(9), 750-758.

Patel, J. B. (2001) 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnostics 6*, 313-321.

Quiroz-Castañeda, R. E, Mendoza-Mejía, A., Obregón-Barboza, V., Martínez-Ocampo, F., Hernández-Mendoza, A., Martínez-Garduño, F., Guillén-Solís, G., Sánchez-Rodríguez, F., Peña-Chora, G., Ortíz-Hernández, L., Gaytán-Colín, P., Dantán-González, E. (2015) Identification of a new *Alcaligenes faecalis* strain MOR02 and assessment of its toxicity and pathogenicity to insects. *Biomed Research International* 570243. doi: 10.1155/2015/570243

Ramjeet, M., Cox, A. D., Hancock, M. A., Mourez, M., Labrie, J., Gottschalk, M., & Jacques, M. (2008) Mutation in the LPS outer core biosynthesis gene, galU, affects LPS interaction with the RTX toxins ApxI and ApxII and cytolytic activity of *Actinobacillus pleuropneumoniae* serotype 1. *Molecular Microbiology* 70(1), 221-235.

Razia, M., Karthik Raja, R., Padmanaban, K., Chellapandi, P., & Sivaramakrishnan, S. (2011) 16S rDNA-Based Phylogeny of Non-Symbiotic Bacteria of Entomopathogenic Nematodes from Infected Insect Cadavers. *Genomics Proteomics Bioinformatics 9*(3), 104-112. doi: 10.1016/S1672-0229(11)60013-2

Rehfuss, M., & Urban, J. (2005) *Alcaligenes faecalis* subsp. phenolicus subsp. nov. a phenol-degrading, denitrifying bacterium isolated from a graywater bioprocessor. *Systematic and Applied Microbiology* 28(5), 421-429. doi: 10.1016/j.syapm.2005.03.003

Teyssier, C., Marchandin, H., Simeon De Buochberg, M., Ramuz, M., & Jumas-Bilak, E. (2003) Atypical 16S rRNA gene copies in *Ochrobactrum intermedium* strains reveal a large genomic rearrangement by recombination between rrn copies. *Journal of Bacteriology* 185(9), 2901-2909.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25(24), 4876-4882.

Whalen, J. G., Mully, T. W., & English, J. C. (2007) Spontaneous *Citrobacter freundii* Infection in an Immunocompetent Patient. *Archives of Dermatology* 143(1), 124-125.

Yadav, S., Shokal, U., Forst, S., & Eleftherianos, I. (2015) An improved method for generating axenic entomopathogenic nematodes. *BMC Res Notes* 8(1), 461. doi: 10.1186/s13104-015-1443-y

Received: 11 March 2016
Accepted: 10 September 2016