# Isolation & identification of *Yersinia* & *Pseudomonas* sp. from Australian milk & salad using 16s rDNA

Malik Adil Nawaz[1*], Rewati Raman Bhattarai[2]
[1]School of Agriculture and Food Sciences, The University of Queensland, St Lucia, Qld 4072, Australia.
[2]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

---

**Correspondence:** Malik Adil Nawaz, School of Agriculture and food Sciences, The University of Queensland, St Lucia, Qld, 4072, Australia
Email: malik.nawaz@uqconnect.edu.au
Phone: +61 426244515

## Abstract

A systematic research was conducted for the isolation and identification of undesirable microorganisms in food products using molecular approach. The result revealed the presence of *Yersinia aleksiciae* and *Pseudomonas fluorescens* strains in Australian pasteurized milk and mixed salad respectively. *Y. aleksiciae* grouped as lacking virulence marker and is regarded non-pathogenic. *P. fluorescens* has been identified as a marker of spoilage of ready-to-eat vegetable salads. This study confirms the rapidity and sensitivity of 16s rDNA analysis in identifying strains which contribute in early monitoring, accurate analysis and control of microbial risks in food products. The described methodology has special relevance in food quality control and safety.

**Key words:** Milk, Mixed salad, *Yersinia*, *Pseudomonas*, PCR

## Introduction

Control of microbial spoilage of agro-products is crucial for the quality and safety of foods (Zhang, *et al*., 2014) which requires an understanding of a number of factors including the knowledge of possible hazards, their likely occurrence in different products, their physiological properties and the availability and effectiveness of different preventative measures (Blackburn, 2006). The rapid, accurate and reliable identification of spoilage microorganisms through PCR is very important in the efficient monitoring of microbiological quality, especially in raw and ready-to-eat foods (Arakawa, *et al*., 2008).

Milk is a significant food of human nutrition owing to its high nutritional value. It is naturally a good medium for growth of microorganisms. Quality control of milk and milk products is therefore of paramount importance (Bashir, *et al*., 2014). Recent outbreaks of food-borne illnesses associated with milk and dairy products consumption have been found to be contaminated with pathogenic microorganisms such as *Listeria* spp., *Salmonella* spp., *Campylobacter* spp. and *Yersinia* spp. (Gram, *et al*., 2002). Microbial analysis of milk and dairy products therefore has a critical role to play in the quality evaluation of these products, promoting public health safety (Nicolaon and Goodacre, 2008; Bashir, *et al*., 2014).

The popularity of fresh cut fruits and vegetables mixed salad is increasing day by day. The incidence of food borne illness associated with fresh-cut salads is very low relative to the quantity consumed; the increased use of these products has been accompanied by an increase

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.820v1 | CC-BY 4.0 Open Access | rec: 7 Feb 2015, publ: 7 Feb 2015

in reported outbreaks associated with their consumption (Palumbo, *et al*., 2007). The predominant microbiological populations in ready-to-eat salads are psychrotrophs including *Pseudomonas* spp. and *Erwinia* spp.. Pectolytic strains of Pseudomonas have been reported to cause vegetable deterioration during storage (Willocx, *et al*., 1993). Recent statistics shows that consumption of milk and salad is increasing in Australia (Moore, 2014). In this context, the present investigation will be highly beneficial to mitigate the problems associated with consuming contaminated food. Not only will the consumers, food industries also be equally profited.

Molecular approaches, especially those based on the use of rRNA genes (DNA) and related techniques, have provided the opportunity to analyse complex communities on the basis of sequence diversity. Bacterial species can be identified by generating clone libraries of the 16S rDNA followed by sequencing and comparison with databases containing thousands of ribosomal sequences to allow a phylogenetic affiliation to cultured, as well as uncultured microorganisms (Ercolini, 2004). Unlike conventional cultivation methods which are time consuming and labour intensive, molecular diagnosis tools being highly powerful, sensitive and rapid are gaining popularity for microbial identification (Böhme *et al.*, 2014). To obtain knowledge on the bacterial communities of pasteurized milk and fresh cut salad, packaged under ordinary packaging, in the present study a polyphasic characterization mainly based on molecular approaches, was applied. Therefore, the aim of this study was to identify the foodborne pathogens especially *Yersinia* and *Pseudomonas* spp. from milk and salad through 16s rDNA using PCR.

## Material and Methods

The experiment was conducted at the School of Agriculture and Food Sciences, University of Queensland, Australia.

**Isolation and Identification of Bacterial Species** Two food samples were brought in for isolation and identification of bacteria. The first food sample was Australian Lite Milk. The other food sample was Australian Mixed Salad. 10 samples of each food product (milk and salad) were purchased from the local supermarket. The samples were streak plated on the nutrient agar and incubated at 37°C for 24 hrs. The single and isolated colonies were re-streaked on nutrient agar plates to obtain pure cultures.

The well isolated colonies (one round and white colony from each sample) were inoculated into 10 mL of nutrient broth and incubated at 37$^o$C. The gDNA was extracted; PCR and sequencing were performed as described by (Matrone, *et al*., 2009). 16s rDNA was amplified from genomic DNA of *P. fluorescens* with the primers 5′TGGCTCAGATTGAACGCTGGCGG-3′ and 5′-GATCCAGCCGCAGGTTCCCCTAC-3′ and for *Y. aleksiciae* 5′-GAATATTGCACAATGGGCGCA-3’ and 5’-AACAAACCGCCTGCGTGCGC-3’ primers were used.

The PCR reaction was carried out in 10X PCR buffer containing 10 mM dNTPs, 10 µM each primer, 50mM $MgCl_2$ and 1µl gDNA. The PCR program was: 95°C for 2 min; 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 2 min and extra 72°C for 5 min . The resulting PCR products were observed on 1% agarose gel electrophoresis in 1X TAE buffer at 120 V for 60 min then the gel was stained with ethidium bromide solution for 10-15 min and photographed on gel documentation system (Bio-Rad). The PCR products were purified with high pure PCR product purification kit (Roche).

## Results and Discussion

The initial 16 streak results showed round white colonies produced in milk sample while three different colonies (round yellow, round orange and round white) produced in salad sample. Well isolated round white colonies from both samples were isolated.

Agarose gel of bacterial genomic DNA revealed that both samples comprised of high

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.820v1 | CC-BY 4.0 Open Access | rec: 7 Feb 2015, publ: 7 Feb 2015

molecular weight DNA and thus was suitable for PCR. The only problem was in mixed salad sample in which the DNA was bit degraded. From the result of agarose gel of PCR product (Figure 1), comparing to the DNA marker, it can be the sample DNA has about 1400 base pairs.

A partial DNA sequence from 20 to 800 was analysed in BLAST website. Both samples show a high ratio match to the database. The result from BLAST database showed the DNA sequence of bacteria isolated from milk sample had 100% matches (Figure 2) with the DNA sequence of *Y. aleksiciae* strain DSM 14987 (Accession FJ717341) while the result from salad sample had 100% matches (Figure 2) with the DNA sequence of *P. fluorescens* strain 1408 (Accession GU726880).

The genus *Yersinia* is composed of 12 species and belongs to the *Enterobacteriaceae* family. The most recent addition to the genus *Yersinia* is *Y. aleksiciae*. The species name *Y. aleksiciae* was proposed by Sprague and Neubauer (2005) for a group of strains isolated from diverse origins (human faeces, rats, moles, reindeer and pigs, and from dairy products) formerly classified as *Y. kristensenii* serotype O:16. *Y. aleksiciae* is a Gram-negative, motile, coccoid rods. *Y. aleksiciae* can also be grouped with *Y. enterocolitica*–like species. *Y. enterocolitica*–like bacteria lack the classical *Yersinia* virulence markers and thus have generally been regarded as non-pathogenic. *Y. enterocolitica* like bacteria have been isolated from almost any possible environmental source imaginable (Sulakvelidze, 2000). Ibrahim, *et al*., (1997) isolated thirty-eight bacterial isolates from raw milk samples in Queensland, Australia which were identified as members of the genus *Yersinia* on the basis of biochemical profile, and comparative 16s rDNA sequence analysis. Likewise Prasad and Turner (2011) reported *Y. aleksiciae* from expired milk highlighting low level of pathogen are generally nonproblematic and strain isolated may not contain virulence factor.

*P. fluorescens* is a spoilage-causing bacterium present in a variety of food related environments (Sillankorva, *et al*., 2008). *P. fluorescens* has been identified as a marker of spoilage of ready-to-eat vegetable salads (Wei, *et al*., 2006). Prasad & Turner (2011) isolated this bacterium from raw milk and mixed salad. Moreover, Randazzo, *et al*., (2009) also reported that *Pseudomonaceae* was the major group of microorganisms contaminating fresh cut salad products. Members of this family are typically present in soil and plants and their distribution are affected by farming procedures (Behrendt, *et al*., 2001). Many species of the genus *Pseudomonas* have been previously isolated in field-grown samples such as salad vegetables (Robbs, *et al*., 1996). These bacteria are tightly associated with plant matrices and are not removed during washing procedures. Among *Pseudomonaceae* family, *P. fluorescens* is an opportunistic pathogens, which shows resistance to different antibiotics (Hamilton-Miller and Shah, 2001). Although the behaviour of psychrotrophic bacteria is not always comparable due to the variability of fresh produce, the quality of vegetable salad may be inferior, both through tissue disruption and/or the production of polysaccharides (Tallgren, *et al*., 1999). Storage at refrigerated temperatures generally selects in favour of the growth of psychrotrophic bacteria, including the pectinolytic *Pseudomonas* (Nguyen-The and Carlin, 1994). Different species of *Pseudomonas* are able to produce pectinolytic enzymes which could influence textural changes in fresh-cut vegetables by degrading the middle lamella and the primary cell wall (Ragaert, *et al*., 2007). Moore (2014) reported milk as an indispensable item in Australian diet and more than 75% of population consuming vegetables including salads. Regular consumption of healthy foods (including milk and salad) have beneficial effects on human health.

## Conclusion

The results of this study confirm the sensitivity of 16s rDNA analysis in identifying strains to the species level, and as an additional, indispensable tool for differentiation among strains expressing heterogeneous biochemical profiles. Such rapid and sensitive detection of microorganisms helps to early detect and control microbial risks in food products.

## References

Arakawa K, Kawai, Y, Iioka, IH, Tanioka, M, Nishimura, J, Kitazawa, H, Tsurumi, K, Saito, T (2008) Microbial Community Analysis of Food-Spoilage Bacteria in Commercial Custard Creams Using Culture-Dependent and Independent Methods. J Dairy Sci 91:2938-2946.

Bashir, S, Awan, MS, Khan, SA, Suthar, V, Khan, MM, Khan, MT (2014) Microbiological Quality Evaluation of Raw Milk Consumed in and Around Rawalakot, Azad Jammu and Kashmir. Intl J Microbial Res 5:112-116.

Behrendt, U, Ulrich, A, Schumann, P, (2001) Description of *Microbacterium foliorum* sp. nov. and *Microbacterium phyllosphaerae* sp. nov. isolated from the phyllosphere of grasses and the surface litter after mulching the sward and reclassification of *Aureobacterium resistens* as *Microbacterium resistens* comb. Nov.. Int J Sys Evo Microbiol 51:1267– 1276.

Blackburn, C (2006) *Food Spoilage Microorganisms*, Woodhead Publishin Limited.

Böhme, K, Cremonesi, P, Severgnini, M, Villa, TG, Fernández-No, IC, Barros-Velázquez, J, Castiglioni, B, Calo-Mata, P, (2014) Detection of Food Spoilage and Pathogenic Bacteria Based on Ligation Detection Reaction Coupled to Flow-Through Hybridization on Membranes. BioMed Res Int 2014: 1-11 doi:10.1155/2014/156323

Ercolini, D (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food: a review. J Microbiol Methods 56:297–314.

Gram, L, Ravn, L, Rasch, M, Bruhn, JB, Christensen, AB, Givskov, M (2002) Food spoilage – interactions between food spoilage bacteria. Int J Food Microbiol 78:79-97.

Hamilton-Miller, JMT, Shah, S (2001) Identity and antibiotic susceptibility of enterobacterial flora of salad vegetables. Int J Antimicrobial Agents 18:81–83.

Ibrahim, A, Liesack, W, Steigerwalt, AG, Brenner, DJ, Stackebrandt, E, Robins-Browne, R M (1997) A cluster of atypical *Yersinia* strains with a distinctive 16S rRNA signature. FEMS Microbiol Letters 146:73–78.

Matrone, M, Keid, LB, Rocha, VCM, Vejarano, MP, Ikuta, Cy, Rodriguez, CAR, Ferreira, F, Dias, RA, Ferreira Neto, JS (2009) Evaluation of DNA extraction protocols for *Brucella abortus* PCR detection in aborted foetuses or calves born from cows experimentally infected with strain 2308. Brazil. J Microbiol 40:480-489.

Moore, G. (2014). Australian eating habits survey – the results? Could do better…(Accessed on 9/10/2014 from http://www.sumosalad.com/australian-eating-habits-survey-the-results-could-do-better/)

Nguyen-The, C, Carlin, F (1994) The microbiology of minimally processed fresh fruits and vegetables. Crit Reviews Food Sci Nut 34:371–401.

Nicolaon, N, Goodacre, R (2008) Rapid and quantitative detection of the microbiological spoilage in milk using Fourier transform infrared spectroscopy and chemometrics. The Analyst 133:1424-1431.

Palumbo, MS, Gorny, JR, Gombas, DE, Beuchat, LR, Bruhn, CM, Cassens, B, Delaquis, P, Farber, JM, Harris, LJ, Micheal, KI, Osterholm, T, Smith, M, Swanson, KMJ (2007) Recommendations for handling fresh-cut leafy green salads by consumers and retail food service operators. Food Protection Trends 27:892-898.

Prasad, P, Turner, MS (2011) What bacteria are living in my food?: An open-ended practical series involving identification of unknown foodborne bacteria using molecular techniques. Biochem Mol Bio Edu *39*:384-390.

Randazoo, CL, Caggia, C, Neviani, E (2009) Application of molecular approaches to study lactic acid bacteria in artisanal chesses. J Microbial Meth 78:1-9.

Ragaert, P, Devlieghere, F, Debevere, J (2007) Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. Postharvest Bio Technol, 44:185–194.

Robbs, PG, Bartz, JA, Sargent, SA, McFie, G, Hodge, NC (1996) Potential inoculums sources for decay of fresh-cut celery. J Food Sci 61:449–452.

Sillankorva, S, Neubauer, P, Azeredo, J (2008) *Pseudomonas fluorescens* biofilms subjected to phage phiIBB-PF7A. BMC Biotechnol 8:1-12.

Sprague, LD, Neubauer, H (2005) *Yersinia aleksiciae* sp. nov.. Int J Sys Evo Microbiol 55:831-835.

Sulakvelidze, A (2000) *Yersiniae* other than *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*: the ignored species. Microbes and Infection 2:497–513.

Tallgren, AH, Airaksinen, U, von Weissenberg, R, Ojamo, H, Kuusisto, J, Leisola, M (1999) Exopolysaccharide-producing bacteria from sugar beets. Applied Envir Microbiol 65:862–864.

Wei, H, Wolf, G, Hammes, WP (2006) Indigenous microorganisms from iceberg lettuce with adherence and antagonistic potential for use as protective culture. Innovative Food Sci Emerging Technol 7:294-301.

Willocx, F, Mercier, M, Hendricks, M, Tobback, P (1993) Modelling the influence of temperature and carbon dioxide upon the growth of *Pseudomonas fluorescens*. Food Microbiol 10:159-173.

Zhang, SB, Zhai, HC, Hu, YS, Wang, L, Yu, GH, Huang, SX, Cai, JP (2014) A rapid detection method for microbial spoilage of agro-products based on catalase activity. Food Control, 42:220-224.

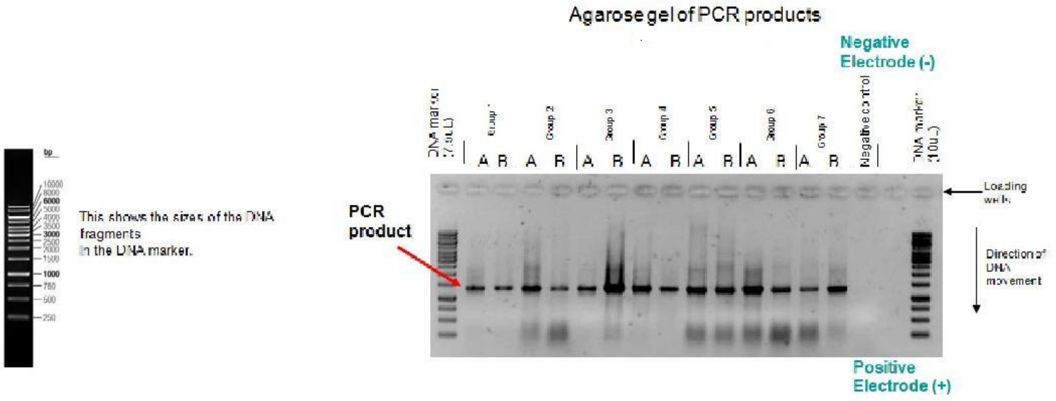
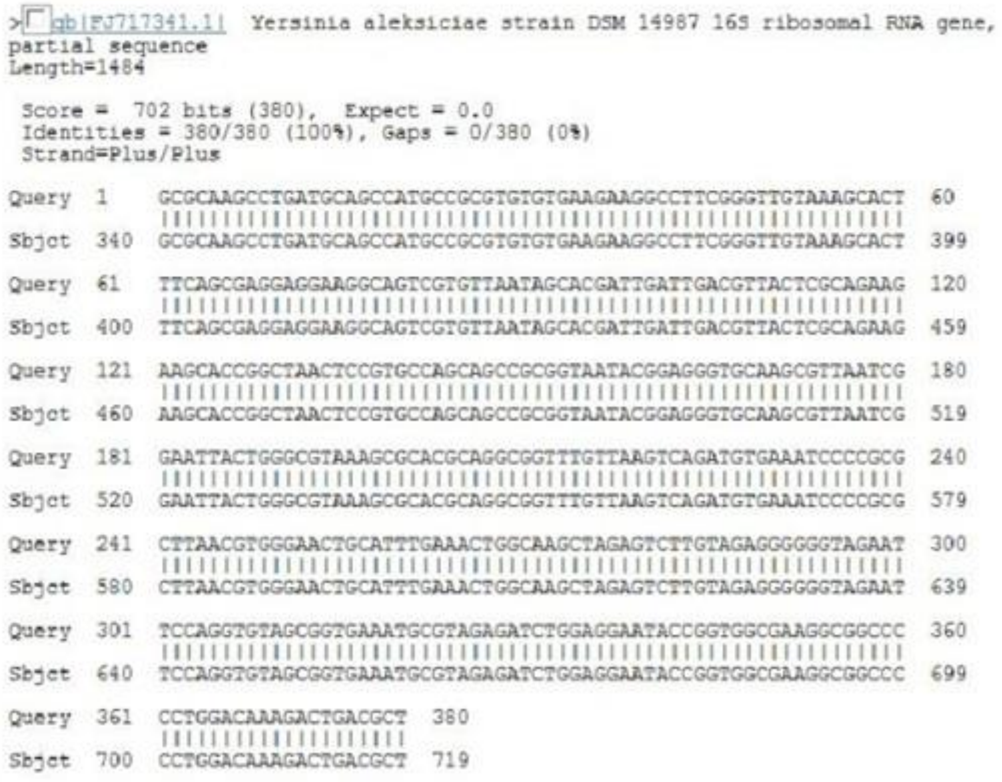
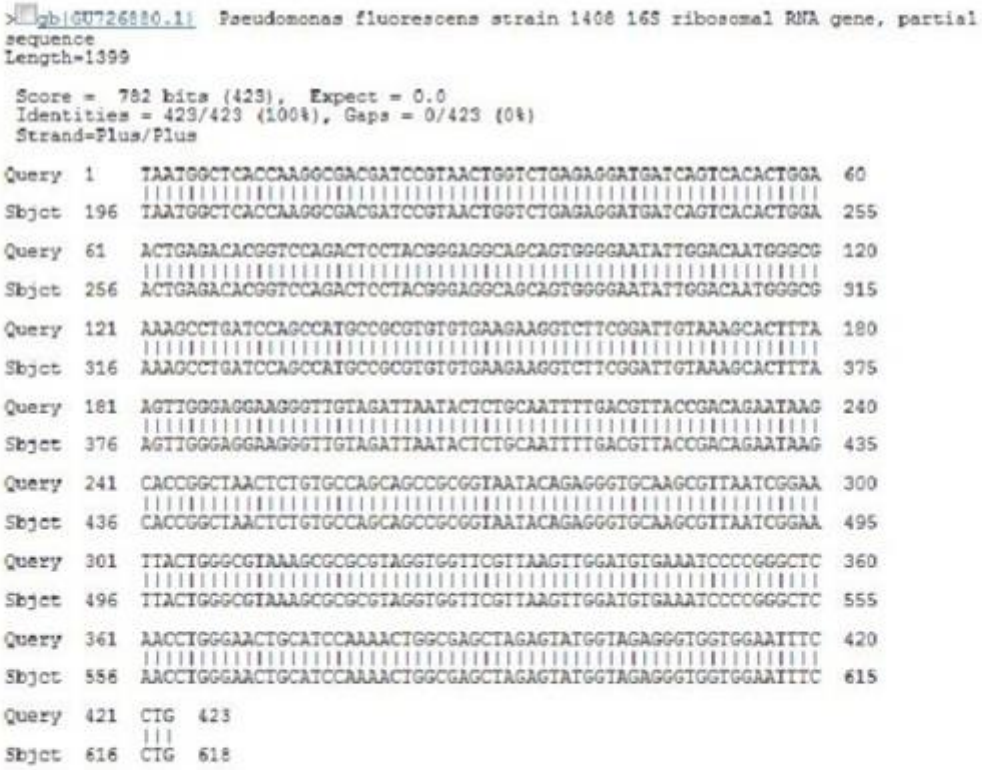


**Figure 1 -** Agarose Gel of PCR Products.



```
>gb|FJ717341.1|  Yersinia aleksiciae strain DSM 14987 16S ribosomal RNA gene,
partial sequence
Length=1484

 Score =  702 bits (380),  Expect = 0.0
 Identities = 380/380 (100%), Gaps = 0/380 (0%)
 Strand=Plus/Plus

Query  1    GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT  399

Query  61   TTCAGCGAGGAGGAAGGCAGTCGTGTTAATAGCACGATTGATTGACGTTACTCGCAGAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  TTCAGCGAGGAGGAAGGCAGTCGTGTTAATAGCACGATTGATTGACGTTACTCGCAGAAG  459

Query  121  AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460  AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG  519

Query  181  GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  520  GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCG  579

Query  241  CTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  580  CTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAAT  639

Query  301  TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  640  TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC  699

Query  361  CCTGGACAAAGACTGACGCT  380
            ||||||||||||||||||||
Sbjct  700  CCTGGACAAAGACTGACGCT  719
```



```
>gb|GU726880.1|  Pseudomonas fluorescens strain 1408 16S ribosomal RNA gene, partial
sequence
Length=1399

 Score =  782 bits (423),  Expect = 0.0
 Identities = 423/423 (100%), Gaps = 0/423 (0%)
 Strand=Plus/Plus

Query  1    TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA  255

Query  61   ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG  315

Query  121  AAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  AAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA  375

Query  181  AGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  AGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAG  435

Query  241  CACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  CACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAA  495

Query  301  TTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  TTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTC  555

Query  361  AACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  AACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTC  615

Query  421  CTG  423
            |||
Sbjct  616  CTG  618
```

**Figure 2 -** BLAST Nucleotide Sequence.