Pseudomonas fluorescens-like bacteria from the stomach: A microbiological and molecular study

Saurabh Kumar Patel, Chandra Bhan Pratap, Ajay Kumar Verma, Ashok Kumar Jain, Vinod Kumar Dixit, Gopal Nath

Saurabh Kumar Patel, Chandra Bhan Pratap, Ajay Kumar Verma, Gopal Nath, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi UP 221005, India
Ashok Kumar Jain, Vinod Kumar Dixit, Department of Gastroenterology, Institute of Medical Sciences, Banaras Hindu University, Varanasi UP 221005, India
Author contributions: Patel SK performed the major part of the experimental work, interpreted the results and prepared the manuscript; Pratap CB and Verma AK assisted in performing experiments; Jain AK and Dixit VK coordinated and helped in collection of human material, and also helped in shaping the manuscript; Nath G was responsible for genesis of the hypothesis, designing of the experiment, analysis of data and shaping of the manuscript; all authors read and approved the final draft.

Supported by Department of Biotechnology, Government of India, No. 102/IFD/SAN/PR1310/2006-07; Council of Scientific and Industrial Research, New Delhi, India, in the form of Senior Research Fellowship (to Patel SK)
Correspondence to: Dr. Gopal Nath, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Pandit Madan Mohan Malviya Rd, Varanasi UP 221005, India. gopalnath@gmail.com
Telephone: +91-542-6703484 Fax: +91-542-2367568
Received: July 19, 2012 Revised: September 13, 2012 Accepted: September 22, 2012 Published online: February 21, 2013

Abstract

Aim: To characterize oxidase- and urease-producing bacterial isolates, grown aerobically, that originated from antral biopsies of patients suffering from acid peptic diseases.

Methods: A total of 258 antral biopsy specimens were subjected to isolation of bacteria followed by tests for oxidase and urease production, acid tolerance and aerobic growth. The selected isolates were further characterized by molecular techniques viz. amplifications for 16S rRNA using universal eubacterial and HSP60 gene specific primers. The amplicons were subjected to restriction analysis and partial sequencing. A phylogenetic tree was generated using unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. Assessment of acidity tolerance of bacteria isolated from antrum was performed using hydrochloric acid from 10^{-7} mol/L to 10^{-1} mol/L.

Results: Of the 258 antral biopsy specimens collected from patients, 179 (69.4%) were positive for urease production by rapid urease test and 31% (80/258) yielded typical Helicobacter pylori (H. pylori) after 5-7 d of incubation under a microaerophilic environment. A total of 240 (93%) antral biopsies yielded homogeneous semi-translucent and small colonies after overnight incubation. The partial 16S rRNA sequences revealed that the isolates had 99% similarity with Pseudomonas species. A phylogenetic tree on the basis of 16S rRNA sequences denoted that JQ927226 and JQ927227 were likely to be related to Pseudomonas fluorescens. On the basis of HSP60 sequences applied to the UPGMA phylogenetic tree, it was observed that isolated strains in an aerobic environment were likely to be P. fluorescens, and HSP60 sequences had more discriminatory potential rather than 16S rRNA sequences. Interestingly, this bacterium was acid tolerant for hours at low pH. Further, a total of 250 (96.9%) genomic DNA samples of 258 biopsy specimens and DNA from 240 bacterial isolates were positive for the 613 bp amplicons by targeting P. fluorescens-specific conserved putative outer membrane protein gene sequences.

Conclusion: This study indicates that bacterial isolates from antral biopsies grown aerobically were P. fluorescens, and thus acid-tolerant bacteria other than H. pylori can also colonize the stomach and may be implicated in pathogenesis/protection.

© 2013 Baishideng. All rights reserved.
Key words: Antral biopsy; Helicobacter pylori; Pseudomonas fluorescens; HSP60; Nested polymerase chain reaction; Acid-tolerant bacteria

Patel SK, Pratap CB, Verma AK, Jain AK, Dixit VK, Nath G. Pseudomonas fluorescens-like bacteria from the stomach: A microbiological and molecular study. World J Gastroenterol 2013; 19(7): 1056-1067 Available from: URL: http://www.wjg.net.com/1007-9327/full/v19/i7/1056.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i7.1056

INTRODUCTION

Helicobacter pylori (H. pylori) is a gram-negative, microaerophilic bacterium found primarily in the stomach. It has been implicated in chronic gastritis, gastric ulcers, duodenal ulcers and stomach cancers that were previously believed to be of non-microbial origin[1-3]. There was a misconception that no bacteria could live in the stomach because of its highly acidic environment. For the first time, Steer and Colin-Jones[4] published their results regarding the presence of gram-negative, oxidase- and urease-producing bacteria, but they proposed that it was Pseudomonas (P), a contaminant and not related to peptic ulcer. In an effort to grow H. pylori from an antral biopsy, we could see that a peculiar type of bacterial colony was growing consistently after overnight incubation while H. pylori was taking 3-5 d to grow. These colonies were also oxidase and urease producers, but growing in an aerobic environment. There are reports that show the presence of a variety of bacteria in the stomach by isolation, DNA profiling and polymerase chain reaction (PCR)-based analysis, and some of them are urease producers hindering the specificity of the urea breath test[5-7]. Therefore, we aimed to characterize this type of bacterial isolate and to analyze whether they are colonizers or contaminants.

MATERIALS AND METHODS

Collection of specimens

The study subjects were patients attending inpatient services of the Department of Gastroenterology, University Hospital of Banaras Hindu University, Varanasi, Uttar Pradesh, India. This hospital provides tertiary-level health services for the eastern part of Northern India. The culture isolation, phenotypic and molecular characterizations were carried out in the Department of Microbiology, Institute of Medical Sciences.

Patients and samples

A total of 258 patients suffering from upper gastrointestinal (UGI) diseases like non- ulcer dyspepsia (NUD), peptic ulcer diseases (PUD) including gastric ulcer and duodenal ulcer, and gastric carcinoma were enrolled during a period of 3 years (2007-2010) and three antral biopsy pieces from each patient were collected. Before taking a biopsy, the endoscope was rinsed with detergent followed by water, and disinfected with 2% alkaline glutaraldehyde for 30 min then rinsed with sterile water. In a similar way, biopsy forceps were washed and sterilized and one biopsy forceps was used for one patient exclusively. The biopsy specimens were collected by endoscopic forceps from each individual with full aseptic precautions after taking well-informed consent. The work was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University. Patients with mucosal breaks greater than 5 mm in size with apparent depth were diagnosed as having ulcer and those with ulcerative-infiltrative lesions with positive histology/brush cytology were considered as having stomach carcinoma. The patients not having ulcerative lesions but suffering from dyspeptic symptoms were diagnosed as NUD. Individuals with normal endoscopic findings without gastroduodenal symptoms but having other gut problems were treated as healthy controls. In the present study, those patients were excluded who had a history of previous gastric surgery, active UGI bleeding, chronic alcoholism, intake of antibiotics and proton pump inhibitors during the last 4 wk or those taking non-steroidal anti-inflammatory drugs. Further individuals less than 18 years of age, pregnant or lactating mothers or those having illnesses like cirrhosis, chronic renal failure or ischemic heart disease were also excluded.

Microbiological processing

The three biopsy pieces were pooled and homogenized into phosphate saline buffer together in an all glass disposable homogenizer and were divided into three aliquots. The first aliquot of the tissue homogenate was transferred immediately into a rapid urease test (RUT) medium and the second was plated within 30 min of collection onto the media used for bacterial culture [Mueller Hinton agar without supplement and media containing brain heart infusion agar (Difco, Becton Dickinson, Sparks, MD, United States), supplemented with 7% sheep blood, 0.4% IsoVitaleX, and Skirrow selective supplement (vancomycin 10 μg/mL; polymixin B sulfate 2.5 IU/mL; trimethoprim lactate 5 μg/mL) (Difco, Becton Dickinson, Sparks, MD, United States)]. The non-enriched plate was incubated at 37 °C in an aerobic atmosphere while the other was incubated in the presence of 5% O2, 10% CO2, and 85% N2 for 3-7 d. Several small colonies could be seen after overnight incubation in the first plate. The other plate was examined every alternate day after 3-7 d to see if colonies other than those observed after overnight incubation developed. Small translucent colonies developed after 5-7 d of incubation other than those originally after observed overnight incubation on non-enriched nonselective medium. These colonies were further subjected to morphological and biochemical tests viz motility, oxidase, catalase, and urease. All the isolates were divided into two groups on the basis of the incubation period, i.e., those isolates obtained after overnight incubation designated as group A and the other group B possessing those strains isolated after 5-7 d of incubation. The third aliquot was subjected to genomic DNA extraction.
Table 1 Primers used to study Helicobacter pylori and Pseudomonas fluorescens isolates

| Target gene    | Primer name | Primer sequence (5’-3’) | PCR condition (number of cycles, size of product) |
|----------------|-------------|-------------------------|--------------------------------------------------|
| 16S rRNA       | 16S F       | TGCTAGAAGTGTCTTCTGCCCC  | 94 °C, 30 s; 59 °C, 30 s; 72 °C, 30 s (30, 115 bp) |
|                | 16S R       | ACGTCATCCACCTTTTCTC    |                                                  |
| HSP60          | Primary     | AAAGGCGTCAAGTTGATAGAGGCT | 94 °C, 30 s; 56 °C, 30 s; 72 °C, 30 s (35, 594 bp) |
|                | Nested      | TTGATAGAAGCTACCTTACCCTC | 94 °C, 30 s; 56 °C, 30 s; 72 °C, 30 s (35, 501 bp) |
| mupV           | Primary     | TCTKRYCRRMAGATACRARACWRYC | 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35, 704 bp) |
|                | Nested      | TGGGYWWMMWWCCYWRWWCCWTGA | 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35, 613 bp) |
| PFMP           | Primary     | TGAAGTTCTGATGTGACCTGCTGTG | 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35 cycles, 722 bp) |
|                | Nested      | AACTCGCCAGATGCTGAAGTACAC |                                                  |
| HSPN1          |             | TGTCATAATCCCGGCTGTCGCC |                                                  |
| HSPN2          |             | ACGTGAATCCCGGCTGTCGCC  |                                                  |
| Putative membrane-bound |             | ACGTGAATCCCGGCTGTCGCC  |                                                  |
| protein (PFMP) | Primary     | AKCABGGTSGCGMVCCRRBGC   |                                                  |
|                | Nested      | ATGCATCTGCTGCCACACCTGATC |                                                  |

PCR: Polymerase chain reaction.

**RUT**

For the RUT, biopsy specimens were inoculated into 1 mL of 10% urea in deionized water (pH 6.8), to which two drops of 1% phenol red solution was added, and incubated at 37 °C for 24 h. A positive result was recorded when the color changed from yellow to pink within 30 min. If there was mild color change within 30 min, the RUT tubes were incubated for a further 24 h.

**Subculture at different temperatures**

The organisms which appeared after overnight incubation were tested for growth at 4 °C, 35 °C and 42 °C onto Muller Hinton agar; incubation was maintained for 14 d to distinguish Pseudomonas spp.8,9. Those organisms showing growth at 4 °C were sub cultured twice and incubated at the appropriate temperature for more than 10 d each time.

**Assessment of acidity tolerance of bacteria isolated from gastric niche**

Five isolates were suspended into different molar concentrations of hydrochloric acid (10⁻³ to 10⁻⁷ mol/L corresponding to pH 1.0 to 7.0) and a CFU was maintained as 10⁶ CFU/mL. After the intervals of 0 min, 5 min, 10 min, 20 min, 30 min and 60 min, 100 μL acidic suspension (10⁷ CFU) were transferred into 3 mL BHI broth. After overnight incubation optical density was recorded with the help of a spectrophotometer at 600 nm wavelength and bacterial count was expressed in CFU/mL. This experiment was repeated twice.

**Preparation of genomic DNA for PCR assay**

Extraction of genomic DNA from both types of bacterial isolates (A total of 320 strains including groups A + B) as well as from tissue homogenate was performed using a standard proteinase K and phenol-chloroform method18. To exclude the possibility of cross contamination of DNA during DNA extraction, one set of double distilled was included in each batch of DNA extraction.

**Detection of H. pylori by nested PCR**

Confirmation of H. pylori was done at a molecular level by nested PCR targeting the conserved HSP60 gene and its restriction fragment length polymorphism. The reaction was performed in 25 μL final volume containing 10 ng of DNA, 1 U of Taq polymerase (Bangalore Genie, India), 200 mmol/L (each) deoxynucleotide triphosphate (MBI, Fermentas) and 1.5 mmol/L MgCl₂ in standard PCR buffer and 10 pmol of each primer as described by Singh et al13. Primer sequences and PCR conditions are displayed in Table 1. For the internal amplification, the PCR product from the primary cycle was diluted 1/50 and 1 μL was used as the template in the nested PCR. The conditions for the PCR amplification, first and nested reactions were the same. DNA from H. pylori reference J99 and a tube containing water in place of DNA were assayed in each PCR run as positive and negative controls, respectively.

After amplification, the PCR products (501 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 μL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme HindIII in appropriate buffered solution recommended by the manufacturer (Bangalore Genie, India) and incubated for 3 h at 37 °C. The digested DNA fragments were analyzed by electrophoresis on 2% agarose gels (Bangalore Genie, India) containing 0.5 μg of ethidium bromide per mL. The gel was run at 70 V with TBE (Tris Boric acid EDTA) buffer for 3 h and was examined by a transilluminator and photographed. The sizes of digested DNA fragments were estimated from distances of molecular weight standards and compared with in silico restriction digestion.
Amplified rDNA restriction analysis
For each group of isolates, the 16S rRNA from 8 randomly selected isolates was amplified using forward primer-16SF and reverse primer-16SR.[2] PCR amplification was performed in a thermocycler (Biometra, Germany) according to standard procedures (Table 1). After amplification by universal eubacterial primers, the PCR products (1155 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 μL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme EcoR1, in appropriate buffered solution recommended by the manufacturer (Bangalore Genie) and incubated for 3 h at 37 °C. The visualization of the restriction fragment was done by the same method as described in the previous paragraph.

Sequencing
The amplified PCR products were purified from salts and primers using QIA quick PCR purification kit (Qiagen, United States). A total of 8 purified amplicons generated targeting HSP60 and 2 amplicon 16S rRNA genes were outsourced for partial sequencing to Bangalore Genie, India. Sequences were analyzed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) to verify the identity of the sequences: whether H. pylori or some other microorganism.

Sequence analyses
Reference sequences of Pseudomonas group and other enteric pathogens used for phylogenetic analyses were retrieved from Genbank. The partial 16S rRNA sequences and HSP60 sequence for the strains were aligned with reference sequences using Clustal X version 1.81, with default parameters[13]. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4.[14] The phylogenetic tree was generated using the unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. The degree of statistical support for branches was determined with 500 bootstrap replicates.

Primers designed for Pseudomonas fluorescens targeting putative membrane-bound protein
The putative membrane-bound protein coding gene present in all the strains of P. fluorescens is available in NCBI Genbank. Due to a similarity of sequences of about 55% in P. fluorescens, we therefore planned to design nested degenerate primers to amplify the partial sequence of putative membrane bound protein so that it was able to amplify all strains of P. fluorescens. Forward and reverse oligo-nucleotide degenerate primers derived from the region located between bases 68796 and 68822 of the mupirocin biosynthetic gene cluster of Pseudomonas fluorescens (NCIMB 10586). An internal primer was derived from the region between bases 68796 and 68796 (GenBank Accession number AF318063.2; gene GI: 20150006). PCR amplification was similar to amplification of HSP60 gene and conditions are described in Table 1.

Randomly amplified polymorphic DNA PCR
Fingerprinting of 71 randomly selected strains from group A was performed based on randomly amplified polymorphic DNA (RAPD) PCR methods by using primers RAPD3 5’-TACAGCTCG-3’and RAPD5 5’AGCACT-GGCT-3’ (this study). PCR was carried out in 25 μL volume using 10 ng of genomic DNA, 1 U of Taq polymerase (Bangalore Genie, India), and 15 pmol of each primer (Bangalore Genie), 200 mmol/L (each) deoxynucleotide triphosphate (Bangalore Genie, India) and 2 mmol/L MgCl2 in standard PCR buffer. Amplification reactions were carried out in a thermal cycler (Biometra, Goettingen, Germany).

The gel images were analyzed under ultraviolet light in a gel documentation system (Alpha Innotech, United States). Cluster analysis of all the isolates was done on the basis of the fingerprint generated. Based on the presence or absence of different DNA fragments in the fingerprints of the P. fluorescens strains, a binary data matrix was created. Overall similarity between the pair of strains was calculated from the binary data matrix using the simple matching-dice coefficient. The resulting similarity matrix was used as the input data for cluster analysis by NTSYS pc2.0 programme of UPGMA.[16]

Statistical analysis
The level of significance between the two proportions, i.e., culture rates and molecular detection rates, was calculated by Fischer’s Exact Probability test.

RESULTS
Bacteriological findings
Of the 258 antral biopsy specimens collected from patients, 69.4% (179/258) were found to be positive by RUT and 31% (80/258) by culture for typical H. pylori, after 5-7 d of incubation under a microaerophilic environment; these were gram-negative curved rods and were positive for oxidase, catalase and urease. However, 258 antral biopsies yielded 240 (93%) homogeneous semi-translucent and small colonies after overnight incubation. These isolates also grew aerobically but the colonies had
Table 2  Comparative isolation rates and prevalence of *Helicobacter pylori* and *Pseudomonas fluorescens* against rapid urease test in antral biopsies by nested polymerase chain reaction targeting HSP60 gene and membrane bound protein n (%)

| Diseases | Antral biopsies | RUT, positivity | H. pylori HSP60, positivity | P value<sup>1</sup> | P. fluorescens putative outer membrane protein, positivity | P value<sup>2</sup> | Isolation of different types of bacteria, positivity | P value<sup>3</sup> |
|----------|----------------|----------------|-----------------------------|----------------|---------------------------------------------------|----------------|---------------------------------|----------------|
|          |                |                |                             |                |                                                   |                |                                  |                |
| PUD      | 65             | 51 (78.5)      | 59 (90.8)                   | < 0.001        | 63 (98.9)                                         | < 0.010        | 61 (93.8)                       | 23 (35.4)      | < 0.001 |
| NUD      | 123            | 92 (74.8)      | 109 (88.6)                  | < 0.001        | 121 (98.4)                                        | < 0.001        | 119 (96.7)                      | 39 (31.7)      | < 0.001 |
| CA       | 49             | 23 (46.9)      | 24 (48.9)                   | < 0.050        | 46 (93.9)                                         | < 0.001        | 43 (87.5)                       | 12 (24.5)      | < 0.001 |
| Normal   | 21             | 13 (61.9)      | 19 (90.4)                   | < 0.001        | 20 (95.2)                                         | 0.001          | 17 (80.9)                       | 6 (28.6)       | < 0.001 |
| Total    | 258            | 179 (69.4)     | 211 (81.8)                  | < 0.001        | 250 (96.9)                                        | < 0.001        | 240 (93.0)                      | 80 (31.0)      | < 0.001 |

<sup>1</sup>P < 0.001 between *Helicobacter pylori* (H. pylori) and nested polymerase chain reaction (PCR); <sup>2</sup>P < 0.001 between *H. pylori* and *Pseudomonas fluorescens* (P. fluorescens); <sup>3</sup>P < 0.001 between colonies appearing after overnight incubation group (group A) and colonies appearing after 3-7 d of incubation group (group B). Group A: Non-ulcer dyspepsia (NUD) vs gastric carcinoma (CA), P = 0.02; NUD vs normal, P < 0.02; H. pylori: Peptic ulcer diseases (PUD) vs CA, P < 0.001; NUD vs CA, P < 0.001; CA vs Normal, P = 0.001; Overall: Culture of P. fluorescens vs nested PCR, P < 0.001. Normal: Patients whose endoscopic findings were normal; RUT: Rapid urease test; RUT: Rapid urease test.

an opaque, small character contrary to the translucent one which is typical for *H. pylori* (Table 2).

**Biochemical characterization**

A total of 100 isolates randomly selected from group A were subjected to extensive phenotypic characterization. All of them were gram-negative, oxidase- and catalase-negative and urease-positive. All were non-fermenters, showed variable nitrate reduction and failed to utilize simple sugars (glucose, lactose, sucrose, mannitol and maltose). Citrate was utilized by all of them. All the tested strains were Methyl Red negative and Voges-Praskauer negative or equivocal. All the strains were oxidase- and catalase-positive. Indole test was negative but on mixing with the Kovac’s reagent a typical greenish color developed. All these isolates were able to multiply at 4°C. These findings intimated that those isolates which appeared after overnight incubation were *P. fluorescens*.

**Acid tolerance assay**

The bacterial count of *P. fluorescens* was 7.3 × 10⁸, 6.9 × 10⁸, 7.1 × 10⁸, 6.1 × 10⁸, 7.6 × 10⁸, 6.5 × 10⁸ and 9.8 × 10⁸ CFU/mL for 0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min after acid exposure (pH 1.0). Average bacterial counts were 9.3 × 10⁹, 9.8 × 10⁹, 1.0 × 10⁹, 9.5 × 10⁸ and 9.8 × 10⁸ CFU/mL for acid tolerance of low pH 2, pH 3, pH 4, pH 5, pH 6 and pH 7, respectively, for different time intervals (0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min). Similarly, bacterial growth was approximately the same in control experiments where acidic solution was replaced by LB Broth. The exposure to acidic pH showed that P. fluorescens growth was not killed by exposure to lower pH 1.0 for an hour.

**Amplification and RLFP of HSP60 gene**

Isolates from group B, which grew under microaerophilic environment, were subjected to amplification by primers specific for HSP60 gene of *H. pylori*. All the 80 isolates from 258 patients were positive for the 501 bp of amplicon for the corresponding gene. However, 211 (81.8%) of 258 antral biopsies were positive for *H. pylori* DNA as the 501 bp amplicon was produced by nested PCR. All

**Phylogenetic sequence analysis**

The partial nucleotide sequence of 16S rRNA of 2 isolates (GenBank accession number JQ927226 and JQ927227) from group A represented no restriction site for *Pseudomonas*.

**Identification on the basis of 16S rRNA and HSP60 gene sequence**

The partial nucleotide sequence of 16S rRNA of 2 isolates (*Pseudomonas* sp) from group A was 74% similarity with *Pseudomonas fluorescens*. Similarly, partial nucleotide sequence of unrestricted amplified HSP60 gene (590 bp) of the isolates that grew after overnight incubation aerobically also showed 96% similarity with *P. fluorescens*.
P. fluorescens

HSP60 data, h-clusters. The tree showed, on the basis of 16S rRNA sequences, it can be grouped clearly into three r-clusters on the basis of number of bootstraps i.e., 90, 78 and 60. Cluster r-1 represents a fluorescens group along with two sequences submitted to gene bank (accession number as JQ927226 and JQ927227 for RCa25 and RCa24 respectively). Cluster r-2 represents Pseudomonas aeruginosa, Pseudomonas syringae, Pseudomonas stutzeri, Pseudomonas chlororaphis, Pseudomonas putida, Pseudomonas pertucinogena group and 4 P. fluorescens reference strains, and r-3 cluster represents other microbes and enteric pathogens rather than non-Pseudomonas spp. From cluster analysis it is clear that JQ927226 and JQ927227 are related to P. fluorescens (Figure 4).

The tree shows that, on the basis of HSP60 sequences, three h-clusters could be observed (bootstraps number, 99, 63 and 71): the sequences of seven isolated strains from the gastric niche could be grouped in h-cluster I along with P. fluorescens strains while cluster II represents non-fluorescens pseudomonas species with the exception of P. fluorescens F113 (CP003150). The h-cluster III grouped non-Pseudomonas sp. including enteric pathogens along with H. pylori.

Interestingly, one strain of P. fluorescens RCa 24 fell into this III cluster with closeness to Stenotrophomonas maltophilia and Bordetella pertussis (Figure 5).

Pseudomonas fluorescens specific PCR

One specific pair of primers targeting putative outer membrane protein was used to identify P. fluorescens. The other specific primers targeting mupirocin biosynthetic gene were used to screened out whether any P. fluorescens-like isolate was producing mupirocin. Genomic DNA extracted from group-A isolates and all biopsies was used as template for PCR amplification. A total of 250 (96.9%) out of 258 biopsy specimen genomic DNA samples and 240 bacterial isolates were positive for the amplification of the corresponding gene, i.e., the putative outer membrane protein gene sequences (Table 2 and Figure 6). Although putative outer membrane protein gene targeting primers were degenerate, they were unable to produce the 613 bp amplicon from Pseudomonas aeruginosa and Pseudomonas putida at similar PCR conditions. The mupirocin biosynthetic gene targeting primers were unable to produce amplification of the 722 bp or 611 bp amplicon either in primary or secondary round PCR, respectively, from any of the isolates (data not shown).

RAPD

All the 71 strains tested from group-A yielded significant PCR products with RAPD primers. The strains generated approximately 3-13 well-defined bands between 150 bp to 2.5 kb sizes (9 bands on average) with each isolate yielding a unique profile of products. Cluster analysis with a RAPD-PCR based method showed that only a few isolates exhibited an identical profile. Nearly all the isolates appeared as dissimilar from each strain, but 5 strains isolated from cancer patients showed similar banding pattern at 0.0 coefficient (Figures 7 and 8).

DISCUSSION

Traditionally, the human stomach has been viewed as an...
unrooted tree was generated using un-agar without an antibiotic supplement. The small white *P. fluorescens* pylori and gastric carcinoma patients with the belief that only the bacteria from NUD, gastric ulcer, duodenal ulcer was the first report of its kind showing the presence of a bacteria adapted to this human niche seems quite plausible. This mechanisms by which these organisms adapt to the gastric tric helicobacters, and subsequent insight into the mecha-

Our acidic environment along with several other antimicrobial factors. With the discovery of its acidic environment because of its inhospitable environment for microorganisms because of its acidic environment along with several other antimicrobial factors. With the discovery of *H. pylori* and other gastric helicobacters, and subsequent insight into the mecha-

**Figure 4** Phylogenetic affiliation of the *Pseudomonas fluorescens*-like isolates (n = 2; JQ927226 and JQ927227). The unrooted tree was generated using un-weighted pair group method with arithmetic mean from evolutionary distance computed with bootstrap test of phylogeny using MEGA version 4 by aligning published sequences from GenBank of 16S rRNA genes from 34 reference strains representative of the principal *Pseudomonas* (P) phyta (accession number followed by species name in parentheses). For checking relatedness with other genera, we included 16S rRNA gene sequences from GenBank of 12 bacterial pathogens namely *Stenotrophomonas maltophilia*, *Burkholderia bronchiisephta*, *Wolinella succinogenes*, *Helicobacter pylori*, *Helicobacter acinonychis*, *Escherichia coli* (E. coli), Enterobacter spp., *Campylobacter jejuni*, *Burkholderia pseudomallei*, *Alviniconcha hessleri*, *Helicobacter bovis* and *Mycobacterium tuberculosis*.

Branches found by maximum likelihood are labeled with asterisks: one asterisk if bootstrap values = 90%, two asterisks if = 78% and three asterisks if = 60%.

in its native acidic environment. Colonies grew on a simple medium like Mueller-Hinton agar without an antibiotic supplement. The small white colonies had Gram-negative slightly curved rods and produced oxidase, urease and catalase enzymes. These colonies appeared after overnight incubation in an aerobic environment. These isolates exhibited growth at 4 ℃, which is one of the key characteristics of *P. fluorescens*. When these isolates were exposed to acidic pH as low as pH 1.0 for 1 h, the subsequent growth of the bacteria was not affected, indicating that they were acid tolerant. However, due to deviation from the classical *H. pylori* growth characteristics, we were prompted to consider them as non-*H. pylori* and submit them for further character-

For the purpose of this study, specific primers for amplifying *HSP60* gene-specific sequences were applied. However, an approximate 600 bp sized amplicon
Lanes 1 and 9: Molecular lates which grew after 5-7 d of incubation yielded 501 bp was excluded. On the other hand, the typical ers. Thus, the possibility of these isolates being yield a 501 bp amplicon for in these amplicons. Further, none of these isolates could was produced by the first round of PCR, but a restriction site specific for H. pylori for HindIII enzyme was absent in these amplicons. Further, none of these isolates could yield a 501 bp amplicon for H. pylori by nested PCR primers. Thus, the possibility of these isolates being H. pylori was excluded. On the other hand, the typical H. pylori isolates which grew after 5-7 d of incubation yielded 501 bp

amplicon by nested PCR protocol targeting the HSP60 gene.

Identification based on partial nucleotide sequencing of 16S rRNA of the representative isolates showed 99% sequence similarity with γ-protobacteria. On the basis of enzyme restriction analysis as well as partial nucleotide sequencing of 16S rRNA, the strains isolated from the acidic environment of the stomach either could be grouped in the genus Pseudomonas or with a closely related new genus. Further, on the basis of blasting of partial nucleotide sequences of HSP60 gene sequences on the NCBI gene data bank, isolated strains could be grouped by partial 16S rRNA sequences on the NCBI gene data bank, isolated strains could be grouped in the genus Pseudomonas or with a closely related new genus.
UPGMA phylogenetic tree, it may be concluded that the HSP60 gene sequence has better discriminatory power than 16S rRNA. Further, as one of the isolates could be grouped with *Stenotrophomonas maltophilia* and *Bordetella pertussis*, the possibility of the presence of bacteria other than *P. fluorescens* may not be denied.

The relatively conserved 16S rRNA gene of *P. fluorescens* has been targeted for PCR-based amplification in culture isolates, but with variable specificity\(^\text{[18]}\). Therefore, we decided to target a conserved putative membrane-bound protein gene of *P. fluorescens*. The putative membrane-bound protein specific primer screened in the present study was found to be specific for *P. fluorescens* and it yielded an amplicon of 613 bp, confirming the isolates as being *P. fluorescens*. Mupirocin is known for its antibacterial activity and has been reported in only one strain of *P. fluorescens* (NCIMB 10586). A mupirocin-producing gene cluster could not be traced in any of the *P. fluorescens* isolates.

---

**Figure 8** Dendrogram generated by unweighted pair-group method, arithmetic mean on basis of banding pattern of randomly amplified polymorphic DNA for *Pseudomonas fluorescens*. A: 1-35 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A (\(n = 35\)); B: 36-71 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A (\(n = 36\)).
isolate despite confirmation by 3 genes, i.e., 16S RNA, HSP60 and putative membrane bound protein genes. None of our isolates showed the presence of \textit{mapV} (mu-pirocin). It is quite possible that this gene might be either absent in all the strains or have too much polymorphism at the loci of primer annealing\cite{39}. We could isolate the \textit{P. fluorescens} strain from 93% of antral biopsies of the patients suffering from gastric diseases. These isolates were further confirmed by PCR-based amplification targeting 3 conserved genes. In DNA of antral biopsy tissue also, 98.4% of NUD and 93.9% of gastric cancer patients were found to be positive for the \textit{P. fluorescens} DNA by nested PCR, while 95.2% of normal stomach had \textit{P. fluorescens} and 96.9% of patients with PUD were found to have the bacterium. Thus our observation indicates a high prevalence and density of these non-\textit{H. pylori} bacteria in the gastric mucosa of patients. Further, to ascertain that the isolated \textit{P. fluorescens} were not contaminants, we carried out whole genome fingerprinting of the 71 randomly selected isolates by using RAPD\textsuperscript{20,21} with two RAPD primers. Cluster analysis indicated that almost all the strains had different banding patterns with only a few exceptions, confirming that the isolates were of different clones and thus ruling out the possibility of cross contamination. Moreover, from time to time, we did specific PCR amplification for the \textit{P. fluorescens} on DNA extracted from samples of tap water and washouts of the endoscope, which never yielded the required amplicon even by nested protocol for putative outer membrane protein.

It is interesting to mention that before the implication of \textit{H. pylori} in acid peptic diseases and stomach cancer, Steer and Collin-Jones\textsuperscript{2} reported that 80% of antral biopsies have \textit{Pseudomonas} spp. There are reports showing the presence of non-\textit{Helicobacter} bacteria in gastric biopsies of patients suffering from gastric atrophy\textsuperscript{22,23}. In cases of reduced gastric acidity due to antacids, it also has been reported that there is presence of several other bacteria\textsuperscript{25-28}. Two more studies based on profiling of bacterial flora by temperature gel electrophoresis, 16S rRNA sequence analysis\textsuperscript{17} and molecular analysis of the bacterial microbiota\textsuperscript{24} have shown presence of several bacterial species including \textit{Pseudomonas} in the stomach. This bacterium seems to be able to colonize the stomach due to its ability to produce urease enzyme. Similarly, it was reported that a non-\textit{H. pylori} bacterium, \textit{Ochrobactrum anthrops}, could be implicated in causation of gastritis in the Squirrel monkey\textsuperscript{26}.

Such a high prevalence of \textit{P. fluorescens} in human stomach raises many questions: Is it prevalent in the stomach of patients of other subcontinents and continents? Does it have any pathogenic role? Does it have some protective role? Is it simply a part of commensal flora of human stomach? There is a study from Venezuela\textsuperscript{25} reporting that \textit{Pseudomonas} strains may interfere with the identification of \textit{H. pylori}. They suggested that one should not rely on rapid urease, catalase and oxidase tests for identification of \textit{H. pylori}. However, studies are needed to observe and identify the presence of \textit{P. fluorescens} in other parts of the world also. With regard to its pathogenic potential, \textit{P. fluorescens} is known as an unusual pathogen of humans. It has been reported as causing septicemia in humans, especially associated with transfusion and cancer\textsuperscript{28-30}.

Moreover, there is a report indicating that \textit{P. fluorescens} encodes the Crohn’s disease-associated \textit{F} sequence and T cell super antigen, thus implicating it in the pathogenesis of Crohn’s disease\textsuperscript{31}.

However, its commensal state in the stomach may be speculated strongly due to its prevalence in stomach at such a high level and density (it could be isolated in the majority of the antral biopsy). Assumptions may be made that \textit{P. fluorescens} might be producing some antibacterial substances, such as is produced by \textit{P. aeruginosa}. \textit{P. aeruginosa} is known for producing 4-hydroxy-2-alkylquinoline which is inhibitory in vitro to \textit{H. pylori}\textsuperscript{32}. Moreover, one of the \textit{P. fluorescens} strains is already known for production of mupirocin which is very effective against meticillin-resistant \textit{Staphylococcus aureus}. This bacterium has been stated to have a probiotic role in the gills of fish\textsuperscript{33}. Furthermore, low isolation of \textit{H. pylori} and fewer incidences of acid peptic diseases including gastric cancer in North Indians may also be speculated on the basis of the probiotic activity of \textit{P. fluorescens} in the stomach. In addition, there are reports of the unique property of \textit{P. fluorescens} to inhibit the growth of other bacteria, fungi and nematodes causing plant pathology\textsuperscript{34-40}

In view of the suggestions made by previous studies\textsuperscript{41-43}, there is a strong need to explore the exact role of \textit{H. pylori} in stomach diseases because the commensal role of \textit{H. pylori} cannot be rejected outright. In a similar way, the observations made in the present study strongly indicate that further exploration of the different aspects of associations of \textit{P. fluorescens} with human disease and health should be carried out. The pathogenic potential may be explored in animal models like gerbils.

This study concludes that \textit{P. fluorescens} is as common as \textit{H. pylori} in the stomach of humans. Colonies that appeared on enriched BHI agar after 72 h of strictly microaerophilic incubation were \textit{H. pylori} while those growing faster in an aerobic atmosphere were different. These different growths could be identified as \textit{P. fluorescens}. The activity of \textit{P. fluorescens} in the stomach may be speculated to be either pathogenic or probiotic.

\textbf{COMMENTS}

\textbf{Background}

Although \textit{Helicobacter pylori} (\textit{H. pylori}) has been implicated in acid peptic diseases along with stomach cancer, there are reports indicating the presence of several other bacterial species in the stomach. Antral biopsies from North Indian subjects frequently yielded a bacterial growth on selective, non-enriched simple medium in an aerobic environment at 37 °C of small, low convex, and pinhead-size translucent colonies. Presence of these types of growth provoked questions about its characterization and its status: whether it was a contaminant from the environment during antral biopsy collection.

\textbf{Research frontiers}

The big question to be answered is further characterization of these isolates and to ensure that they are actual colonizers of the stomach. Do these acid tolerant isolates have pathogenic potential if they are real colonizers?
Innovations and breakthroughs

Pseudomonas fluorescens (P. fluorescens)-like bacteria colonize the stomach quite frequently of North Indian patients at high density, as polymerase chain reaction (PCR)-based detection and isolation rates were both comparable. In contrast, the density of H. pylori seems to be quite low as nested PCR-based detection is significantly high as compared to the isolation rate of the bacterium. P. fluorescens isolates are urease producers and acid tolerant. Although the murpinoic gene could not be detected in any of the P. fluorescens isolates, the probiotic (inhibitory to H. pylori) role of the bacterium in the stomach may be speculated.

Applications

The potential of P. fluorescens as a probiotic may be explored because despite very high prevalence of H. pylori in India the incidences of acid peptic diseases and stomach cancer are quite low.

Terminology

Nested polymerase chain reaction is a modified technique of PCR intended to increase sensitivity and specificity of primer and to reduce the contamination in amplicons due to the amplification of undesired primer annealing sites. Phylogenetics is the study of evolutionary relations among groups of organisms such as strains or species, which are based on molecular sequencing data matrices. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, e.g., lactic acid bacteria.

Peer review

The study is well carried out from the methodological perspective. This manuscript characterized the bacterial isolates growing aerobically from antral biopsies were P. fluorescens, which was the acid tolerant bacteria other than H. pylori. On the basis of 16S rRNA and HSP60 sequence and from phylogenetic sequence analysis these organisms were closely related to P. fluorescens. The authors also confirmed this unknown bacterium as P. fluorescens by PCR positivity of P. fluorescens specific conserved putative outer membrane protein gene. Finally, the authors concluded that P. fluorescens is as common as H. pylori in the human stomach.

REFERENCES

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984; 1: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]
2. Cover TL, Blaser MJ. Helicobacter pylori and gastrroduodenal disease. Annu Rev Med 1992; 43: 135-145 [PMID: 19080578 DOI: 10.1146/annurev.me.43.021592.001311]
3. McGowan CC, Cover TL, Blaser MJ. Helicobacter pylori and gastric acid: biological and therapeutic implications. Gastroenterology 1996; 110: 926-938 [PMID: 8608904 DOI: 10.1053/gast.1996.v110.58608904]
4. Steer HW, Colin-Jones DG. Mucosal changes in gastric ulceration and their response to carbenoxolone sodium. Gut 1975; 16: 590-597 [PMID: 810934 DOI: 10.1136/gut.16.8.590]
5. Dominguez-Bello MG, Reyes N, Teppa-Garrán A, Romero R. Interference of Pseudomonas strains in the identification of Helicobacter pylori. J Clin Microbiol 2000; 38: 937 [PMID: 10722321]
6. Khanolkar-Gaitonde SS, Reubish GK, Lee CK, Stadtländer CT. Isolation of bacteria other than Helicobacter pylori from stomachs of squirrel monkeys (Saimiri spp.) with gastritis. Dig Dis Sci 2000; 45: 272-280 [PMID: 10711437]
7. Monstein HJ, Tiveljung A, Kraft CH, Borch K, Jonasson J. Profiling of bacterial flora in gastric biopsies from patients with Helicobacter pylori-associated gastritis and histologically normal controls by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. J Med Microbiol 2000; 49: 817-822 [PMID: 10966230]
8. Blazevic DJ, Koeppke MH, Matson JM. Incidence and identification of Pseudomonas fluorescens and Pseudomonas putida in the clinical laboratory. Appl Microbiol 1973; 25: 107-110 [PMID: 4631431]
9. Gilligan PH. Pseudomonas and Burkholderia. 6th ed. In: Murray PR, Baron EJ, Pfaffer MA, Tenover FC, Yolken RH. Manual of clinical microbiology. Washington: ASM Press, 1995: 509-519
10. Ho SA, Hoyle JA, Lewis FA, Secker AD, Cross D, Mapstone NP, Dixon MF, Wyatt JI, Tompkins DS, Taylor GR. Direct polymerase chain reaction test for detection of Helicobacter pylori in humans and animals. J Clin Microbiol 1991; 29: 2543-2549 [PMID: 1720372]
11. Singh V, Mishra S, Rao GR, Jain AK, Dixit VK, Gulati AK, Mahajan D, McClelland M, Nath G. Evaluation of nested PCR in detection of Helicobacter pylori targeting a highly conserved gene: HSP60. Helicobacter 2003; 8: 30-34 [PMID: 18205663 DOI: 10.1111/j.1525-373X.2008.00573.x]
12. Carroll NM, Jaeger EE, Choudhury S, Dunlop AA, Matheson MM, Adamson P, Okhravi N, Lightman S. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using nested PCR. J Clin Microbiol 2000; 38: 1753-1757 [PMID: 10790093]
13. Thompson JD, Gibson TJ, Plewniak F, Jeannougin F, Higgin DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-4882 [PMID: 9396791 DOI: 10.1093/nar/25.24.4876]
14. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596-1599 [PMID: 17488738 DOI: 10.1093/molbev/msm092]
15. Grahn N, Olofsson M, Ellnebo-Svedlund K, Monstein HJ, Jonasson J. Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by pyrosequencing of cloned amplifies. Femsi Molec Micr Biotech 2003; 21: 87-91 [PMID: 12594028 DOI: 10.1093/femsmb/21.2.87]
16. Romesbrug HC. Cluster Analysis for Researchers. Belmont (CA): Lifetime Learning Publications, 1984
17. Merrell DS, Goodrich ML, Otto G, Tompkins LS, Falkow S. pH-regulated gene expression of the gastric pathogen Helicobacter pylori. Infect Immun 2003; 71: 3529-3539 [PMID: 12761138 DOI: 10.1128/IAI.71.3529-3539.2003]
18. Kumar NR, Thirumalai V Arasu, Gunasekaran P. Genotyping of antifungal compounds producing plant growth promoting rhizobacteria, Pseudomonas fluorescens. Curr Sci 2002; 82: 1463-1468
19. Fuller AT, Mellows G, Woolford M, Banks GT, Barrow KD, Chain EB. Pseudomonic acid: an antibiotic produced by Pseudomonas fluorescens. Nature 1971; 234: 416-417 [PMID: 503547]
20. Charan AR, Reddy VP, Reddy PN, Reddy SS. Assessment of genetic diversity in Pseudomonas fluorescens using PCR-based methods. Biores Bioiol Bioassay 2011; 5: 10-16
21. Manceau C, Horvais A. Assessment of genetic diversity among strains of Pseudomonas syringae by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on P. syringae pv. tomato. Appl Envir Microbiol 1997; 63: 498-505 [PMID: 9023928]
22. Dobby JM, Webster AD, Borriello SP, Barclay FE, Bartholomew BA, Hill MJ. Bacterial colonization and nitrite concentration in the achlorhydric stomachs of patients with primary hypogammaglobulinaemia or classical pernicious anaemia. Scand J Gastroenterol 1984; 19: 105-110 [PMID: 6710072]
23. Jonkers D, van den Broek E, van Dooren I, Thijis C, Dorant E, Hageman G, Stobberingh E. Antibacterial effect of garlic and omeprazole on Helicobacter pylori. J Antimicrob Chemother 1999; 43: 837-839 [PMID: 10404325 DOI: 10.1093/jac/43.6.837]
24. Mauritz W, Graninger W, Schindler I, Karner J, Zadrobilek E, Sporn P. Pathogenic flora in the gastric juice and bronchial secretion of long-term ventilated intensive-care patients. Anaesthesist 1985; 34: 203-207 [PMID: 4003748]
25. Howden CW, Hunt RH. Relationship between gastric secretion and infection. Gastroenterology 1987; 92: 96-107 [PMID: 3564004]
26. Houben GM, Hooi J, Hameeteman W, Stockbregger RW.

Patel et al. *Pseudomonas fluorescens* from antral biopsies
Twenty-four-hour intragastric acidity: 300 mg ranitidine b.d., 20 mg omeprazole o.m., 40 mg omeprazole o.m. vs. placebo. Aliment Pharmacol Ther 1995; 9: 649-654 [PMID: 8824652 DOI: 10.1111/j.1365-2036.1995.tb00434.x]

27 Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, Perez-Perez G, Blaser MJ, Relman DA. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci USA 2006; 103: 732-737 [PMID: 16407106 DOI: 10.1073/pnas.0506651010]

28 Murray AE, Bartzokas CA, Shepherd AJ, Roberts FM. Blood transfusion-associated Pseudomonas fluorescens septicaemia: is this an increasing problem? J Hosp Infect 1987; 9: 243-248 [PMID: 2886528 DOI: 10.1016/0195-6701(87)90120-4]

29 Hsueh PR, Teng LJ, Pan HJ, Chen YC, Sun CC, Ho SW, Luh KT. Outbreak of Pseudomonas fluorescens bacteremia among oncology patients. J Clin Microbiol 1998; 36: 2914-2917 [PMID: 9738043]

30 Gershman MD, Kennedy DJ, Noble-Wang J, Kim C, Gulion J, Kacica M, Jensen B, Pascoe N, Saiman L, McHale J, Blaser MJ. Role of fluorescent siderophore production in biological control of Pythium ultimum by a Pseudomonas fluorescens strain. Phytopathology 1996. United Kingdom: Chapman and Hall, Ltd.: 187-236 [DOI: 10.1007/978-1-4613-1213-0_6]

31 Wei B, Huang T, Dalwadi H, Sutton CL, Bruckner D, Braun J. Pseudomonas fluorescens encodes the Crohn's disease-associated I2 sequence and T-cell superantigen. J Infect Dis 2008; 197: 1372-1379 [PMID: 18397575 DOI: 10.1086/592968]

32 Lacey SL, Mehmet S, Taylor GW. Inhibition of Helicobacter pylori growth by 4-hydroxy-2-alkyl-quinolines produced by Pseudomonas aeruginosa. J Antimicrob Chemother 2002; 50: 656-661 [PMID: 12060405 DOI: 10.1093/jac/50.3.656]

33 Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF. Inhibition of vibrio anguillarum by Pseudomonas fluorescens AH2, a possible probiotic treatment of fish. Appl Environ Microbiol 1999; 65: 969-973 [PMID: 10049849]

34 Haas D, Défago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 2005; 3: 307-319 [PMID: 15759041 DOI: 10.1038/nrmicro1129]

35 Haas D, Keel C. Regulation of antibiotic production in root-colonizing Pseudomonas spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 2003; 41: 117-153 [PMID: 12723089 DOI: 10.1146/annurev.phyto.41.052002.095656]

36 Sharifi-Tehrani A, Zala M, Natsch A, Loccoz YM, Defago G. Biocontrol of soil-borne fungal plant diseases by 2,4-di-acetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. Er J Plant Pathol 1998; 104: 631-643

37 Thomasow LS, Weller D. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Stacey G, Keen NT, editors. Plant-microbe interactions 1996. United Kingdom: Chapman and Hall, Ltd.: 187-236 [DOI: 10.1007/978-1-4613-1213-0_6]

38 Toyoda H, Katsuragi K, Tamari T and Ouchi S. DNA sequence of genes for detoxification of fusaric acid a wilt-inducing agent produced by Fusarium spp. Journal of Phytopathology 1991; 133: 265-277 [DOI: 10.1111/j.1439-0434.1991.tb00126.x]

39 Loper JE. Role of fluorescent siderophore production in biological control of Pythium ultimum by a Pseudomonas fluorescens strain. Phytopathology 1988; 78: 166-172 [DOI: 10.1094/Phyto-78-166]

40 Neillands JB, Leong SA. Siderophores in relation to plant growth and disease. Ann Rev Plant Physiol 1986; 37: 187-202 [DOI: 10.1146/annurev.pp.37.060186.001155]

41 Blaser MJ. Not all Helicobacter pylori strains are created equal: should all be eliminated? Lancet 1997; 349: 1020-1022 [PMID: 910641 DOI: 10.1016/S0140-6736(96)90133-7]

42 Blaser MJ. Helicobacters are indigenous to the human stomach: duodenal ulceration is due to changes in gastric microecology in the modern era. Gut 1998; 43: 721-727 [PMID: 9824358 DOI: 10.1136/gut.43.5.721]

43 Blaser MJ. Hypothesis: the changing relationships of Helicobacter pylori and humans: implications for health and disease. J Infect Dis 1999; 179: 1523-1530 [PMID: 10228075 DOI: 10.1086/314785]

P-Reviewers Slomiany BL, Wang JT
S-Editor Gou SX L-Editor Logan S E-Editor Lu YJ