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Full Length Research Paper

Sequencing of 16S rRNA gene for identification of *Staphylococcus* species in water sample

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A bacterial strain Bz19 was isolated from a water sample collected from river Gomati at the Indian city of Lucknow. We characterized the strain using 16S rRNA gene sequence. Phylogenetic analysis showed that the strain formed a monophyletic clade with members of the genus *Staphylococcus*. The closest phylogenetic relative was *Staphylococcus arlettae* with 99% 16S rRNA gene sequence similarity. It is proposed that the identified strain Bz19 be assigned as the type strain of a species of the genus *Staphylococcus* (*Staphylococcus* sp. Bz19) based on phylogenetic tree analysis together with the 16S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession number HM488958.

Key words: 16S ribosomal RNA gene, *Staphylococcus*, polymerase chain reaction (PCR), phylogenetic analysis, DNA isolation, DNA sequencing.

INTRODUCTION

Accurate and definitive bacterial identification is essential for correct disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with bacterial infections. Characterization of bacterial species using classical methods is not as specific as the genotyping methods. Genotypic techniques involve the amplification of a phylogenetically informative target, such as the small-subunit (16S) rRNA gene (Woese and Fox, 1977). rRNA is essential for the survival of all cells, and the genes encoding the rRNA are highly conserved in the bacteria and other kingdoms. The sequences of the rRNA and proteins comprising the ribosome are highly conserved throughout evolution, because they require complex inter- and intramolecular interactions to maintain the protein-synthesizing machinery (Sacchi et al., 2002; Hillis et al., 1991; Woese 1987).

There are over thirty different species of *Staphylococcus* bacteria that can cause infections ranging from mild to life threatening. Most *Staphylococcus* infections are caused by a genus known as *Staphylococcus aureus*. *Staphylococcus* cause illness directly by infection, or indirectly through products they make, such as the toxins responsible for food poisoning and toxic shock syndrome and cause thousands of deaths every year. The exfoliative (epidermolytic) toxins of *S. aureus* are the causative agents of the staphylococcal scalded-skin syndrome (SSSS), a blistering skin disorder that predominantly affects children (Ladhani et al., 1999). In the generalized forms of SSSS, widespread involvement of the entire skin surface can occur but the mucous membranes are usually spared (Melish et al., 1974; Pollack, 1996). The disease usually follows a localized infection of the upper respiratory tract, inner ear, conjunctiva, or umbilical stump (Bailey et al., 1995), although rare cases of SSSS caused by staphylococci isolated from patients with pneumonia, septic arthritis (Melish, 1982), pyomyositis (Wong et al., 1993), and maternal breast abscesses (Raymond et al., 1997) have been reported.

In this study, we use the method of isolation and identification of an unknown bacterium from the Indian River Gomati using 16S rRNA gene sequence as describe in previously published work (Srivastava et al.,

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2008; Yadav et al., 2009) to characterize the strain Bz19 as a member of the *Staphylococcus*. The Gomati River has received great attention from the public, due to its potential for biodiversity and biological conservation. We initiated a systematic screening programme to catalogue the microbial composition of Gomati River water at the Indian city Lucknow.

MATERIALS AND METHODS

Culturing of bacteria

Water sample collected from the Gomati River was serially diluted and spread onto peptone/Beef extract/Nacl/Agar- Agar plates followed by for incubation at 30°C under anaerobic con ditions. Single colonies of bacterial strains were picked and further grown and sub-cultured several times to obtain a pure culture.

DNA isolation of bacteria

Pure culture of the target bacteria was grown overnight in liquid NB medium for the isolation of genomic DNA using a method described by Hiney et al. (1992).

Polymerase chain reaction (PCR) amplification 16S rDNA gene

PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CTTGTGCGGGCCCCCGTCAATTC-3') were used for the amplification of the 16S rDNA gene fragment. The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of 10 × PCR amplification buffer (100 mM Tris-HCl, , 500 mM KCl pH-8.3) , 200 µM dNTP, 10 p moles each of the two universal primers and 1.5 mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing temperature o f primers was 55°C for 30 s and extension at 72°C for 1 min. T he final extension was conducted at 72°C for 10 min.

Agarose gel electrophoresis

Ten microlitre of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator.

Purification of PCR product

The PCR product was purified by Qiagen gel extraction kit using the following protocol described below. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. Then the gel slice was weighed in an eppendorf. We then added 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 µl). The mixture was then incubated at 50°C for 10 min. The gel was dissolved by vortexing the tube every 2 to 3 min during the incubation until the mixture color is uniformly yellow. We then added 1 gel volume of Iso-propanol to the sample and mixed. A QIAquick spin column is then placed in a 2 ml collection tube provided. The sample is applied to the QIAquick column followed by centrifugation for one minute so that DNA binds to the column. The flow-through is discarded and the QIAquick column is placed back in the collection tube. We then added 0.75 ml of buffer PE to QIAquick column and centrifuged for 1 min to wash. The flow through is again discarded and the QIAquick column centrifuged for an additional 1 min at 10,000 × g. The QIAquick column is now placed into a clean 1.5 ml eppendorf. We then added 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 min to elute DNA.

DNA sequencing of the 16S rDNA fragment

The 16S rDNA amplified PCR product (100 ng concentration) was used for the sequencing with the single 16S rDNA 27F Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' by ABI DNA Sequencer (Applied Biosystem Inc).

Sequence analysis

A comparison of the 16S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) as a database was done using BLAST (Zhang et al., 2000). A number of sequence of *Staphylococcus* were selected on the basis of similarity score (<99% and ≥ 95%) of the determined sequence with a reference sequence. Multiple sequence alignment of these selected homologous sequences and 16S rRNA gene sequence of test strain was performed using ClustalW (Thompson et al., 1994). Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics analysis) version 4.0 (Tamura et al., 2007). We again compared the 16S rRNA gene sequence of test strain with different set of sequence databases such as small subunit ribosomal RNA (ssu rRNA) and large subunit ribosomal RNA (lsu rRNA) using Ribosomal RNA BLAST (Altschul et al., 1997). 16S rRNA gene sequence of test strain is also compared against those sequences in Ribosomal Database Project (Cole et al., 2009) by using the RDP Classifier check Program (Wang et al., 2007). The annotated information for the sequence in the database to which 16S rRNA aligns is used for the bacterial identification.

RESULTS AND DISCUSSION

Rapid identification of microorganisms is necessary in the clinical laboratory to take decision for installment of antibiotic therapy. The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains. The genomic DNA was extracted from isolated bacterial strain Bz19 and universal primers 27F and 939R were used for the amplification and sequencing of the 16S rRNA gene fragment. A total of 849 bp of the 16S rRNA gene was sequenced and used for the identification of isolated bacterial strain. Subsequently, a 16S rRNA gene sequence based phylogenetic tree showing the relationships between the test strain Bz19 and selected representatives of the genus *Staphylococcus* is given in Figure 1. It is evident from phylogenetic analysis of 16S rRNA gene that the isolate Bz19 represents a genomic species in the genus *Staphylococcus.* Comparison of test

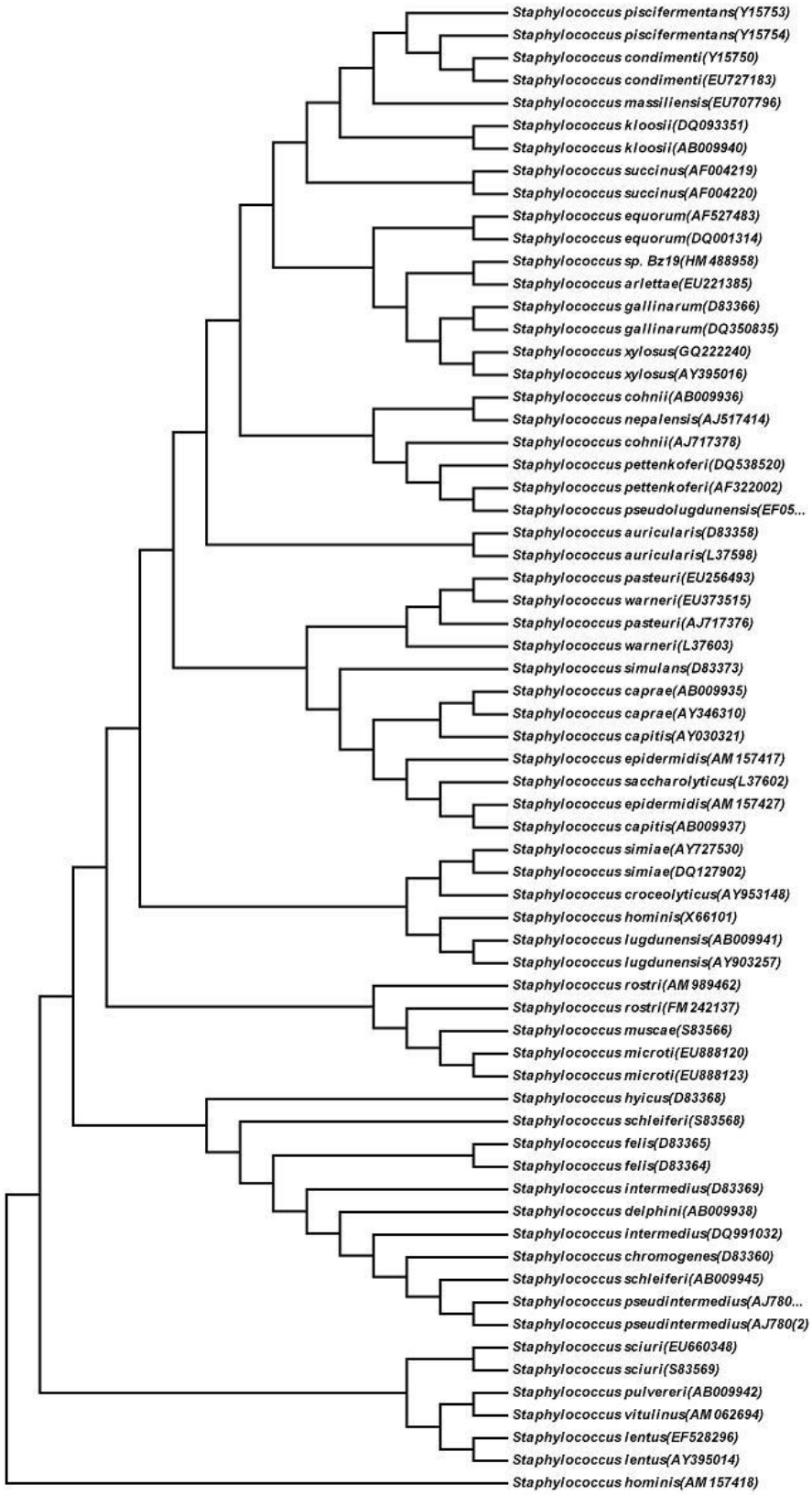

Figure 1. Neighbour joining tree of selected 16S rRNA gene sequences of the genus *Staphylococcus* obtained from BLAST search of the Bz19 strain sequence for phylogenetic inference.

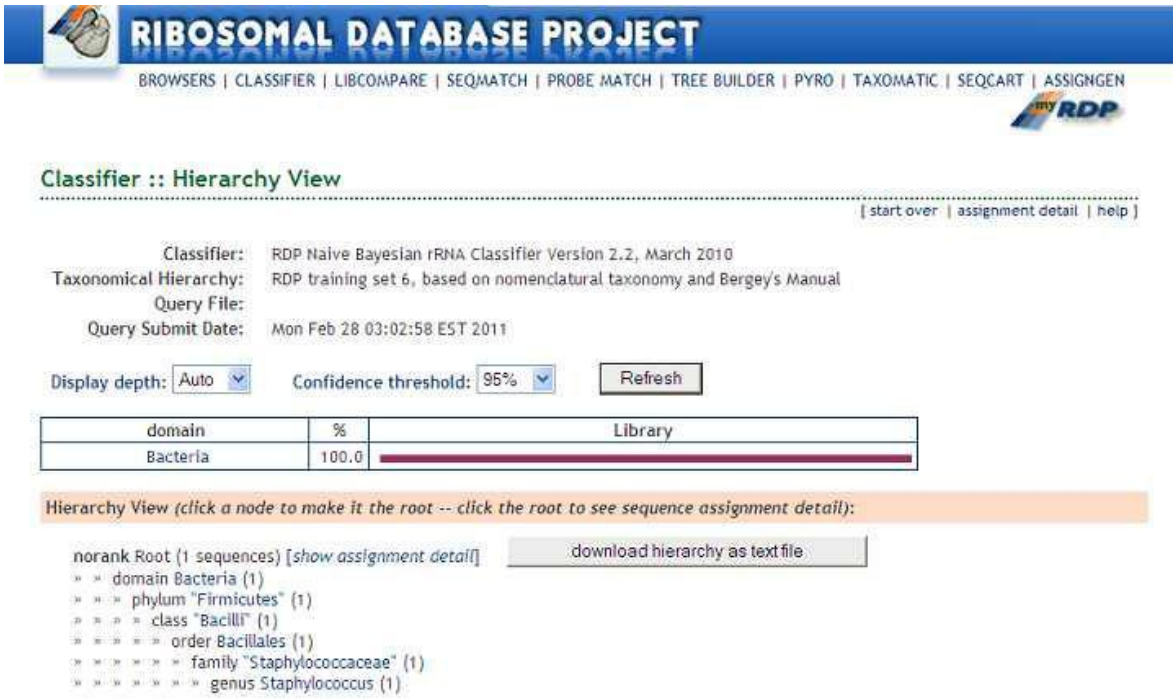

Figure 2. Result of RDP Classifier to assign 16S rRNA gene sequence of isolate Bz19 to the new phylogenetically consistent higher-order bacterial taxonomy.

strain against known sequences of ssu rRNA and lsu rRNA databases showed that the gene sequence of isolate Bz19 has 99% sequence similarity (Score=1608 bits, Expect=0.0) with 16S rRNA gene sequence of *Staphylococcus arlettae* (Genbank Acc. No.: EU221385). Thus, data shows that the isolate Bz19 is a member of the genus *Staphylococcus*. Similarity rank program classifier (Wang et al., 2007) available at the Ribosomal Database Project (Cole et al., 2009) classified the isolate Bz19 as a novel genomic species of the genus *Staphylococcus* with a confidence threshold of 95% (Figure 2). The 16S rRNA gene sequence of isolate Bz19 was deposited in GenBank with the accession number HM488958.

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