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CHARACTERIZATION OF *SERRATIA FONTICOLA*, AN OPPORTUNISTIC PATHOGEN ISOLATED FROM DRINKING WATER

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Abstract – We characterized the ST2 strain of *Serratia fonticola* isolated from drinking water of a capping spring on Mt. Vlasina. The ST2 strain isolated from bottled water showed the characteristics of Enterobacteriaceae family but not of the *Serratia* genus. *S. fonticola* belongs to a group of opportunistic pathogens and can cause illness in people with weak or damaged immune systems. A biochemical characterization of the strain was made by using the identification system API (bioMèrieux®). Molecular characterization was done by PCR amplification of 16S rDNA gene using the thermal cycling sequencing method and by sequencing. By comparing the obtained 1016 nucleotide sequence with the NCBI collection of all deposited sequences for 16S rDNK, and by using the BLAST search service, the highest identity (98% uniformity) was obtained with the *S. fonticola* strain, designated as LMG 7882 (gi|15054669|gb|AF286869.1). The identity of 16S rDNA between the referent strain and ST2 is not absolute, indicating an autochthonous origin of strain ST2.

Key words: Serratia fonticola, *Citrobacter*-like, 16S rRNA gene, drinking water, Serbia.

INTRODUCTION

In this paper, we monitored the isolate *Serratia fonticola* ST2 that showed characteristics that classified it into the family Enterobacteriaceae, but it does not conform to the present definition of the genus *Serratia*. Water springs, which are used for the bottling of analyzed water, are located on the mountain, Vlasina, southeast Serbia, at an altitude of about 1500 m at 42^0 38' 14" north latitude and 22^0 16' 35" east longitude.

Serratia species have been isolated from water, soil, animals (including man), and from the surfaces of plants (Grimont et al., 1992).

In 1965, Leclerc and Buttiaux described a new group of Enterobacteriaceae which seemed to be related to the genus *Citrobacter* on the basis of its IMViC characteristics (indole positive, methyl red positive, Voges-Proskauer positive, Simmons citrate positive) but which differed from *Citrobacter* in other significant properties such as lysine decarboxylase production (Leclerc et al., 1965).

A group of strains called "*Citrobacter* lysine$^+$" was found to be related significantly to the genus *Serratia* in DNA/DNA hybridization studies (Crosa et al., 1974). In (DNA)-DNA, the hybridization study established that this group of "*Citrobacter*-like" bacteria, referred to as class C, are more closely related

to *Serratia* than to *Citrobacter* or any other Enterobacteriaceae (Steigerwalt et al., 1976).

In 1979, *Serratia fonticola* was described as a new species of *Serratia*. The strains were isolated from fresh water and soil (Gavini et al., 1979). This genospecies has recently been named *Serratia fonticola*. However, the problem is that *S. fonticola* does not have the key characteristics of the genus *Serratia.* They conform to the definition of the family Enterobacteriaceae, but not to the present definition of the genus *Serratia – Species Incertae Sedis* (Grimont et al., 1984).

In 1985, Farmer et al. reported the isolation of *S. fonticola* as a contaminant of wounds and from the respiratory tract (Farmer et al., 1985). In addition, Muller et al. isolated 18 strains of *S. fonticola* from fecal specimens of 90 wild European birds (Muller et al., 1986). *S. fonticola* was also isolated from leg abscess in a patient following an accident (Bollet et al., 1991).

Because of the evidence that some species of the genus *Serratia* have medical significance as opportunistic pathogens, we conducted a biochemical and molecular characterization of the strain ST2.

MATERIALS AND METHODS

Isolation of pure cultures

The isolation of the strain *Serratia fonticola* ST2 was performed by using the membrane filtration technique (MF): 100 ml of water was filtered through a membrane filter disk of 0.2 μm (manufacturer PALL-Gelman). After filtration, the filter disk was transferred to Endo Agar, Biolife S.r.l. for 48 h at 37^{0}C. Pure cultures were obtained by multiple subsequent dilution using inoculation loop streaking on nutrient agar.

The preliminary identification of the isolated strains was done manually using the commercial identification system API 20E – bioMérieux (Smith et al., 1972). Assessment of the identification was obtained by database software identification (Boeufgras et al., 1987).

DNA extraction

For the isolation of bacterial DNA, overnight cultures were used. After centrifugation, the supernatant was discarded and the bacterial pellet was resuspended in 400 μl of Solution E1 (50 mM Tris i 10 mM EDTA) adjusted to pH = 8 (Better et al., 1983). Nucleic acids were released from the cells using 250 μl of 2% aqueous solution of N-lauryl sarcosine. Then 200 μl of pronase solution (final concentration 5 mg/ml) was added, mixed well and incubated for 30 min at 37^{0}C. After that, 150 μl of neutral phenol was added, mixed by vigorous vortexing and centrifuged at maximal speed. This step was repeated until a clean supernatant was obtained. Precipitation of DNA was done by adding 1/10 of sodium acetate 3M and 0.6 of isopropanol in the aqueous phase. After centrifugation, the pellet of DNA was washed with 500 μl of 75% ethanol and dried in vacuum evaporators. The dried pellet was resuspended in 50 μl of water containing RNA-se A and incubated for 15 min at 37^{0}C. Isolated DNA was stored at -20^{0}C prior to use.

The quantity and quality of the isolated DNA was checked by agarose gel electrophoresis (Agarose Low EEO) in a 1x TAE buffer at a constant voltage of 5 V/cm. For the comparison of DNA fragment size and intensity, the standard DNA Ladder Mix (Fermentas) was used. The visualization of DNA was performed by adding ethidium bromide (0.5 μg /ml) into the gel and exposure to a UV illuminator.

Amplification of 16S rDNA by PCR

For the amplification of 16S rDNA the Hot Master Taq polymerase was used, and it is most effective at 68^{0}C. The 16S rDNA gene fragments were amplified by PCR using the primers BAC16S1 (5'-GTT TGA TCC TGG CTC AG-3') and BAC16S2 (5'-GAC GGG CGG TGT GTA CAA-3').

The amplification of 16S rDNA was done by touchdown PCR. The following thermocycling pro-

gram was used: 3 min initial denaturation at 95^{0}C; amplification was done in two phases: first, a PCR program consisting of 15 subsequent cycles of 40 s denaturation at 95^{0}C, 1 min annealing at 60^{0}C; 2.5 min extension at 68^{0}C; second the PCR consisting of 10 cycles of 40 s denaturation at 95^{0}C, 1 min annealing at 45^{0}C; 2.5 min extension at 68^{0}C; and a final extension step of 10 min at 68^{0}C.

DNA sequencing

The sequencing of bacterial DNA was performed by the thermal cycling sequencing method. The termination method involves a random incorporation of 2', 3' dideoxy-nucleotide triphosphate (ddNTP) enduring the *in vitro* synthesis of DNA.

Prior to sequencing, amplified DNA was first purified using the QIAquick PCR Purification Kit (QIAGEN-GmbH). After that, the primer BAC16S was added to the purified DNA and the DNA mix was dried. The sequencing of the samples was done by the CRIBI Sequencing Service, at the University of Padua, Italy.

Database searches for similar sequences were performed using the BLAST program at the National Center for Biotechnology Information (Altschul et al., 1997).

Nucleotide sequence accession number

The nucleotide sequences for 16S rDNA of *Serratia fonticola* ST2 was submitted to EMBL GenBank under the accession number FR832379 (Tasić et al., 2009).

RESULTS

The spring water from which *Serratia fonticola* ST2 was isolated, is characterized by a uniform flow (0.3 l/s), low mineralization (< 50 mg/l), pH=6.4 and low temperature, 5.8^{0}C (Tasić et al., 2012). According to the chemical characteristics, the water belongs to the class of hydrocarbonic waters with complex cationic composition. Tests for total α and β activities and gamaspectroscometric analysis showed that the radioactivity of the water was within permissible limits (Tasić, 2007).

The growth of the strain ST2 occured between 4^{0}C and 37^{0}C. After 24 h, the strain produced smooth colonies on Endo agar. No growth was observed at 41^{0}C. Motility of bacterial cells was detected at 37^{0}C.

The strain had the biochemical profile number 5304753 when tested by the API 20E system and profile number 16474557323 when tested by the API ID32 E (bioMérieux® sa, France), and it was classified as *S. fonticola* by the database APILAB Plus V 3.3.3, bioMérieux. Multiple biochemical tests were subsequently performed to confirm the identification of the organism. Strain ST2 was metabolically very active (Tasić et al., 2008).

Positive biochemical tests of the isolated strain ST2 were obtained for D-dulcitol, *β*-galactosidase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, methyl red test (37^{0}C), glucose, mannitol, inositol, sorbitol, rhamnose, melibiose, amygdaline, arabinose, galacturonate, phenol red, *β*-glucosidase, maltose, adonitol, palatinose, malonate, arabitol, *α*-galactosidase, trehalose and L-aspartic acid arylamidase.

Negative tests were obtained for Voges-Proskauer (VP), arginine dihydrolase, H_2S production, urease, tryptophan deaminase, indole production, acetoin production, gelatinase, sucrose, L-fucose, sorbose, inulin, melezitose, cytochrome oxidase, 5-ketogluconate, lipase, *β*-glucuronidase, N-acetyl-*β*-glucosaminidase, *α*-glucosidase, cellobiose and *α*-maltosidase.

The chromosomal DNA of strain ST2 was isolated and used for PCR amplification. The quality and quantity of the isolated chromosomal DNA was analyzed on agarose gel, and it was found to be very good.

Using universal primers for bacterial 16S rDNA and chromosomal DNA of strain ST2 in the PCR re-

action, the DNA molecule of expected size of about 1300 bp was obtained. Electrophoresis of the amplified DNA on 1% agarose gel showed that the PCR reaction was higly specific (without nonspecific products) (Fig. 1).

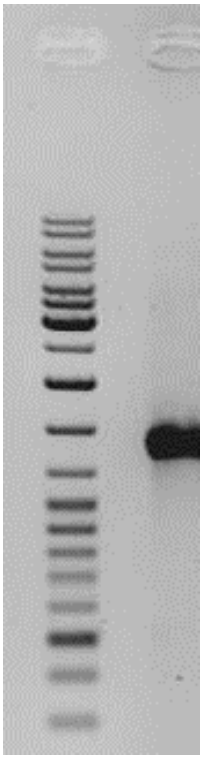

Fig. 1. Agarose gel electrophoresis of amplified DNA of 16S rDNA of the *Serratia fonticola* ST2 on 1% agarose gel. (1. marker, 2. amplified 16S rDNA for ST2); DNA marker contains bands (from bottom to the top) of 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1031 bp, 1200 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 5000 bp, 6000 bp, 8000 bp and 10000 bp.

Using one sequencing reaction, a sequence of 1016 nucleotides was obtained (Fig. 2).

DISCUSSION

Because the results obtained in the biochemical determination of isolate ST2 were ambiguous, we decided to perform a molecular characterization. The method used here was the sequencing of the gene for 16S rRNA.

The sequence of 16S rDNA of isolate ST2 from oligomineral bottled drinking water of 1016 nucleotides was compared with sequences in the NCBI gene bank data for 16S rRNA, and the greatest similarity (98% identity) was that of strain ST2 with the strain *Serratia fonticola*, designated as *LMG 7882* (gi|15054669|gb|AF286869.1). Having in mind that the identity of 16S rRNA genes between these two strains was not 100%, it could be postulated that strain ST2 is an autochthonous isolate.

From the results of DNA alignments of the 16S rDNA sequence of strain ST2 and the genes for 16S rDNA deposited in all DNA databases, it was possible to conclude that the isolate from the bottled oligomineral drinking water is *Serratia fonticola* ST2.

In this case, the molecular method of identification was very successful. Which of the applied methods for identification will give more successful results of identification depends on the isolated bacteria. We propose to use both biochemical and molecular genetics methods.

Bearing in mind the health aspect of drinking water, it is essential to understand the accurate differentiation between *Serratia fonticola*, *Serratia liquefaciens* and *Serratia marcescens*. In contrast to the rest of the pathogenic species of the genus *Serratia* for which the Voges-Proskauer test is usually positive, this test is negative for *S. fonticola*. The positive D-dulcitol test for *S. fonticola* is very important because it differentiates this species from the following biochemically similar pathogenic taxons: *Serratia* species, *Enterobacter aerogenes*, *E. agglomerans, E. cloaceae, Klebsiella pneumoniae* and the pectinolytic *Erwinia sp.*

In 1979, *Serratia fonticola* was described as a new species of *Serratia*. The strains of this species were isolated from fresh water and soil. Strain ST2 was isolated from oligomineral bottled water. The strain showed the ability to survive in specific conditions that characterize this bottled water (especially the very low mineralization <50 mg/L, which is an unfavorable medium for most bacterial species).

GCTAGAGCGTTGTAGTCGTGCAGATGTCCCGCGCAAGCGGTGAGAGAAAGCCATTGACGC
TCTGGACGTTGGCACACCGCCCGTCAGAGGGGGATAACTACTGGAAACGGTAGCTAATAC
CGCATAACGTCTTCGGACCAAAGTGGGGGACCTTCGGGCCTCACACCATCGGATGTGCCC
AGATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTC
TGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAA
GGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGT
TCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTA
AGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTT
GTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGG
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAGCGGTGGAGCATGTGGTT
TAATTCGATGCAACGCGAAGAACCGTAACCTACTCTTGACTCCACAGAACTTTCCACAGA
TGGATGGTGCCGTCCTGGAACTGTCGANACAGGTGCTGCATGCTGTCGTCAGCTCC

Fig. 2. Sequence of the 16S rRNA gene of the *Serratia fonticola* ST2 strain

Strain ST2 grew on different media for *Enterobacteriaceae* between 4^{0}C and 37^{0}C. No growth at 41^{0}C was observed, which is a typical characteristic of the genus *Serratia*.

Because of the uncertain systematic status of strain ST2 obtained by biochemical tests, the molecular characterization of this strain was performed. Using the sequencing method of 16S rDNA, amplified by PCR, strain ST2 was identified as *Serratia fonticola*. The comparison with the NCBI collection of deposited sequences identified the greatest similarity (98% of identity) with the strain *Serratia fonticola* marked as LMG 7882 (gi|15054669|gb|AF286869.1). The obtained homology clearly determined strain ST2 as *Serratia fonticola,* but as the identity was not a 100% match, it could mean that it is an indigenous strain.

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