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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *CHRYSEOBACTERIUM VRYSTAATENSE* ST1 ISOLATED FROM OLIGOMINERAL WATER OF SOUTHEAST SERBIA

S. TASIĆ[1], M. KOJIĆ[2], S. STANKOVIĆ[3] and D. OBRADOVIĆ[4]

[1] *Department of Food Technology, Higher School of Applied Professional Studies,* 17500 Vranje, Serbia
[2] *University of Belgrade, Institute of Molecular Genetics and Genetic Engineering,* 11010 Belgrade, Serbia
[3]*Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia
[4]*Institute of Food Technology and Biotechnology, Faculty of Agriculture, University of Belgrade,* 11080 Belgrade, Serbia

Abstract – The isolation and molecular characterization of bacterial strains isolated from water sources in the Vlasina Mountain in southeast Serbia, confirmed the presence of a new species *Chryseobacterium vrystaatense* ST1. This Gram-negative species showed an extremely low level of biochemical reactivity in biochemical tests. The gene for 16S rRNA was amplified by PCR using universal primers and sequenced. Comparison of 16S rRNA gene sequence and phenotypic features indicated that the isolate ST belonged to *Chryseobacterium vrystaatense.* A BLAST search of sequenced 1088 nucleotides of the 16S rRNA gene with all sequences deposited in the NCBI collection showed the highest similarity (98%) with the strain *Chryseobacterium vrystaatense* sp. nov., designated as strain R-23533. The very high homology of these two strains allowed classification of our strain at the species level, but some differences indicate, and indirectly confirm, that the isolate ST is an authentic representative. On the basis of these results, we could conclude that *Chryseobacterium vrystaatense* ST was for first time isolated in Serbia, which is particularly important when one bears in mind that there are only three sequences of this species deposited in the NCBI collection.

Key words: Chryseobacterium vrystaatense, 16S rRNA gene, oligomineral water

INTRODUCTION

The environment is abundant with microorganisms. Many of them are non-cultivable. Various groups of microorganisms are found in drinking water as part of its indigenous microflora, but many of them, because of a lack of adequate methods, are not isolated or properly identified (Ishida et al., 1982). The standard identification methods used for the isolation and identification of bacteria are limited (Gerhardt et al., 1981). Because of this, most researchers use cultivation-independent molecular techniques for the monitoring of bacteria in the environment. Molecular methods are also used together with cultivation methods in order to prove or to assist in species determination.

The species *Chryseobacterium vrystaatense* sp. nov. was proposed as a novel species by de Beer et al. (2005) and named after the province Vrysstaat (Free State, South Africa) where the bacterial species was isolated for the first time.

The genus *Chryseobacterium* was classified into the fourth group of Gram-negative aerobic/microaerophilic rods and coccoid bacteria (Vandamme et al., 1994). Previously it did not belong to any family and was grouped in the so-called group "other ge-

nus" (Sneath et al., 1986). Today it is grouped within the family *Flavobacteriaceae* (Bernardet et al., 1996).

In this paper, we monitored different water sources for the presence of different bacterial species using cultivable methods in combination with molecular techniques. The object of biochemical and molecular characterization was the isolate ST that belongs to the species *Chryseobacterium vrystaatense* that was isolated from oligomineral water springs in Jakov Do (Tasić, 2008). The water springs are located on Mt. Vlasina, southeast Serbia, at an altitude of about 1500 m at 42^0 38' 14" north latitude and i 22^0 16' 35" east longitude.

The tested strain ST of *Chryseobacterium vrystaatense* is an indigenous oligotroph with extremely low levels of biochemical reactivity in the biochemical tests used for strain identification. For this reason, molecular methods of characterization were performed. The gene for 16S rRNA was amplified by PCR using universal primers, and sequenced. Comparison of the sequenced 1088 nucleotides of the 16S rRNA gene with all sequences deposited in NCBI collection showed a high similarity (98%) with the strain *Chryseobacterium vrystaatense* sp. nov., designated as strain R-23533 (Tasić, 2007). The alignment of these two sequences showed that they are not absolutely identical which means that the tested strain can be classified at the species level, indicating, and indirectly confirming that this is an authentic representative isolated in southeast Serbia.

MATERIALS AND METHODS

Isolation of pure cultures

Isolation of the strain *Chryseobacterium vrystaatense* was done using a 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 kept on nutrient agar (Nutrient Agar, Biolife S.r.l.) for 48 h at 30^0C. Pure cultures were obtained by multiple subsequent dilutions using inoculation loop streaking on solid nutrient medium.

Biochemical characterization of isolates

Preliminary identification of the isolated strains was done manually using a commercial identification system API 20 NE – bioMérieux (Smith et al., 1972). In conventional tests of the API 20 NE system a bacterial suspension in 0.85% NaCl medium, turbidity 0.5 (McFarland standard) was used. For the assimilation tests, an AUX medium was used. After inoculation, the incubation box was incubated at 30^0C for 48 h.

Assessment of the identification was obtained by the use of 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 was 200 μl of pronase solution (final concentration 5 mg/ml) added, mixed well and incubated for 30 min at 37^0C. After this, 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 of 1/10 volume of sodium acetate 3M and 0.6 volume of isopropanol into 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-ze A and incubated 15 min to 37^0C. The isolated DNA was stored at -20^0C prior to use.

The quantity and quality of the isolated DNA was checked by agarose gel electrophoresis (Agarose Low EEO) in 1x TAE buffer at constant voltage 5 V/cm. For comparison of the DNA fragments' size and intensity, the standard DNA Leder Mix was used (Fermentas). Visualization of DNA was done 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, Hot Master Taq polymerase was used, which 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'). Amplification of 16S rDNA was done by PCR touchdown. The following thermocycling program was used: 3 min initial denaturation at 95^{0}C; amplification in two phases – first 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 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 for 10-min at 68^{0}C.

DNA sequencing

The sequencing of bacterial DNA was performed by the thermocycling sequencing method, which is a modification of the Sanger method (Sanger et al., 1975). The termination method involves the incorporation of 2', 3'-dideoxynucleotide triphosphate (ddNTP) in an *in vitro* synthesis of DNA. The amplified DNA was first purified using a QIAquick PCR Purification Kit (QIAGEN-GmbH). Then a BAC16S1 primer was added to the purified DNA and the DNA mix was dried. The sequencing of the samples was done at the University of Padua, Italy. Sequence annotation and a database search for similar sequences were performed using the BLAST site of programs at the National Center for Biotechnology Information (Altschul et al., 1997).

Nucleotide sequence accession number

The nucleotide sequences for 16S rDNA of *Chryseobacterium vrystaatense* ST1 was submitted to the EMBL GenBank under accession number FR714871.

RESULTS

Physical and chemical characteristics water

The spring water from which *Chryseobacterium vrystaatense* ST1 was isolated is characterized by its uniform flow (0.3 l/s), low mineralization (< 50 mg/l), pH=6.4 and low temperature (5.8^{0}C). According to the chemical characteristics, the water belongs to the class of hydrocarbonic waters with a complexed cationic composition. Tests for total α and β activities and gamma spectroscometric analysis showed that the radioactivity in the water was within permissible limits.

Isolation of Chryseobacterium vrystaatense ST1 from water

Using a membrane filtration technique for concentrating the bacteria in the water and growth of filter on nutrient agar for 48 h at 30^{0}C, ten different types of bacteria were isolated according to different characteristics of colonies. After repurification, five of them were used for further analysis. Among them was the bacterium *Chryseobacterium vrystaatense* with unusual characteristics.

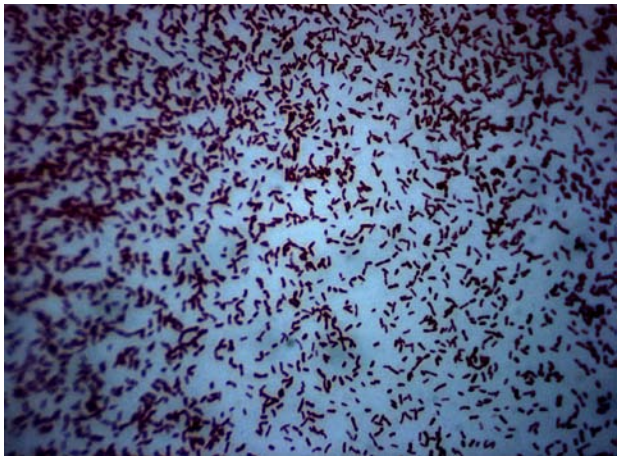

Fig. 1. Isolate *Chryseobacterium vrystaatense* ST1, microscopic appearance of Gram stained bacteria, magnification 1000x.

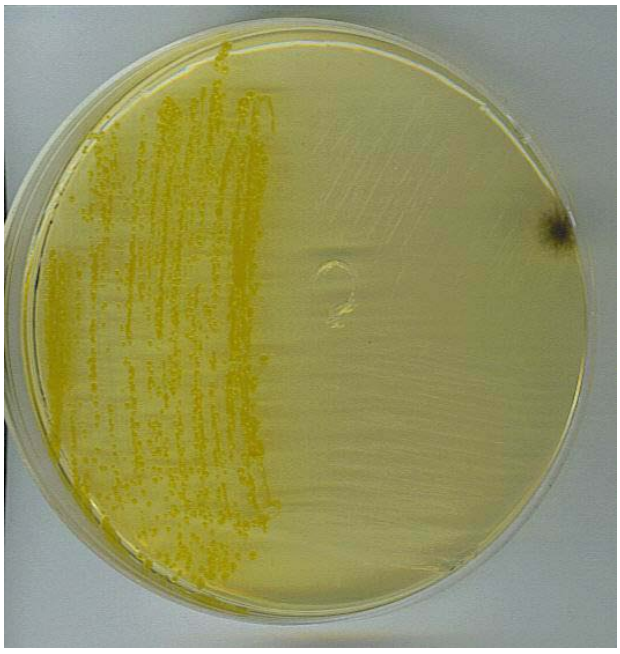

Fig. 2. Growth of *Chryseobacterium vrystaatense* ST1 on nutrient agar (*Biolife S.r.l.*) after incubation for 48 h at 30^{0}C.

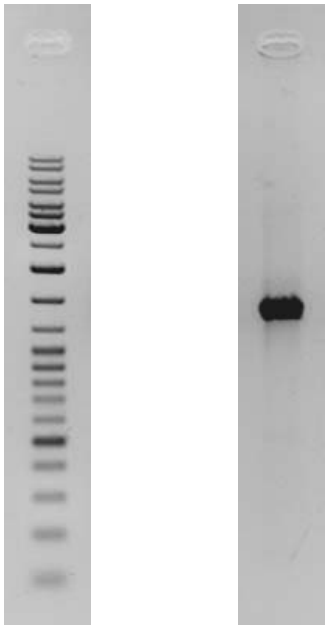

Fig. 3. Agarose gel electrophoresis of amplified DNA of 16S rDNA (1. marker, 2. amplified 16S rDNA for ST1) DNA marker contains bands (from botton to the top) of 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1031 bp, 1200 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 3500 bp, 4000 bp, 5000 bp, 6000 bp, 8000 bp and 10000 bp.

General characteristics of isolate Chryseobacterium vrystaatense ST1

Chryseobacterium vrystaatense ST1 comprises non-motile short rods of Gram-negative bacterium with rounded ends and a size of 0.5 x 2.0 μm. Intracellular granules of poly-β-hydroxybutyrate are absent and it does not form endospores (Fig. 1**).**

Chryseobacterium vrystaatense ST1 is an aerobic species that has a strict respiratory type of metabolism. On nutrient agar it showed good growth during the incubation at 22^{0}C and 30^{0}C. Colonies were yellow pigmented, opaque, round (1-2 mm in diameter), more or less convex, smooth, shiny with sharp edges (Fig. 2).

Biochemical analysis

In order to identify the strain biochemical analysis was carried out. The strain showed extremely low levels of biochemical reactivity (Table 1). After using the API 20NE identification system the profile 2610004 was obtained. Database software (APILAB Plus V 3.3.3, bioMérieux) identified this strain as *Chryseobacterium indologenes* (Id = 99.5%, T = 0.94) (Table 1).

Positive biochemical tests of isolated strain ST1 were: the production of indole from tryptophan (TRP), hydrolysis of urea (URE), esculin hydrolysis (ESC), hydrolysis of gelatin (GEL) and test oxidase (OX).

Negative tests were the reduction of nitrate (NO3), assimilation of D-glucose (GLU), catabolism of arginine (ADH), the activity of β-galactosidase (PNPG), acidification of glucose (GLU), assimilation of arabinose (ARA), assimilation of mannose (MNE), assimilation mannitol (MAN), assimilation of N-acetyl-glucosamine (NAG), assimilation of maltose (MAL), assimilation of gluconate (GNT), assimilation caprate (CAP), assimilation of adipate (ADI), assimilation malate (MLT), assimilation of citrate (CIT) and assimilation of phenyl acetate (PAC).

Table 1. Biochemical characteristics of *Chryseobacterium vrystaatense* ST1 (API 20NE, profile 2610004).

Tests	Substrates	Reaction/Enzymes	Results
NO_3	potassium nitrate	reduction nitrates to nitrites	-
TRP	tryptophane	indole production	+
HGLU	glucose	acidification	-
ADH	arginine	arginine dihydrolase	-
URE	urea	urease	+
ESC	esculin	hydrolisis (β-glucosidase)	+
GEL	gelatine	hydrolisis (protease)	+
PNPG	*p*-nitrophenyl-β-D-galactopyranoside	β-galactosidase	-
/GLU/	glucose	assimilation	-
/ARA/	arabinose	assimilation	-
/MNE/	mannose	assimilation	-
/MAN/	mannitol	assimilation	-
/NAG/	N-acetyl-glucosamine	assimilation	-
/MAL/	maltose	assimilation	-
/GNT/	gluconate	assimilation	-
/CAP/	caprate	assimilation	-
/ADI/	adipate	assimilation	-
/MLT/	malate	assimilation	-
/CIT/	citrate	assimilation	-
/PAC/	phenyl-acetate	assimilation	-
OX	tetramethyl-*p*-phenylene diamine	cytochrome oxidase	+

Molecular characterization of Chryseobacterium vrystaatense ST1

Because of the unclear results obtained by biochemical determination of the isolate ST1, we decided to use molecular characterization. The method used here was sequencing of gene for 16S RNA. Chromosomal DNA of strain ST1 was isolated to be 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 (data not shown).

Using PCR with universal primers for bacterial 16S rDNA, amplified DNA molecules were obtained with the expected size of about 1300 bp. Electrophoresis of the amplified DNA on 1% agarose gel showed that the PCR reaction was highly specific (without nonspecific products) (Fig. 3).

The amplified DNA was purified and sequenced using the primer that was also used for PCR amplification. Using one sequencing reaction, it a sequence of 1088 nucleotides in length was obtained (Fig. 4).

The sequence of 16S rDNA of isolate ST1 from Jakov Do which was 1088 nucleotides long was compared with the sequences in the NCBI gene bank data for 16S rRNA and it was the highest similarity (98%) of strain ST1 with the strain *Chryseobacterium vrystaatense* was obtained, designated as R-23533 (gi|60099259|emb|AJ71398.1).

From the results of DNA alignments of 16S rDNA of strain ST1 and genes for 16S rRNA deposited in all DNA databases, it was possible to conclude that the isolate from Jakov Do is *Chryseobacterium vrystaatense* ST1.

DISCUSSION

Chryseobacterium vrystaatense sp. nov. is a bacteria that acquired the status of new species in September 2005. The species was named after the province

NNNNNNNGNNGCTACCATGCAGCCGAGCGGTAGGTTTCCTTCGGGAGACTGAGAGCGGCG
CACGGGTGCGGAACACGTGTGCAACCTGCCTTTATCAGGGGGATAGCCTTTCGAAAGGAA
GATTAATACCCCATAACATTTTAAGTGGCATCACTTTAAATTGAAAACTCCGGTGGATAA
AGATGGGCACGCGCAGGATTAGATAGTTGGTAGGGTAACGGCCTACCAAGTCAACGATCC
TTAGGGGGCCTGAGAGGGTGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTAC
GGGAGGCAGCAGTGAGGAATATTGGACAATGGGTGAGAGCCTGATCCAGCCATCCCGCGT
GAAGGACGACGGCCCTATGGGTTGTAAACTTCTTTTGTATAGGGATAAACCTACTCTCGT
GAGAGTAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCTGAT
TTGTAAGTCAGTGGTGAAATCTCACAGCTCAACTGTGAAACTGCCATTGATACTGCAAGT
CTTGAGTGTTGTTGAAGTAGCTGGAATAAGTAGTGTAGCGGTGAAATGCATAGATATTAC
TTAGAACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGACGCTGATGGACGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCTAACTCGT
TTTTGGGTTTTCGGATTCAGAGACTAAGCGAAAGTGATAAGTTAGCCACCTGGGGAGTAC
GGACGCAAGTCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTG
GTTTAATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGGGAAATGACAGGTTTA
GAAATAGACTTTTCTTCGGACATTTTTCAAGGTGCTGCATGGTNGTCGTCAGCTCGTGCC
GTGAGTGTTAGGTTAAGTCCTGCAACGAGCGCACCCCTGTCACTAGTGCATCATCAGTGG
GGACTCTAGTGAGACTGCNACGCAGTAGANANNNNGTGGGGGATGACGTCAATCATCNAC
GGGCCTTTACGCCTTGGGNNNN

Fig. 4. Sequence of the 16S rRNA gene of the *Chryseobacterium vrystaatense* ST1 strain.

Vrystaat (Free State Province in South Africa) from where the bacterium was first isolated (the strain was isolated from raw chicken).

The strain *Chryseobacterium vrystaatense* ST1 was isolated from water that is characterized by very low mineralization (< 50 mg/l) and low temperature (5.4^{0}C), which is an unfavorable medium for most bacterial species. The strain showed good growth on nutrient agar at 30^{0}C.

In the biochemical tests, the strain showed atypical and low biochemical reactivity. This result may explain why this species was identified and systematized so late. Results obtained using the API 20NE identification system and database software APILAB Plus V 3.3.3 bioMérieux allowed identification of this strain as *Chryseobacterium indologenes* (Id = 99.5%, T = 0.94), which is a successful identification up to the level of the family.

It was necessary to carry out molecular characterization of the isolate ST1. Using a sequencing method of 16S rDNA, amplified by PCR, the strain ST1 was identified as *Chryseobacterium vrystaatense*. In this case, molecular identification was very successful. Which method will give the more successful results of identification depends on the isolated bacteria. We propose to use both biochemical and molecular genetic methods.

Comparing the sequences of the 16S rDNA of the strain *Chryseobacterium vrystaatense* ST1 with NCBI gene bank data showed the highest similarity (98%) with the strain designated as R-23533 of new species *Chryseobacterium vrystaatense* sp. nov. This is particularly important when one bears in mind that in the NCBI collection there are only four deposited sequences of *Chryseobacterium vrystaatense*, of which one is ours.

In addition, the results of comparable analysis showed that the species *Chryseobacterium vrystaatense* is phylogenetically closest to *Chryseobacterium joostei* (96.9%), *Chryseobacterium indologenes* (97.1%) and *Chryseobacterium gleum* (96.1%), (Hugo et al., 2003).

In this paper, we report for the first time the isolation of the species *Chryseobacterium vrystaatense* in Serbia from water sources at Jakov Do. This finding is particularly important when considering that this is the fourth isolate of the species *Chryseobacterium vrystaatense* in the world.

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