Serinicoccus kebangsaanensis sp. nov: A new bacterium isolated from the toxic diatom, *Nitzschia navis-varingica*

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Abstract

Serinicoccus kebangsaanensis sp. nov strain P2D13-UKM is a new species of Gram-positive bacteria isolated from a toxic diatom, *Nitzschia navis-varingica*. It is a halophilic aerobic, oxidase-negative, catalase-positive, circular, and colonies with white colour. Based on the 16S rRNA gene, the closest species were *Serinicoccus profundi* MCCC 1A05965 strain 0714S6-1, 97.41%, and *Serinicoccus hydrothermalis* strain JLT9, 97.35%. This bacteria's predominant cellular fatty acids were iso-C15: 0 (30.1 %) and iso-C16: 0 (16.2 %). The polar lipids identified in this bacterium were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, and an unknown glycolipid. The whole-genome sequence analysis of strain P2D13-UKM showed less than 85% similarity from other *Serinicoccus* species. The genomic DNA G + C content is 72.2 %. Here, we report the main characteristic of strain P2D13-UKM as a new species of bacteria according to its draft genome sequence, which was deposited in Gene Bank and is publicly available under the accession number VSLG00000000.

Keywords: Diatom, *Nitzschia navis-varingica*, phytoplankton, *serinicoccus*
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Introduction

Serinicoccus sp. is a non-motile Gram-positive bacteria and a member of the Ornithinimicrobiaceae family, order of Micrococcales, class of Actinomycetia and the phylum of Actinomycetota [1]. It is moderately halophilic aerobic, catalase-positive, oxidase-negative, non-spore-forming and nonacid-fast bacteria. Most Actinomycetota members are organotrophic bacteria involved in decomposing complex and poorly accessible substrates at later stages of microbial succession, synthesising and decomposing humic substances [2]. Many bacteria in this phylum also have the ability to produce spores in response to starvation or harsh chemical or physical conditions [3–5]. Several *Serinicoccus* sp. has been found to produce phenazine, a large group of nitrogen-containing heterocyclic compounds for biofilm formation [6].

Nitzschia navis-varingica (NNV) strain P2CC7 is a pennate shaped diatom that can produce neurotoxin, known as domoic acid (DA) [7]. However, certain bacteria can influence DA production [8]. Diatoms are the most dispersed phytoplankton, essential for various biogeochemical cycles and maintaining the ecological system [9]. Therefore, the interaction between bacteria and phytoplankton is vital for the dynamic of the aquatic environment [10]. Furthermore, these ecological interactions are complex and occur through several distinct mechanisms, including quorum sensing in bacteria, diatom cell to cell signalling or interkingdom signalling [11]. For example, the mutualistic interaction occurs between both groups as the bacteria consume algae-derived organic compounds and remineralise them back to their inorganic substituents, which in turn internalise algae for their growth [12]. Proteobacteria is the most abundant phylum that co-exists with diatom, followed by Cyanobacteria, Bacteroidetes, Planctomycetes, Firmicutes, and Actinobacteria [13].

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Herein, we report that *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM is a new species according to its 16S rRNA gene and comparative genome analysis. Therefore, understanding the interaction between this species and its interacting diatom is vital in modelling and predicting changes in the marine environment.

Strain isolation and identification

The diatom culture used was obtained from the culture collection of the Marine Microbiology and Biotechnology Laboratory, Universiti Kebangsaan Malaysia, namely *Nitzschia navis-varingica* strain P22C7. Culture in the exponential phase (two weeks) has been used for bacterial isolation where the domoic acid concentration is 2.03 pg/cell. A total of 1 mL of diatom culture medium was taken, and the serial dilution was made up to 10^{-7}. Next, 100 µL of the sample was taken from each dilution and spread on the marine agar (Difco, USA). The marine agar plate is kept at 26 °C for ten days. The colony that grows on the agar is to be observed based on colony morphology, which is colour, shape, periphery and elevation. Then the colonies were sub-cultured on the new marine agar plates to obtain a pure culture. The gram staining procedure was performed at the magnification of 100x using a light microscope. For electron microscopy analysis, the samples have been dried on a coverslip and coated with gold-palladium according to the protocol by Fourie [14]. The taxonomy of bacterial interest was validated by PCR and amplification of 16S rRNA using primer pair 27F and 1525R [17]. The PCR products were sent for sanger sequencing at the Apical Scientific Sdn. Bhd. Selangor, Malaysia. Data were manually edited by BioEdit Sequence Alignment Editor v. 7.2.5 [15] and BLASTed against NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast) [16]. Phylogenetic analysis was performed using MEGA 7 software [17]. The strain P2D13-UKM was selected for further identification using *de novo* Bacterial Whole Genome Sequencing (WGS).

Phenotypic characterisation

Different growth conditions were tested to find the optimal salinity, pH, and temperature for the growth of 0–14 % (w/v) NaCl, pH 6-11 and at 10–35 °C. Biochemical tests were carried out using the API 20NE and API 20E (bioMerieux, France), according to the manufacturer's instructions. The kits were incubated at 30 °C for 48 h. The catalase test was done by adding one drop of 0.3 % hydrogen peroxide, and the bubbles indicated positive results. Oxidase activity was detected using N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride as an artificial electron acceptor for the enzyme oxidase. The positive result indicates a coloured compound by indophenol blue.

For analysing the whole-cell fatty acids, cells of strain P2D13-UKM were grown for three days at 30 °C in a marine broth medium with shaking at 150 rpm. The cells were harvested and washed by repeated centrifugation using sterile water. The 100 mg cells were saponified, methylated and the fatty acid methyl esters (FAMEs) analysed using GC-MS [18]. The GC-MS analysis was performed on a 7890A gas chromatograph (Agilent) directly coupled to the mass spectrometer system of a 5975C inert MSD (Agilent) with a triple-axis detector. The MSD Chemstation was used to find all the peaks in the raw GC chromatogram. In addition, a library search for all the peaks was carried out using the NIST/EPA/NIH version 2.0.

Genomic DNA isolation, library preparation and sequencing

The genomic DNA of the bacteria is extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). DNA degradation and potential contamination were examined by 1% of agarose gel electrophoresis, and the purity was determined by NanoDrop TM spectrophotometer (OD260/OD280). The construction of the DNA libraries was done using NEBNext Ultra DNA Library Prep Kit. The qualified libraries are fed into sequencers after pooling according to their effective concentration and expected data volume by Illumina 150 PE (Illumina Inc., San Diego, CA), a high-throughput sequencer with paired-end sequencing runs with the data output of 1Gb and 100x sequencing depth per sample.

Genome assembly and annotation

Illumina SBS technology features error rate grows with sequenced reads extension because of the consumption of sequencing reagent. Therefore, the filtering process is needed to remove reads containing adapters, N > 10% (N represents the base cannot be determined) and low quality (Qscore $\leq$ 5) base, which is over 50% of the total base using bbduk of the BBTools Packages (https://jgi.doe.gov/data-and-tools/bbtools/). QC reads were assembled *de novo* using SPAdes 3.11.1 [19]. The resulting scaffolds were subjected to MEGABLAST against the NCBI nucleotide database to identify the sequence contaminants; Scaffolds with coverage lower than 90 and with hits to non-target were removed. The quality of the genome was assessed using QUAST [20] and the annotation was done using RAST [21]. Barnap version 0.4.2 and tRNAscan-SE version 1.3.1 [22] were used to predict rRNAs and tRNAs, respectively. The circular genome map of strain P2D13-UKM was generated using the CGView server (http://cgview.ca/) [23].

Genomic comparison

Selected genomes of *Serinicoccus* species were obtained from the EzBioCloud (https://www.ezbiocloud.net/genome/). These included; *Serinicoccus profundi* MCCC 1A05965, *Serinicoccus marinus* (DSM 15273, CNJ-927, CUA-874), *Serinicoccus chungangensis* and *Serinicoccus* sp. JLT9. OrthoANI and original ANI values were calculated using the OrthoANI Tools version 0.93.1 [24]. OrthoANI is highly correlated with ANI (using BLASTn), and the former showed approximately 0.1 % higher values than the latter. Values of OrthoANI were calculated using OAT software [24]. Genome to Genome Distance Calculator (GGDC 2.1) from web service (http://ggdc.dsmz.de/distcalc2.php#) is used to calculate the genome to genome distance (GGD) based on DNA–DNA hybridisation (DDH) [25]. Average Amino Acid Identity (AAI) values were calculated using the Web-based AAI tool (http://enve-omics.ce.gatech.edu/aai/) using both best hits (one-way AAI) and reciprocal best hits (two-way AAI) between two genomic datasets of proteins [26].

Results

Strain isolation and phylogenetic analysis

Serinicoccus sp. was isolated on marine agar, and the initial growth was obtained after 72 h of culture in a marine agar in aerobic conditions at 30 °C. The 16S rRNA gene sequence for *Serinicoccus kebangsaanensis* sp. nov strain P2D13-UKM showed the highest similarity carried by *Serinicoccus profundi* MCCC 1A05965 strain 0714S6-1, 97.41%, and *Serinicoccus hydrothermalis* strain JLT9, 97.35%. Based on the phylogenetic analysis using MEGA 7, the tree was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model [11] found; the closely related species is *Serinicoccus profundi* MCCC 1A05965 strain 0714S6-1 (Fig. 1). Previous studies suggested a threshold of 98.7% to 99% 16S rRNA gene sequence similarity for bacterial classification [27]; however, it requires comparative study of DDH if the similarity between two strains is over 97% [28]. For that reason, we proceed with this work using genome sequencing and comparative genomics based on the data available in the GenBank to find evidence for bacterial delineation.

Biochemical properties

For phenotypic characteristics, this strain growth occurs with 0–14% (w/v) NaCl (optimum 3–5 %), at pH 6–11 (optimum pH 7) and at 10–35 °C (optimum 30 °C). Bacterial colonies were white, circular, smooth, and convex with a mean diameter of 0.05 to 0.1 mm. Meanwhile, the Gram staining result indicates Gram-positive bacterium with coccus shaped and non-spore-forming. Strain P2D13-UKM showed catalase-positive and oxidase-negative activities. Biochemical characteristics of strain P2D13-UKM and selected members of the genus *Serinicoccus* is shown in Table 1. The cellular fatty acid profile of strain P2D13-UKM grown on marine agar comprised of iso-$C_{14:0}$ (0.8%), iso-$C_{15:0}$ (30.1 %), iso-$C_{16:0}$ (16.2%), and $C_{16:0}$ (1.5%) (Supplementary Table S1).

Genomic analysis

The genome sequence of *Serinicoccus* sp. showed a raw data set of 7,232,952 reads, and the data filtered by quality control

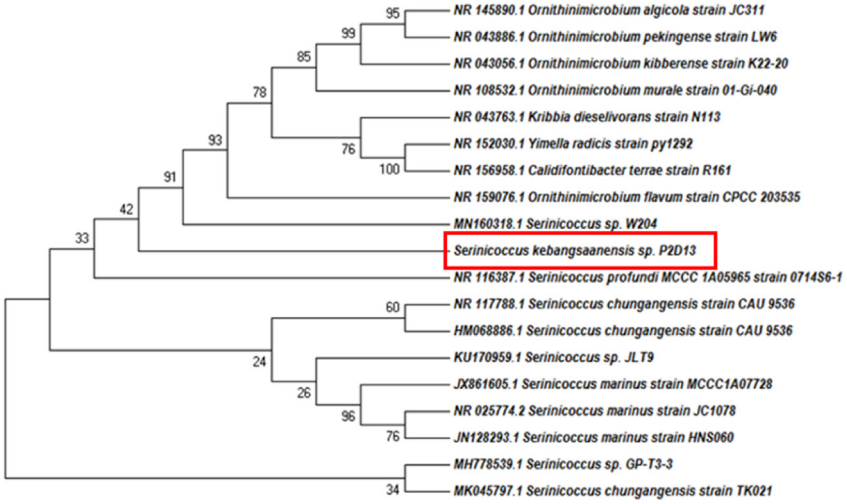

FIG. 1. Phylogenetic tree of *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM. Sequence alignment and phylogenetic inferences were obtained using the maximum likelihood method within MEGA 7. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority of consensus tree.

TABLE 1. Characteristics of *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM and other members of the genus *Serinicoccus*

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	+	+	—	+	+	—	—
Growth with 7 % (w/v) NaCl	+	+	+	—	+	+	+
Growth at 45 °C	—	—	—	+	—	—	ND
Growth at pH 9.0	+	+	—	—	+	—	ND
Hydrolysis of aesculin	+	+	w	w	w	w	+
Enzyme activity:							
Acid phosphatase	+	—	—	—	—	w	—
Alkaline phosphatase	+	+	—	—	—	w	—
α-Chymotrypsin	+	+	—	w	—	—	—
Cystine arylamidase	w	+	—	w	w	—	—
Esterase (C4)	+	+	+	+	w	+	ND
Esterase lipase (C8)	+	+	—	w	—	—	ND
α-Galactosidase	w	—	—	—	—	—	—
β-Glucosidase	w	+	—	—	—	—	—
Leucine arylamidase	+	+	—	+	—	w	—
Naphthol-AS-biphosphohydrolase	w	—	w	w	w	w	—
Trypsin	w	+	—	+	—	—	—
Valine arylamidase	+	+	+	w	—	—	—
DNA G + C content (mol%)	73.5	72	72	70	71	69	72.2

Strains: 1. *Serinicoccus chungangensis* CAU 9536T 2. *Serinicoccus profundi* DSM 21363T 3. *Serinicoccus marinus* KCTC 9980T 4. *Ornithinimicrobium humiphilum* DSM 12362T 5. *Ornithinimicrobium kibberense* DSM 17687T 6. *Kytococcus sedentarius* DSM 20547T 7. *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM +, Positive; -, negative; W, weakly positive. Data 1-6 are from the previous study [31].

showed 7,217,551 clean reads. *Serinicoccus* sp. genome was found to have a total length of 3,503,838 paired-end reads (Table 2). It has 72.2% of G + C content, and the annotation analysis has identified 3368 genes, including 3303 coding genes, three rRNAs and 45 tRNAs (Fig. 2). Annotation by RAST assigned these genes into 264 subsystems with the highest group in carbohydrates (18.5%), amino acid and derivatives metabolism (16.8%), and protein metabolism subsystems (12.8%) (Fig. 3).

In determining the new species of bacteria, apart from the genome sequence data as one of the ultimate tools for taxonomic purposes [29], DNA–DNA hybridisation is widely used besides average nucleotide identity (ANI) and OrthoANI. OrthoANI is a new algorithm that can solve the problem of reciprocal inconsistency in ANI values. The heat map from OAT software (Fig. 4), which consist of both analysis by Original ANI and OrthoANI (Supplementary Table 2) on the genome sequence of strain P2D13-UKM, appears to be less than 85% similarity between their genome and other *Serinicoccus* species, whereby the cut off for species demarcation is 95–96%. The DDH values for strain P2D13-UKM species showed <50% compared to all the genomes analysed; the recommended cut-off point of 70% DDH for species delineation corresponded to 95% ANI and 69% conserved DNA [30]. Therefore, even though the GC content of strain P2D13-UKM is <1% compared to other *Serinicoccus* species, the genomic indices are insufficient to conclude the diversity of this species.

Further analysis using amino acid identity (AAI) value to measure the relatedness of the shared genes and one of the genome-based taxonomy found that strain P2D13-UKM shows the value of less than 84% (Table 3). On the other hand, the value of >90% of AAI shows similarity in species [26].

TABLE 2. *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM genome resources and characteristics

Name	Genome resources/characteristics
1 Bioproject ID	PRJNA561033
2 Biosample ID	SAMN12606013
3 Genome accession number	VSLG00000000
4 Sequence type	Illumina, SBS
5 Total number of reads	3,503,838
6 Overall coverage	>100x
7 GC content (%)	72.2
8 N50	173,418
9 L50	6
10 Number of Contigs (with PEGs)	37
11 Number of subsystems	264
12 Number of coding sequences	3303
13 tRNA coding genes	45
14 rRNA genes (5S,16S,23S)	3

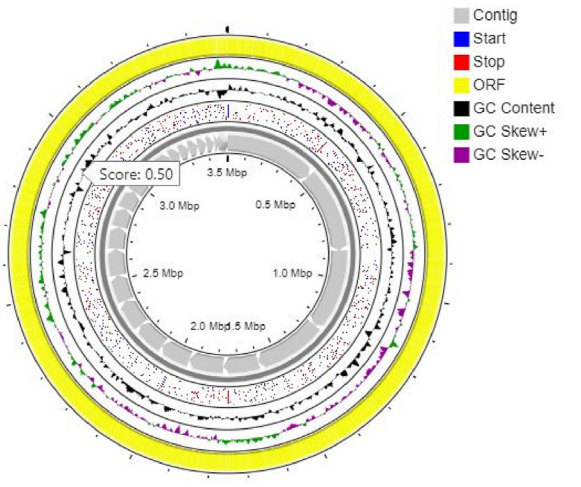

FIG. 2. Circular genome view of *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM.

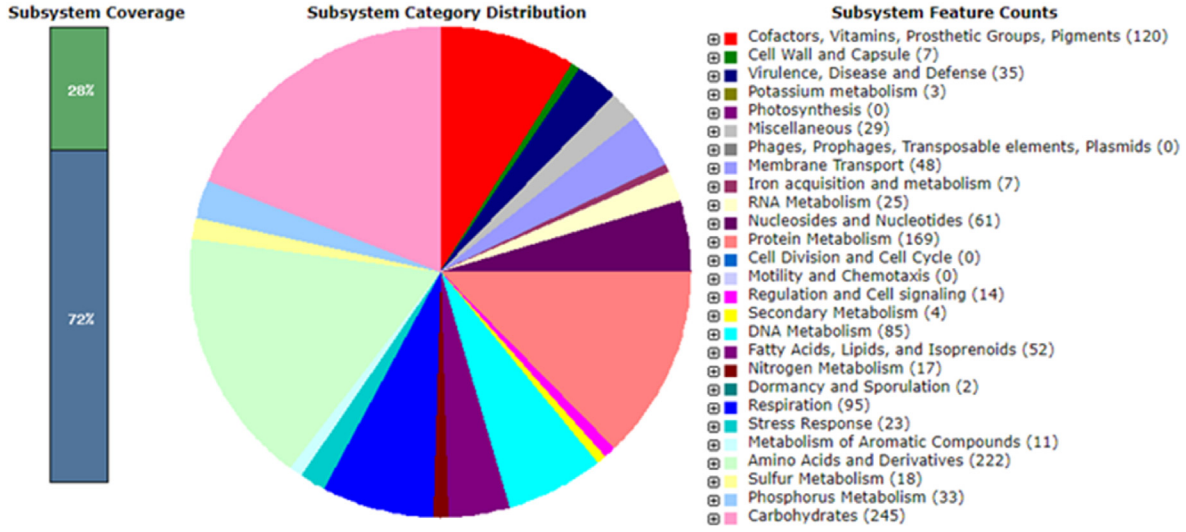

FIG. 3. Annotation of *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM genome using Rapid Annotation using Subsystem Technology (RAST).

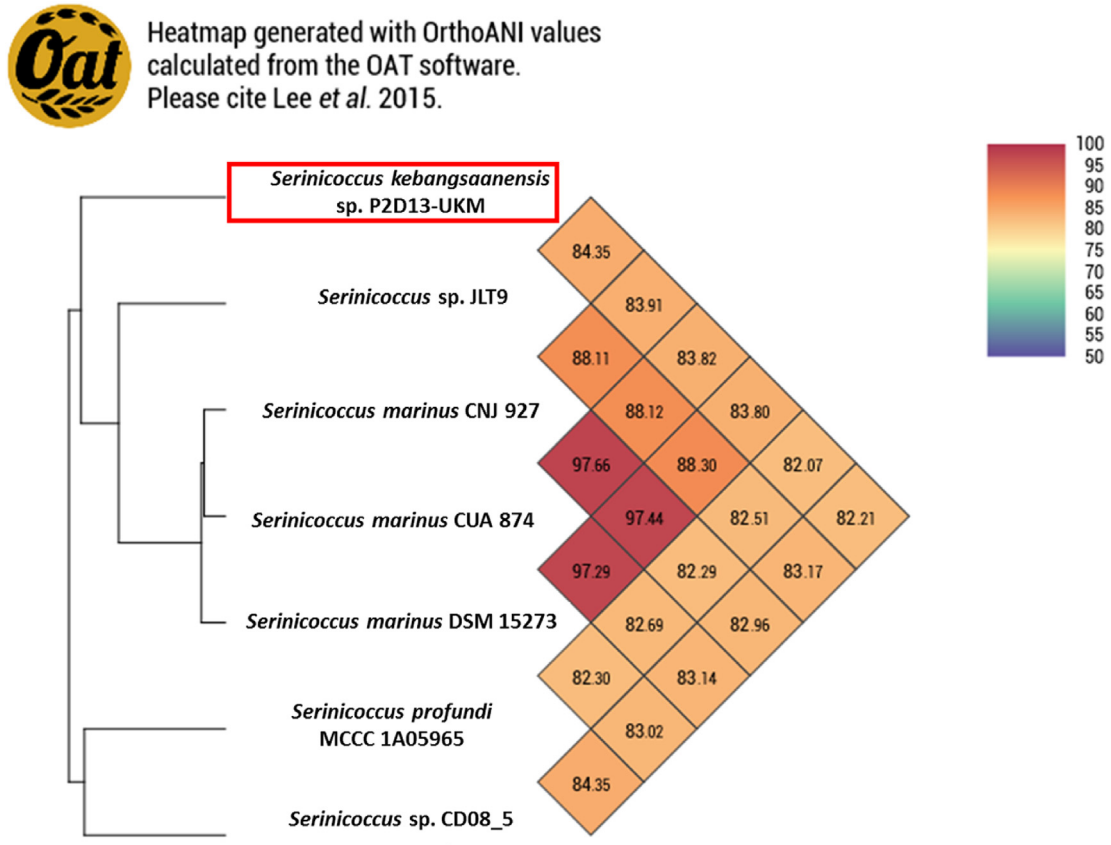

FIG. 4. Heatmap generated with OrthoANI values calculated using the OAT software for of *Serinicoccus kebangsaanensis* sp. strain P2D13-UKM with the closely related species.

TABLE 3. Value of G + C content, DNA–DNA hybridization (DDH) and average amino acid identity (AAI)

Species	Difference in G + C content (%)	DDH	AAI
S. kebangsaanensis sp. P2D13-UKM	0.00	100	100
Serinicoccus sp. JLT9	0.21	49.10% [46–52.1%]	83.53% (SD: 12.09%)
Serinicoccus sp. CD08 5	0.69	24.40% [22–26.8%]	80.21% (SD: 12.87%)
S. marinus CNJ927	0.10	26.90% [24.5–29.3%]	82.92% (SD: 12.63%)
S. marinus CUA 874	0.11	26.70% [24.4–29.2%]	82.90% (SD: 12.82%)
S. marinus DSM 15273	0.00	26.50% [24.2–29%]	82.43% (SD: 13.04%)
S. profundi MCCC 1A05965	0.25	24.40% [22.1–26.8%]	79.96% (SD: 13.22%)

Conclusion

Based on the phenotypic, biochemical, 16S rRNA, and genomic analysis, we propose a new species, *Serinicoccus kebangsaanensis* sp. nov strain P2D13-UKM.

Description of *Serinicoccus kebangsaanensis* sp. nov

Serinicoccus kebangsaanensis sp. nov (ke.bang.sa.a.nen'sis. N.L masc. adj. kebangsaanensis, pertaining to Universiti Kebangsaan Malaysia). Gram-positive, halophilic aerobic, coccus-shaped bacterium. Colony sized 1 mm, cream, round-shaped and convex after three days of incubation on marine agar. This strain growth occurs with 0–14 % (w/v) NaCl (optimum 3–5 %), at pH 6–11 (optimum pH 7) and at 10–35 °C (optimum 30 °C). Oxidase-negative and catalase-positive. Nitrate is not reduced to nitrite. No arginine dihydrolase activity. β-Galactosidase activity is weakly present. Does not produce H_2S on TSI agar, acid from glucose in API 20NE kit or indole from tryptophan. Casein, DNA, aesculin, gelatin, starch, Tween 80 and tyrosine are decomposed; adenine, alginate, cellulose, chitin, hypoxanthine, urea, and xanthine are not. Major fatty acids comprised of iso-$C_{14:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, and $C_{16:0}$. The genome size of *Serinicoccus kebangsaanensis* sp. nov is 3.5 Mbp with 72.2% of G + C content, and the annotation analysis has identified 3368 genes with 3303 coding genes, three rRNA's and 45 tRNA's. The 16S rRNA closest sequence similarity with *Serinicoccus kebangsaanensis* sp. nov strain P2D13-UKM are *Serinicoccus profundi* MCCC 1A05965 strain 0714S6-1, 97.41%, and *Serinicoccus hydrothermalis* strain JLT9, 97.35%. The 16S rRNA and draft genome sequence are deposited in the Genbank database under accession number VSLG00000000.

Nucleotide sequence accession number

The genome sequences were deposited in Genbank under accession number VSLG00000000.

Conflict of interest

None to declare

Author contributions

A.Y carried out the genome analysis and worked with H.B. in its design and coordination. A.Y., F.K.S., A.A., G.U. and H.B. participated in the analysis. A.Y. and N.A.S. drafted the manuscript. All authors have read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2022.101005.

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