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A *Tetragenococcus halophilus* human gut isolate

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ABSTRACT

Tetragenococcus halophilus (*T. halophilus*) is a facultative anaerobic, coccus-shaped halophilic lactic acid-producing bacterium previously detected and cultured in various salty foods and credited for beneficial effects on human health. In this study, we investigated the presence of *T. halophilus* in human samples using a polyphasic approach including scanning electron microscopy, molecular biology methods and microbial culture. This unique investigation yielded the unprecedented presence of *T. halophilus* in human feces samples, thus enriching the repertoire of halophilic microorganisms colonizing the human gastrointestinal tract with the isolation and culture of *T. halophilus* for the first time in humans. Using the E-test strips, the MIC was assessed for T. *halophilus* strain CSURQ6002: rifampicin (MIC at 0.002 µg/mL), benzylpenicillin (MIC at 0.094 µg/mL), amoxicillin (MIC at 0.5 µg/mL), erythromycin (MIC at 2 µg/mL), clindamycin (MIC at 4 µg/mL), and vancomycin (MIC at 8 µg/mL). However, this strain showed a MIC up to 256 µg/mL for ciprofloxacin, fosfomycin, doxycyclin, imipenem, and colistin. *In-silico* profiling derived from whole genome sequencing (NCBI accession number: PRJNA780809), was confirmed. This discovery suggested that *T. halophilus* was part of the human digestive microbiota and that its potential role on human health should be considered.

1. Introduction

Tetragenococcus halophilus (*T. halophilus*) is a gram-positive, non-motile, non-sporulating, facultative anaerobic, coccus-shaped lactic acid-producing bacterium (Chun et al., 2019, Justé et al., 2012). This halophilic microorganism has been initially isolated from miso (Kumazawa et al., 2018) and further detected in various salty foods (Chun et al., 2019, Justé et al., 2012, Guan et al., 2011, Lee et al., 2015, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016), being incorporated into soy sauce, miso, anchovy brines and fermented and smoked fish eggs (Kim et al., 2018, Hanagata et al., 2003, Roling and van Verseveld, 1996, Udomsil et al., 2011, Tanasupawat et al., 2002). Indeed, *T. halophilus* has been shown to produce organic acids, amino acids and flavoring compounds during the fermentation of salty foods (Udomsil et al., 2010, Udomsil et al., 2017, Lee et al., 2018). Moreover, *T. halophilus* has been credited from beneficial effects on human health, in particular an immunomodulatory effect leading to improvement of atopic diseases (Masuda et al., 2008, Ohata et al., 2011). Despite the widespread presence of *T. halophilus* in salty foods, this species has never been described in humans in the many studies which have proven the presence of halophilic bacteria in the human microbiota (Oxley et al., 2010, Seck et al., 2018, Khelaifia et al., 2017, Khelaifia and Raoult, 2016). We herein questioned whether *T. halophilus* could also be detected in the human gut, investigating feces samples collected from apparently healthy individuals exposed to different regions in Europe and Africa. This unique investigation yielded the unprecedented presence of *T. halophilus* in human feces samples, and this finding was confirmed by the sequencing of the first genome of this clinical isolate from the cultivated colonies.

2. Materials and methods

2.1. Sample collection

A series of 184 human anonymized leftover fecal specimens were retrospectively investigated for the prevalence of *T. halophilus*. In France, Mali, and Senegal, where stools samples have been anonymously collected, informed consent was obtained from individuals before

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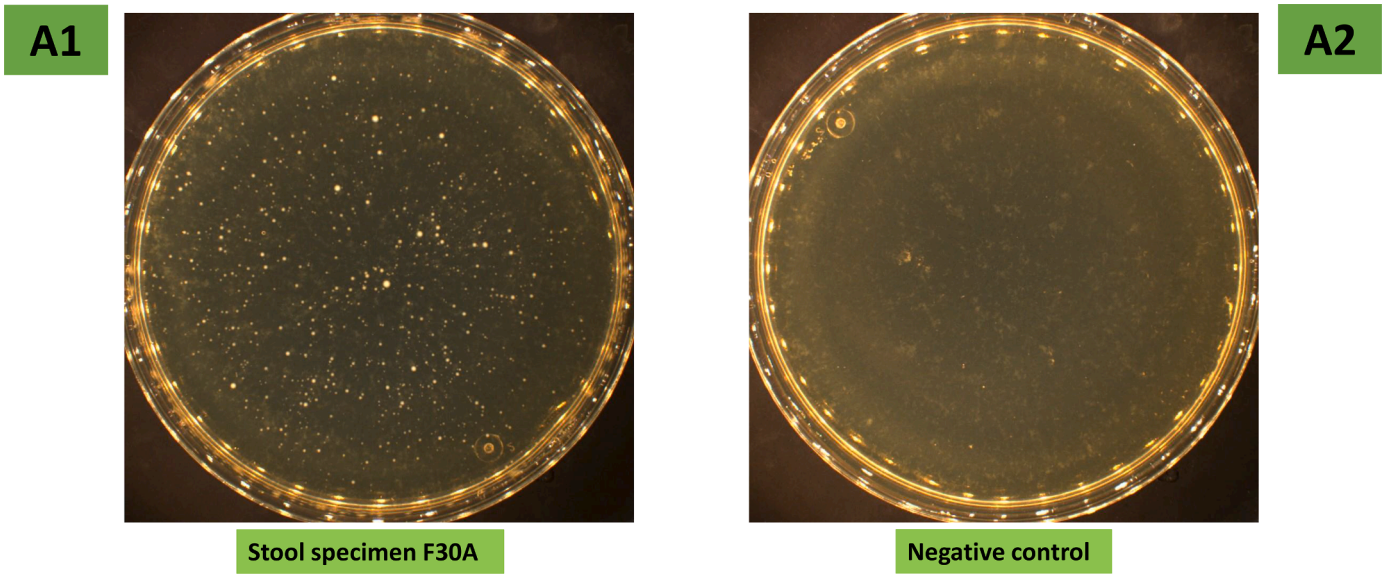

Fig. A. (A1) Spherical white colonies of *T. halophilus* CSURQ6002 growing on agar. **(A2)** Negative control.

participation in this study in agreement with French, Malian and Senegalese laws.

2.2. DNA extraction and PCR assays

Total DNA extracted as previously described (Guindo et al., 2020), was incorporated into PCR for the amplification of the 16S rRNA gene from *T. halophilus* using the primers *T. halophilus* F, 5-ACAGGGGTAGGAGTGAAATCTTG-3, and *T. halophilus* R, 5- TGCTGGCAACAGTGG GCAC-3 (Eurogentec, Angers, France), designed in our laboratory and using a specific program for the amplification reactions (**Appendix A**). Sequencing reactions (Sangers' method) were carried-out as previously described (Guindo et al., 2020).

2.3. Isolation and culture of T. halophilus

We selected 20 human anonymized leftover stool specimens proved to harbor *T. halophilus* following specific DNA sequencing of which five have been collected in five individuals living in Senegal, 13 have been collected in 13 individuals living in Mali and two have been collected in two individuals living in France. Salinity was measured in these 20 stool specimens according to a previously established protocol (Seck et al., 2019). The total mineral salt content of all 20 stool specimens was measured by a salinity refractometer (Thermo Scientific). One gram of each stool specimen was diluted in 10 mL of distilled water (Thermo Scientific, Villebon sur Yvette, France) and vortexed briefly. Then, 100 µL of supernatant was deposited in the refractometer and the result was directly displayed on the screen. One measure was performed per sample.

For stool culturing, we used a specific culture medium containing 100 g of NaCL (**Appendix B**). We established a four-week culture protocol with weekly follow-up. We poured 25 mL of culture medium in bottle serum (Sigma-Aldrich, Saint-Quentin Fallavier, France) and then degassed with 2-bar nitrogen for ten minutes. Then, one gram of each stool specimen was diluted in 10 mL of 10X PBS (Fisher Scientific, Illkirch, France) under anaerobic conditions and subsequently 2 mL of each dilution were injected into the degassed bottle serum and incubated at 37°C during a four-week follow-up. Then, each stool specimen was subcultured in an anaerobic chamber (Don Whitley Scientific, Bingley BD16 2NH, UK) on a solid medium comprising the same components as the liquid medium used and placed in an anaerobic bag (Becton Dickinson, Pont-de-Claix, France) with a CO_2 generator (Becton Dickinson) and incubated at 37°C for ten days.

2.4. Scanning electron microscopy investigations

Morphological features of *T. halophilus* were further assessed using a SU5000 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). Pure cultures were obtained from solid and liquid cultures fixed for three hours with 2.5% glutaraldehyde. Fixed bacteria were cyto-centrifuged on glass slides and platinum sputtered coated (5 µm thick platinum layer) using ion sputter MC1000 (Hitachi). Micrographs were acquired at magnifications ranging from x10,000 to x120,000 with a 10-kV voltage and a spot intensity of 30 using the backscatter electron detector under a high vacuum mode.

2.5. Whole genome sequencing

For whole genome sequencing, *T. halophilus* DNA was extracted flowing an in-house adapted procedure used EZ1 protocol (Qiagen Tissue, Hilden, Qiagen, Germany). Briefly, 200 µL of *T. halophilus* suspension mixed with 200 µL G2 buffer in presence of 20 µL proteinase K (Qiagen) was incubated over night at 56°C. After proteinase K treatment, glass powder was added to the mix and incubated 20 minutes at 100°C and immediately vortexed 90S at 6.5 m/S with FastPrep (MP Biomedical Europe, Illkirch, France). After five-minute centrifugation at 17,000 g, DNA was extracted from 200 µL supernatant and eluted in 50 µL final volume. Extracted DNA was normalized using Qubit dsDNA High Sensitivity Assay Kit (Life Technology, Villebon-sur-Yvette, France). Genome sequencing was performed using 1 ng of normalized DNA following Illumina Nextera-XT paired-end protocol and sequenced on Illumina iSeq 100 and MiSeq instruments (Illumina, San Diego, USA)

Table A
Salinity assessment.

ID	C028	C068	F30A	C036	C014	M334A	C040	C112	C137	C037	C054	C020	C153	C169	C141	S1	S2	S3	S4	S5
Salinity(%)	1.7	2.1	2.2	1.3	0.6	0.6	0.8	0.4	0.6	0.3	0.5	0.46	0.47	0.47	0.47	0.26	0.31	0.21	0.22	0.2

after library preparation, as previously described (Morsli et al., 2021, Morsli et al., 2021, Diop et al., 2016). In parallel, 1 μg of *T. halophilus* DNA was used for Oxford Nanopore library preparation as previously described (Morsli et al., 2021), then sequenced using MinION sequencer (Oxford Nanopore technologies, Oxford, UK). NGS-generated data were analyzed using CLC Genomics Workbench, version 7.5.0 (Qiagen). Phylogenetic tree based on whole genome sequence was generated using Orthologous Average Nucleotide Identity Tool (OAT) software version (0.93.10) (Lee et al., 2016) and pangenome analysis was performed using Roary command after genome annotation on galaxy Europe online software (https://usegalaxy.eu/). Antibiotic resistance and bacteria virulence were analyzed using on-line CGE-database bio-tools (CGE Server "dtu.dk"), and Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi).

3. Results and Discussion

We are reporting on the first isolation and culture of *T. halophilus* from human stools. The results here reported were authentified by the fact that negative controls introduced in all experiments remained negative; in agreement with the fact that *T. halophilus* has never been previously worked in our laboratory and the fact that concordant results were obtained by polyphasic approaches, including molecular methods, culture, and microscopy. The fact that this observation occurred twelve years after the initial detection of food-borne *T. halophilus* in Asia (Kumazawa et al., 2018, Guan et al., 2011, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016), testifies that the search for halophiles is still in its infancy, in clinical microbiology.

In the first step, we searched for *T. halophilus* by PCR-sequencing in stools specimens collected in individuals living in France, Senegal, and Mali. By incorporating 16S rRNA *T. halophilus* gene PCR primers recently designed in our laboratory into PCR-sequencing, we detected the presence of *T. halophilus* DNA in 28/184 (15%) of stools samples here investigated but not in the negative controls (**Appendix C**). All sequences obtained exhibited a 100% similarity and 100% coverage with the sequence of the complete genome of *T. halophilus* strain YJ1 (NCBI accession number: CP046246.1). These data indicated an average prevalence of *T. halophilus* in humans compared to the high prevalence in salty foods (Chun et al., 2019, Justé et al., 2012, Guan et al., 2011, Lee et al., 2015, Udomsil et al., 2010, Jeong et al., 2017, Jung et al., 2016, Jung et al., 2016). This average prevalence could be explained by the generally very low salt diet in the Caucasian population, which represented 67.39% (124/184) of the stool samples here investigated. *A contrario*, the *T. halophilus* strain here reported had been recovered from stool sample collected in an individual exposed to high salt content food.

In a second step, we initiated the isolation by culture of *T. halophilus*, by using a specific salt medium. We used a very rich medium because *T. halophilus* has complex nutritional needs (Collins et al., 1990). After ten days of culture, we observed spherical white colonies suggestive of *T. halophilus* growing on agar (**Fig. A**), as previously described (Vos et al., 2011). This observation was made on 5% (1/20) of the samples cultured after ten-day follow-up. These results suggested the low prevalence of *T. halophilus* in humans regardless of the population studied, but also demonstrated that *T. halophilus* was a slow growing bacterium. The culture-positive sample had a salinity of 2.2% (**Table A**), compatible with the presence of *T. halophilus* in salty stool as defined by a salinity $>$ 1.5% (Seck et al., 2019). However, the mean salinity of here cultured samples was 0.7%, hence the low prevalence of *T. halophilus* in the cultured samples.

To characterize this strain of *T. halophilus*, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Wissembourg, France) on the colonies obtained on agar as previously described (Seng et al., 2009, Seng et al., 2013) and also performed an antibiogram with E-tests (bio-Mérieux, Craponne, France) using 11 of the most prescribed antibiotics in human pathologies (amoxicillin, vancomycin, erythromycin,

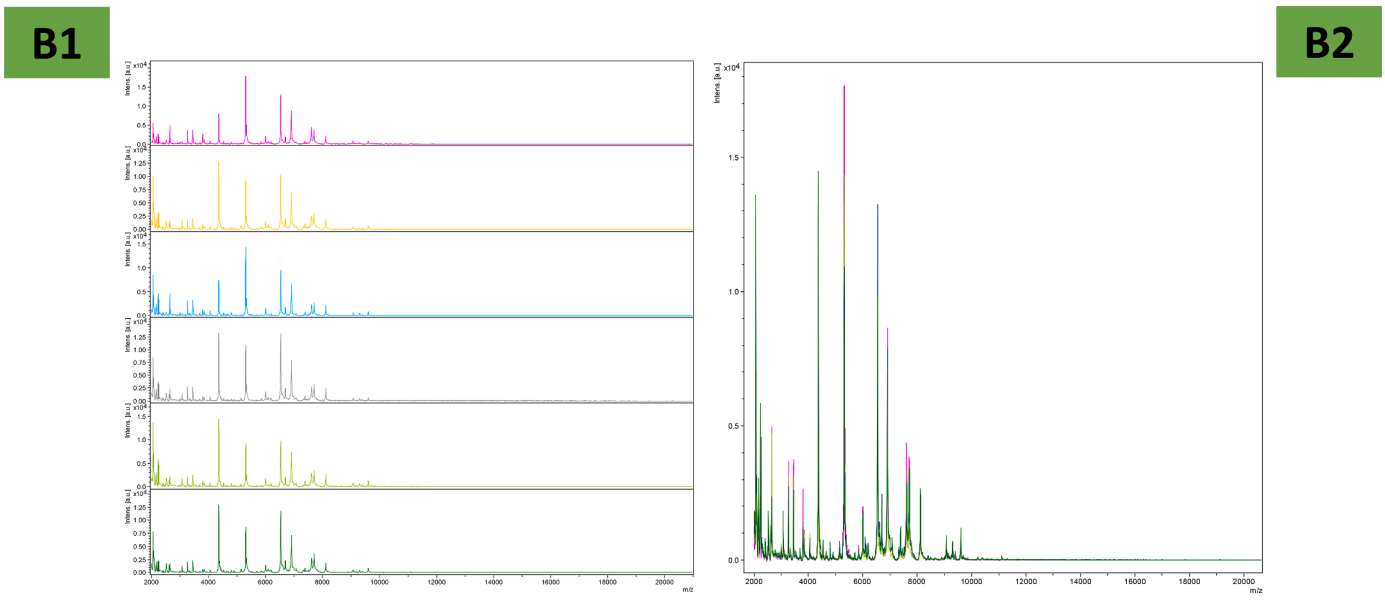

Fig. B. Spectral profiles of *T. halophilus* CSURQ6002. **(B1)** Spectra from six different deposits of *T. halophilus* CSURQ6002. **(B2)** Spectral superposition of *T. halophilus* CSURQ6002.

clindamycin, fosfomycin, ciprofloxacin, benzylpenicillin, rifampicin, doxycyclin, imipenem, and colistin) according to a previously established protocol (Konate et al., 2021). Specifically, to perform E-tests, fresh *T. halophilus* colonies were resuspended in 0.9% NaCl to a 3 McFarland density. We obtained reproducible spectra with intensities at 10^4 **(Fig. B)**. These spectra were added to our MALDI-TOF MS database and are available on the website of the University-Hospital Institute (IHU) Méditerranée Infection on the link https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/urms-data-base/.

Using the E-test strips, the MIC was assessed for *T. halophilus* strain CSURQ6002: rifampicin (MIC at 0.002µg/mL), benzylpenicillin (MIC at 0.094 µg/mL), amoxicillin (MIC at 0.5 µg/mL), erythromycin (MIC at 2 µg/mL), clindamycin (MIC at 4 µg/mL), and vancomycin (MIC at 8 µg/mL). However, this strain showed a MIC up to 256 µg/mL for ciprofloxacin, fosfomycin, doxycyclin, imipenem, and colistin (**Appendix D**).

Using SEM, *T. halophilus* cells appeared as cocci with average diameter of 450 µm, shaped and grape-like clusters (**Fig. C**). At high magnification the surface of cells was not smooth.

Blast nucleotide on NCBI Blast database of the NGS data after assembly using spades on galaxy Europe online software, showed *T. halophilus* strain YJ1 (NCBI accession number: CP046246) at 99.98% sequence similarity, strain isolated from Salty fermented fish sauce in south Korea in 2018 (unpublished data). The *T. halophilus* strain YJ1 complete genome (NCBI accession number: CP046246.1), was used as reference sequence for mapping of total reads by CLC Genomics Workbench software. Mapping MiSeq, iSeq and MinION-generated reads yielded 2,863,767 reads (59%) were matched to the reference genome, generating 2,511,991 nucleotides with 97-read depth. Annotation of identified *T. halophilus* CSURQ6002 (NCBI accession number: PRJNA780809) genome yielded 36.2% GC nucleotides, 0.82 gap ratio and 78% coding ratio including 2,694 encoding gene (CDSs), 15 rRNA genes, 62 tRNA genes and two genes encoding for CRISPRs. Whole genome phylogenetic analysis of the identified *T. halophilus* CSURQ6002 (NCBI accession number: PRJNA780809) with the six hit-blast strains genome recovered from GenBank NCBI database using OrthoANI Tool version (0.93.1), including *Methanohalophilus halophilus* strain Z-7982 (NCBI accession number: NZ_CP017921) as an out of the group, showed that the identified *T. halophilus* genome belonged to *T. halophilus*, sharing 97.37% whole genome sequence similarity with *T. halophilus* strain MJ4, isolated form myeolchi-jeotgal, Korean traditional fermented anchovy (NCBI accession number: NZ_CP012047.1) (Kim et al., 2018), 97.19% whole genome sequence similarity with *T. halophilus* strain KUD23 isolated from Doenjang, fermented soybeans (NCBI accession number: CP020017), and only 97.07% sequence similarity with the first hit-blast *T. halophilus* strain YJ1 (**Fig. D**). This was confirmed by pangenome analysis after annotation of the encoding genome fraction of the identified *T. halophilus* CSURQ6002 (accession number: PRJNA780809) and the first six-blast genomes recovered from GenBank database, yielded that the isolated bacteria strain belonged to the *T. halophilus* species genetically near to *T. halophilus* strain YJ1, first blast-result obtained by blast against GenBank database (**Appendix E**). Based on genomic data analysis, the isolated bacteria probably translocated to the digestive tracts by alimentation, illustrating the role of nutrition in the diversity of the human gut microbiota. Also, *in silico* analysis did not reveal any gene known to encode for virulence and antibiotic resistance, a result somewhat discrepant with E-test results as above, potentially due to the fact that online databases used for such investigations, do not contain halophilic genomes, therefore lacking references for antibiotic resistance.

The *T. halophilus* strain herein isolated has been deposited the Collection de Souches de l'Unité des Rickettsies (CSUR) as *T. halophilus* CSURQ6002.

In conclusion, the present study demonstrated for the first time, isolation by culture of *T. halophilus* from the human digestive tract and enabled the isolation and cultivation of the first strain of *T. halophilus* in humans. However, we observed a low prevalence of *T. halophilus* in humans, and its detection was associated with high stool salinity. This discovery therefore suggested that *T. halophilus* was part of the human

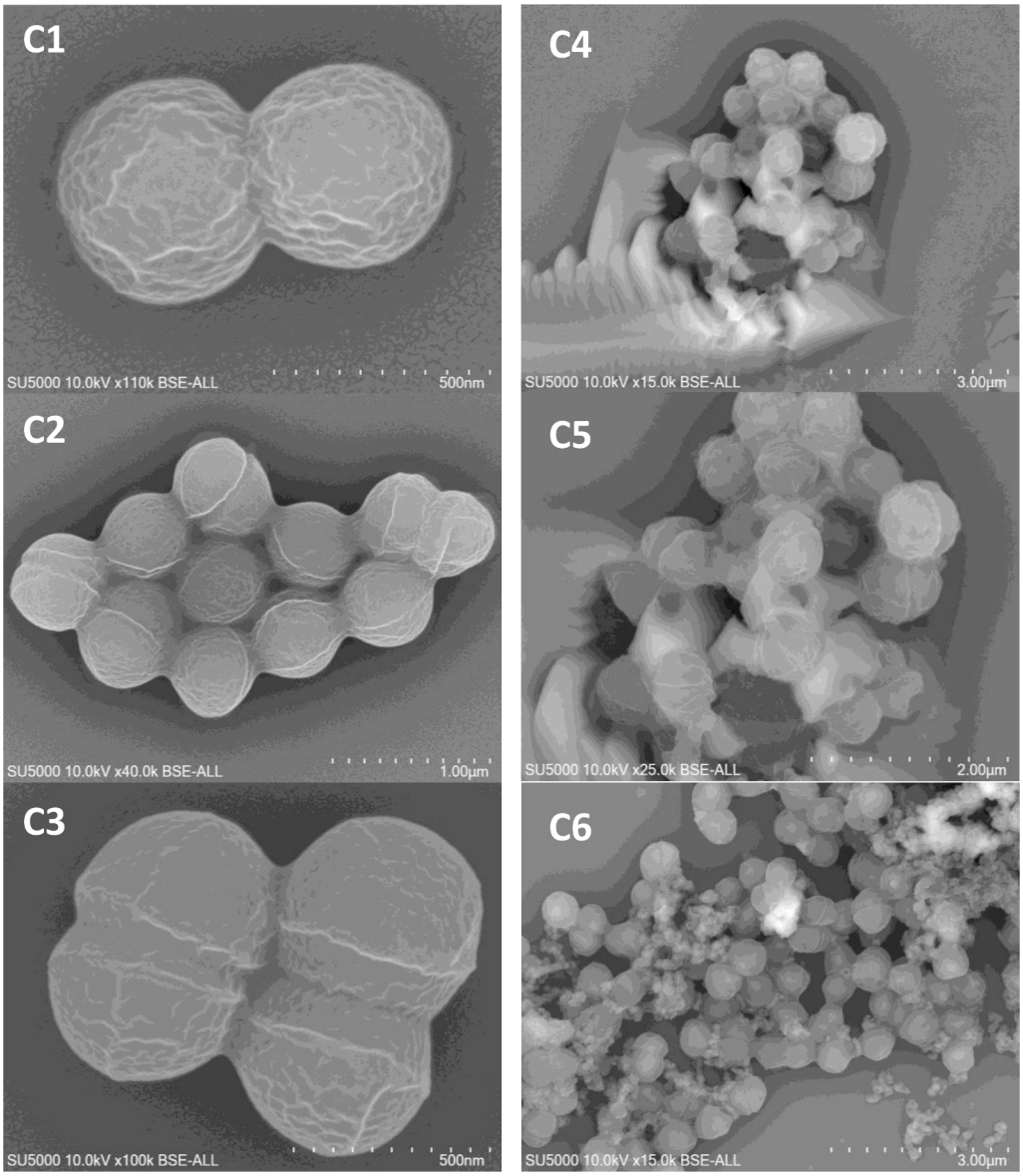

Fig. C. SEM micrographs of *T. halophilus* CSURQ6002. Fresh cultures were obtained from agar plates **(C1, C2, C3)**, as well as from liquid medium **(C4, C5, C6)**.

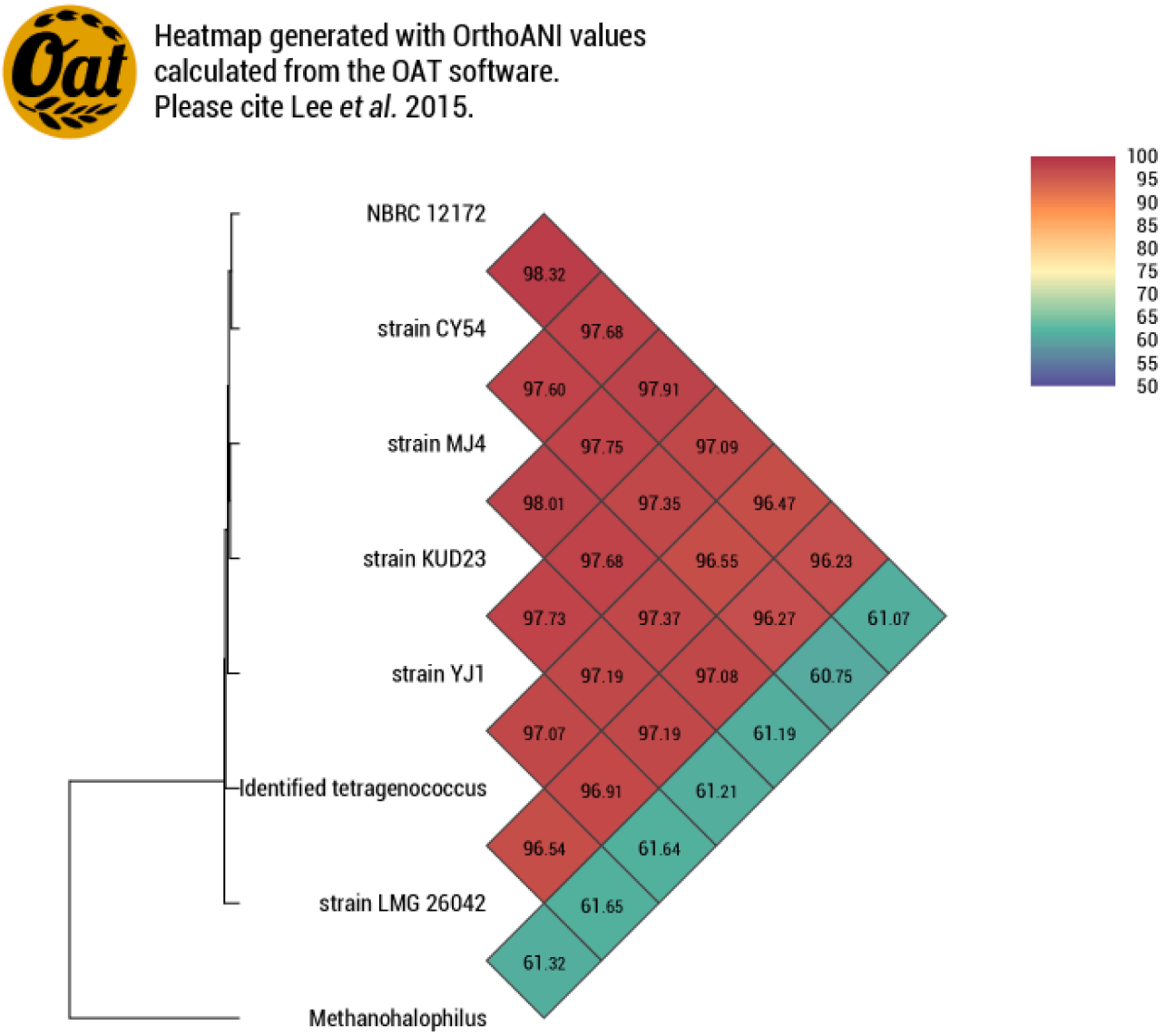

Fig. D. Phylogenetic analysis based on complete genome sequences of the identified *T. halophilus* and the six hit-blast strains recovered from NBCI GenBank nucleotide sequence database (https://www.ncbi.nlm.nih.gov/nucleotide/, accessed on 24 October 2021). The identified *T. halophilus* CSURQ6002 had 97.37% genome identity with *T. halophilus* strain MJ4, 97.19 with *T. halophilus* strain KUD23 and only 97.07% genome identity with the first hit-blast *T. halophilus* strain YJ1. The evolutionary history was inferred in Orthologous Average Nucleotide Identity Tool software used OrthoANI to measure overall similarity between two genome sequences. Unlike the original ANI algorithm, OrthoANI produces identical reciprocal similarities. It has been shown by a large comparison study, values generated by the original ANI and OrthoANI are comparable. The proposed cut-off for species demarcation is 95~96% for both OrthoANI and the original ANI.

digestive microbiota in some individuals, in whom its role on human health should be further considered (Masuda et al., 2008, Ohata et al., 2011).

Ethics approval

The study was previously approved by the Ethics Committee of the University-Hospital Institute (IHU) Méditerranée Infection under n° 2016-011, the Ethics Committee of the Faculty of Medicine and Odonto-Stomatology of Bamako, Mali, under n° 2014/46/CE/FMPOS, and the National Health Research Ethics Committee of Senegal under n°SEN16/45.

Credit author statement

COG contributed to the collection of samples, conducted the experiments, analyzed the data and wrote the paper; MM contributed to the whole genome sequencing and *in silico* data analysis and participated in the writing of the paper; SB contributed to the electron microscopy analysis and participated in the writing of the paper; MD designed the project, participated in the writing of the paper and provided great support carrying out the experiments; GG designed the project, helped conduct the experiments and participated in the writing of the paper. All authors have read and approved the manuscript.

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Declaration of Competing Interest

All the authors declare that there is no conflict of interest.

Acknowledgements

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Appendix

Appendix A. Program for T. halophilus DNA amplification reactions

An initial 15min denaturation step at 95°C was followed by 40 cycles of denaturation of 30s each at 95°C, a 45s annealing step at the appropriate Tm 55°C and a 60s extension at 72°C. Holding at 72°C for 5min allowed the complete extension of the PCR products.

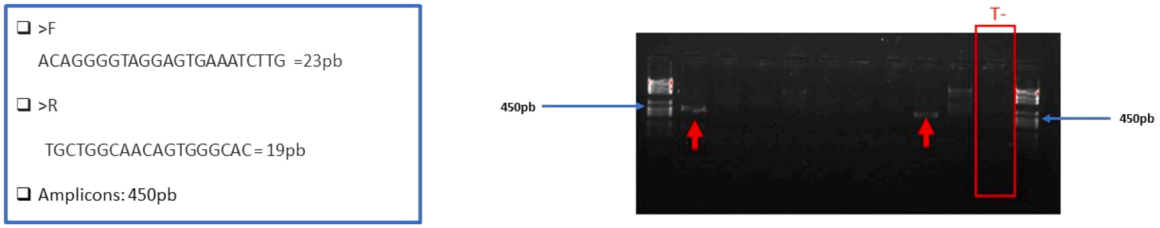
Appendix B. T. halophilus CSURQ6002 culture medium

NaCl	100.0 g
$MgSO_4 \bullet 7H_2O$	3.45 g
$MgCl_2 \bullet 6H_2O$	2.75 g
Sodium acetate	1.0 g
KCl	0.335 g
NH_4Cl	0.25 g
$CaCl_2 \bullet 2H_2O$	0.14 g
$K_2HPO_4 \bullet 3H_2O$	0.14 g
Resazurin	1.0 mg
$NaHCO_3$ solution	80.0 mL
Trimethylamine•HCl solution	20.0 mL
Na_2CO_3 solution	10.0 mL
Trace elements solution	10.0 mL
Vitamin solution	10.0 mL
L-Cysteine•HCl solution	10.0 mL
$Na_2S \bullet 9H_2O$ solution	10.0 mL
Distilled water	860.0 mL

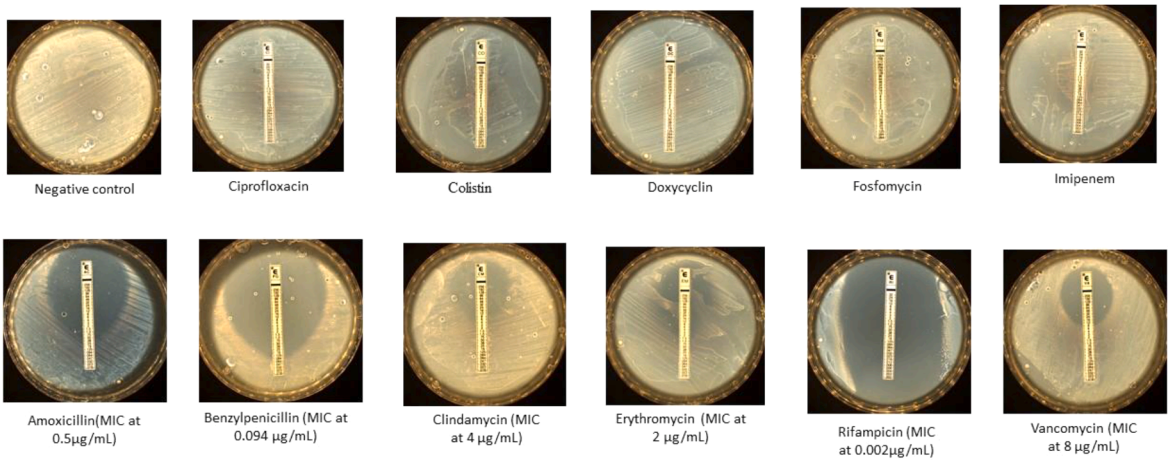
pH 6.9 ± 0.2 at 25°C

Preparation of Medium: Add components, except $NaHCO_3$ solution, trimethylamine•HCl solution, Na_2CO_3 solution, vitamin solution, L-cysteine•HCl solution, and $Na_2S \bullet 9H_2O$ solution, to distilled/deionized water and bring volume to 860.0mL. Mix thoroughly. Sparge with 100% N_2 for 20 min. Then sparge with 80% N_2 + 20% CO_2 for 10 min. Anaerobically distribute into tubes or bottles. Autoclave for 15 min at 15 psi pressure–121°C. Aseptically and anaerobically add 80.0mL of sterile $NaHCO_3$ solution, 20.0mL of sterile trimethylamine•HCl solution, 10.0mL of sterile Na_2CO_3 solution, 10.0mL of sterile vitamin solution, 10.0mL of sterile L-cysteine•HCl solution, and 10.0mL of sterile $Na_2S \bullet 9H_2O$ solution. Mix thoroughly.

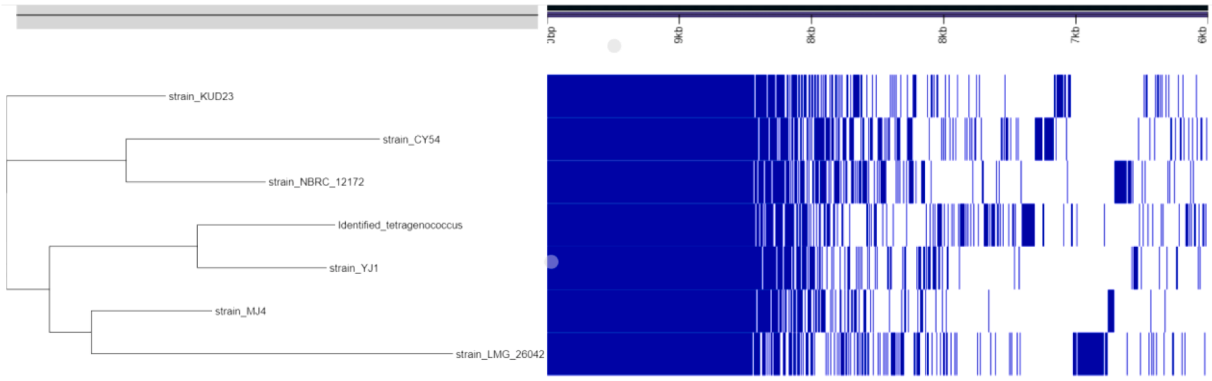
Appendix C. PCR electrophoresis picture of T. halophilus CSURQ6002



Appendix D. Antibiotic susceptibility of T. halophilus CSURQ6002



Appendix E. Pangenome analysis of the identified-Tetragenococcus (T. halophilus CSURQ6002) and the first six hit-blast genomes recovered from GenBank database



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