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, *Just* 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, *Just* 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., 2019, *Just* et al., 2019). This finding was confirmed by the sequencing of the first genome of this human gut isolate (*Kumazawa* et al., 2018, *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, *Khalafia* et al., 2017, *Khalafia* 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...
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- TGCTGGCAACAGTGCGCAC-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-Claiix, France) with a CO₂ 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 protease K (Qiagen) was incubated over night at 56°C. After protease 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).
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 authenticated 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, chloramphenicol, cefazolin, tetracycline, clindamycin, ceferazolin, cotrimoxazol, erythromycin, amikacin, linezolid) and also performed the neisseria test. The colonies obtained exhibited a 100% similarity with 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.
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 10^3 (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 0.002 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 (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 97.57% 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 pangeneome 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 tract 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

![B1](image1.png)  
![B2](image2.png)  

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.
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).
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 Odontology-Stomatolgy 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.

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Appendix

Appendix A. Program for T. halophilus DNA amplification reactions

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

Appendix B. T. halophilus CSURQ6002 culture medium

| Component            | Concentration |
|----------------------|---------------|
| NaCl                 | 100.0 g       |
| MgSO₄•7H₂O           | 3.45 g        |
| MgCl₂•6H₂O          | 2.75 g        |
| Sodium acetate      | 1.0 g         |
| KCl                  | 0.335 g       |
| NH₄Cl               | 0.25 g        |
| CaCl₂•2H₂O          | 0.14 g        |
| K₂HPO₄•3H₂O         | 0.14 g        |
| Resazurin           | 1.0 mg        |
| NaHCO₃ solution     | 80.0 mL       |
| Trimethylamine•HCl solution | 20.0 mL |
| Na₂CO₃ solution     | 10.0 mL       |
| Trace elements solution | 10.0 mL |
| Vitamin solution    | 10.0 mL       |
| L-Cysteine•HCl solution | 10.0 mL |
| Na₂S•9H₂O solution  | 10.0 mL       |
| Distilled water     | 860.0 mL      |

pH 6.9 ± 0.2 at 25°C

Preparation of Medium: Add components, except NaHCO₃ solution, trimethylamine•HCl solution, Na₂CO₃ solution, vitamin solution, L-cysteine•HCl solution, and Na₂S•9H₂O solution, to distilled/deionized water and bring volume to 860.0 mL. Mix thoroughly. Sparge with 100% N₂ for 20 min. Then sparge with 80% N₂ + 20% CO₂ for 10 min. Anaerobically distribute into tubes or bottles. Autoclave for 15 min at 15 psi pressure – 121°C. Aseptically and anaerobically add 80.0 mL of sterile NaHCO₃ solution, 20.0 mL of sterile trimethylamine•HCl solution, 10.0 mL of sterile Na₂CO₃ solution, 10.0 mL of sterile vitamin solution, 10.0 mL of sterile L-cysteine•HCl solution, and 10.0 mL of sterile Na₂S•9H₂O solution. Mix thoroughly.

Appendix C. PCR electrophoresis picture of T. halophilus CSURQ6002

![PCR electrophoresis picture of T. halophilus CSURQ6002](image)
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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