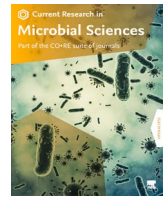


Contents lists available at [ScienceDirect](https://www.sciencedirect.com)

# Current Research in Microbial Sciences

journal homepage: [www.sciencedirect.com/journal/current-research-in-microbial-sciences](https://www.sciencedirect.com/journal/current-research-in-microbial-sciences)

## Introducing clinical nanoarchaeology: Isolation by co-culture of *Nanopusillus massiliensis* sp. nov.

Y. Hassani <sup>a,b</sup>, J. Saad <sup>a,b</sup>, E. Terrer <sup>a,c</sup>, G. Aboudharam <sup>a,c</sup>, B. Giancarlo <sup>d</sup>, F. Silvestri <sup>c</sup>,  
D. Raoult <sup>a,b</sup>, M. Drancourt <sup>a,b,1</sup>, G. Grine <sup>a,c,1,\*</sup>

<sup>a</sup> Aix-Marseille-Univ., IRD, MEPHI, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, Marseille 13005, France

<sup>b</sup> IHU Méditerranée Infection, Marseille 13005, France

<sup>c</sup> Faculté de médecine dentaire, Aix-Marseille Université, Marseille 13005, France

<sup>d</sup> Private practice Marseille France, Marseille, France

### ARTICLE INFO

#### Keywords:

*Nanopusillus massiliensis*

Nanoarchaea

Oral cavity

Classification

major categories

### ABSTRACT

**Background:** Nanoarchaeota, obligate symbiont of some environmental archaea with reduced genomes, have been described in marine thermal vent environments, yet never detected in hosts, including humans.

**Methods:** Here, using laboratory tools geared towards the detection of nanoarchaea including PCR-sequencing, WGS, microscopy and culture.

**Results:** We discovered a novel nanoarchaea, *Nanopusillus massiliensis*, detected in dental plate samples by specific PCR-based assays. Combining fluorescent *in situ* hybridization (FISH) with scanning electron microscopy disclosed close contacts between *N. massiliensis* and the archaea *Methanobrevibacter oralis* in these samples. Culturing one sample yielded co-isolation of *M. oralis* and *N. massiliensis* with a 606,935-bp genome, with 23.6% GC encoded 16 tRNA, 3 rRNA and 942 coding DNA sequences, of which 400 were assigned to clusters of orthologous groups.

**Conclusion:** The discovery of *N. massiliensis*, made publicly available in collection, extended our knowledge of human microbiota diversity, opening a new field of research in clinical microbiology here referred to as clinical nanoarchaeology.

### Introduction

Nanoarchaea are among the smallest known cellular organisms, exhibiting a 400-nm diameter on average, hosting a small, hundreds of mega bases genome featuring an extensive reduction in the number of protein and tRNA genes, resulting in massive loss of biosynthetic capabilities, suggesting a parasitic-type of life (Casanueva et al., 2011; John et al., 2019; Nakai, 2020). The first-ever detected *Nanoarchaeum equitans* was accordingly co-cultured with hosting archaea *Ignicoccus hospitalis* from deep-sea hydrothermal vents at the Kolbeinsey ridge, north of Iceland, and several lines of microscopy observations demonstrated that both organisms were tightly attached to each other (Huber et al., 2002). The discovery of *N. equitans* opened a two-decade long study of nanoarchaea in so-called extreme environments, and nanoarchaea have been detected in extreme hot, acidophilic, and hypersaline environments and in temperate lake and marine sediments (Castelle and Banfield, 2018).

The search for nanoarchaea has been hampered by their extremely small size and nonspecific cocci-like morphology, escaping routine microscopic observations, while the unique structure of the 16S rRNA gene in nanoarchaea made them undetectable by 16S rRNA gene detection (Casanueva et al., 2008; Baker et al., 2003). This situation has continued in clinical microbiology laboratories, which are not archaea-oriented, up to the present work. Indeed, our laboratory and a few others developed microscopy, nucleic acids-based and culture methods targeted to the detection of archaea in clinical samples representative of physiological microbiota, notably those in the oral cavity and gut (Nguyen-Hieu et al., 2013; Grine et al., 2018; Nkamga et al., 2017) and of pathological dysbiosis (Huynh et al., 2017; Belkacemi et al., 2018; Scanlan et al., 2008; Million et al., 2016; Grine et al., 2019), abscesses and more recently archaeamia (Nkamga et al., 2018; Nkamga et al., 2016; Drancourt et al., 2020).

In this report, after having developed laboratory tools geared

\* Corresponding author at: Aix-Marseille-Univ., IRD, MEPHI, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, Marseille 13005, France.

E-mail address: [grineghiles@gmail.com](mailto:grineghiles@gmail.com) (G. Grine).

<sup>1</sup> These authors contributed equally to this work.

<https://doi.org/10.1016/j.crmicr.2021.100100>

Received 11 October 2021; Received in revised form 13 December 2021; Accepted 13 December 2021

Available online 20 December 2021

2666-5174/© 2021 The Authors.

Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

towards the specific detection of nanoarchaea in clinical samples, we report the unprecedented observation of nanoarchaea in the human oral cavity microbiota, culminating with the whole genome sequence-based discovery of the novel *Nanopusillus massiliensis*.

## Materials and methods

**Sample collection.** This study included a series of dental plaque samples collected between December 2019 and August 2020 from patients in the Odontology Department, Timone Hospital, Marseille. Dental plaque samples, considered waste, usually aspirated, and discarded, were collected through the suction cannulas. All patients received information about the study and gave informed consent prior to the investigation. Accordingly, dental plates were collected anonymously using a sterile Gracey dental curette (Hu-Friedy, Rotterdam, Netherlands) or a sterile micro brush. All samples were immediately placed into a Hungate tube (Dominique Dutscher, Brumath, France) containing 5 mL of a specific transport SAB medium previously described (Khelaifia et al., 2013).

**PCR-based detection of nanoarchaea.** Examining the pangenome of 345 archaea and two nanoarchaea *N. equitans* (Waters et al., 2003) (GenBank accession number AE017199.1) and *Candidatus N. acidilobi* (Wurch et al., 2016) (GenBank accession number CP010514.1) using the Roary pan-genome pipeline in Galaxy software (<https://usegalaxy.org.au/>) (SI Appendix, Fig. S1), we determined that the 30S SSU L12 gene conserved among these 347 organisms exhibited a species-specific sequence. More specifically, the 873-bp nanoarchaea 30S SSU L12 gene exhibited sequence similarity < 95% with homologous sequences in methanogenic archaea (here designed as methanogens). A PCR primer pair (forward primer: 5'-TGAAAGCAAAGGGATTTTATTCA-3'; reverse primer 5'-TTGCATGTGGAACAATACCAG-3') (Eurogentec, Seraing, Belgium) was designed using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to specifically amplify a 188-bp fragment of the nanoarchaea 30S SSU L12 gene and specify nanoarchaea species, by sequencing. PCR primers were incorporated into a 50 µL-volume containing 25 µL Amplitaq Gold® (Thermo Fisher Scientific, Illkirch-Graffenstaden, France), 2 µL each primer (10 pM), 16 µL Dnase/Rnase-free distilled water (Gibco, Cergy-Pontoise, France) and 5 µL of extracted DNA. DNA was extracted on dental plates using 15-minute sonication followed by automatic extraction using the EZ1 Advanced extraction kit (QIAGEN, Hilden, Germany) as previously described (Drancourt et al., 2020). The reaction mixture was then subjected to a 40-cycle PCR program comprising a 30-second denaturation step at 95 °C, followed by 45-second hybridization at 60 °C and 1-minute elongation at 72 °C. Each amplification program started with denaturation at 95 °C for 15 min and ended with a final elongation step at 72 °C for 5 min. Non-inoculated transport medium was used as a negative control. PCR products were sequenced as previously described (Dridi et al., 2009). In parallel, samples along with negative controls were assayed for the presence of methanogens by PCR-sequencing targeting the broad range archaeal 16S rRNA gene SDArch0333aS15, 5-TCCAGGCC-TACGGG-3 and SDArch0958aA19, 5-YCCGGCGTTGAMTCCAATT-3) (Grine et al., 2019). PCR products were sequenced using the same primers as used for PCRs, as previously reported (Dridi et al., 2009).

**Microscopic observations of nanoarchaea.** Nanoarchaea PCR-positive clinical samples were examined by microscopy with and without sample deglycosylation aimed at degrading biofilms and facilitating further microscopic observations. As for deglycosylation, 140 µL of sample were incubated for one hour with 20 µL Endo Hf (New England Biolabs, Evry-Courcouronnes, France) at 37 °C. As for scanning electron microscopy, 100 µL of 2.5% glutaraldehyde-fixed sample were cytocentrifuged onto a glass slide (Shandon cytospin, Thermo Scientific, Waltham, MA, USA), stained for 2 min by 1% phosphotungstic acid (Sigma Aldrich, Saint-Louis, MO, USA) at room temperature and observed using a TM4000 Plus scanning electron microscope (Hitachi, Tokyo, Japan). Also, fluorescent *in situ* hybridization (FISH) incorporated the archaea-specific

Arch915 probe Alexa 647 (5'-GTGCTCCCCGCCAATTCCT-3') and the nanoarchaeota 16S rRNA gene probe 515mcr2 probe Alexa488 (5'-CCCTCTGGCCACTGCT-3'), as previously described (Wurch et al., 2016).

**Nanoarchaea genome analyses.** Total DNA extracted from one nanoarchaea PCR positive sample was sequenced three times on the MiSeq platform (Illumina Inc, San Diego, CA, USA) using the Nexera XT DNA sample prep kit (Illumina), with the paired end strategy. The tagmentation step fragmented and tagged each extracted DNA to prepare the paired-end library. A limited PCR amplification (12 cycles) was then performed to complete the tag adapters and to introduce dual-index barcodes. DNA was then purified on AMPure XP beads (Beckman Coulter Inc, Fullerton, CA, USA). In addition, according to the Nextera XT protocol (Illumina), all libraries were normalised on specific beads. We then pooled all libraries into one library for DNA sequencing on MiSeq. The pooled single strand library was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and paired end sequencing with dual index reads were performed in a single 39-hour run in 2 × 250-bp. For each sample/purified nanoarchaea DNA sequence, the quality of each Illumina read was checked by FastQC and trimmed using trimmomatic version 0.36.6. Sequence reads were concatenated and assembled using SPAdes version 3.5.0 software (Bankevich et al., 2012). Finishing was achieved by LongRange PCR (LR-PCR) followed by next-generation sequencing after PCR primers were designed by aligning nanoarchaea on-going genome sequence with *Candidatus N. acidilobi* reference genome, targeting regions to be finished (SI Appendix, Table S5). Purified LR-PCR products were sequenced using the Illumina Iseq technique as previously described (Tulloch et al., 2021). Contigs were identified using Blastn against NCBI database and concatenated reads were mapped using CLCgenomic version 7 against the *Candidatus N. acidilobi* genome sequence. The nanoarchaea genome, annotated using Prokka software (Seemann, 2014), was added to a nanoarchaea pangenome analysis performed using Roary pan-genome pipeline, as above. Genomic similarity of the on-going nanoarchaea with closely related species was estimated using the OrthoANI: An improved algorithm and software for calculating average nucleotide identity | Microbiology Society (2021) and digital DNA-DNA hybridization (dDDH) performed using Type (Strain) Genome Server (TYGS) (<https://tygs.dsmz.de/>) (Meier-Kolthoff et al., 2014). The new nanoarchaea genome was then incorporated into a rhizome representation, using a previously described protocol (Togo et al., 2019). Putative encoded protein functions were searched against the Clusters of Orthologous Groups (COG) database using BLASTP (E-value 1e-03, coverage 0.7 and identity percent 30%). Secretion systems were searched via the Mac-SyDB/TXSSdb database. Further metabolic pathway repertoire was established with a RAST server. Lastly, *in silico* antibiotic resistance profiling of *N. massiliensis* was determined according to Abricate, Resfinder, Card and Argannot databases with a minimum of 10% identity.

**Co-culturing nanoarchaea.** A dental plaque sample positive for methanogens and nanoarchaea by PCR-sequencing was collected in a Hungate tube (Dominique Dutscher) containing 3 mL of transport SAB medium (Khelaifia et al., 2013). As for inoculation, a culture bottle was prepared under a strict anaerobic atmosphere in a BACT/ALERT® blood culture bottle (bioMérieux, Craponne, France) emptied of its original broth and atmosphere, replaced by 30 mL of SAB medium (Khelaifia et al., 2013) used as an antibiotic-free enrichment medium under an 80% H<sub>2</sub>-20% CO<sub>2</sub> atmosphere. Then, one milliliter of inoculated SAB transport medium was inoculated into the conditioned bottle, incubated for 15 days in a Virtuo apparatus (bioMérieux) at 37 °C under constant shaking. After 15-day incubation, CH<sub>4</sub> production was detected in the positive polarity using helium as reference gas in a Clarus 580 gas chromatograph (Perkin Elmer, Villebon-sur-Yvette, France). After methane was detected, 17 mL of broth were ultracentrifuged at 25,000 g for two hours at 4 °C using SORVALL Discovery 90SE with Surespin 630 rotor (Kendro Laboratory Product, Burladingen, Germany). The pellet

was observed by scanning electron microscopy and FISH in the presence of a pure culture of *M. oralis* strain VD9 (*Methanobrevibacter oralis* partial 16S rRNA gene, strain VD9 2015) (demonstrated to be nanoarchaea-free by PCR) used as a nanoarchaea negative control, using methods above described. After observation of pelleted methanogens and nanoarchaea, the pellet was deglycosylated as described above prior to its inoculation in SAB medium supplemented with a vitamin cocktail (Sigma Aldrich, Merck) (SI Appendix, Table S2), 0.1 g D-fructose (Sigma Aldrich), a fatty acid mixture (1 mL of a solution of isobutyric acid, 2-methylbutyric acid, isovaleric acid and valeric acid) (Sigma Aldrich) and 5% of 0.22  $\mu\text{m}$ -filtered bovine rumen. Transport medium inoculated with phosphate-buffered saline was incorporated as a negative control. Co-culture was metagenome-sequenced by using a home-adapted NovaSeq protocol (Illumina Inc.) (SI Appendix, 1) In parallel in the perspective of methanogen isolation, 200  $\mu\text{L}$  of 15-day incubation broth culture were inoculated onto SAB solid medium as previously described (Khelaifia et al., 2013).

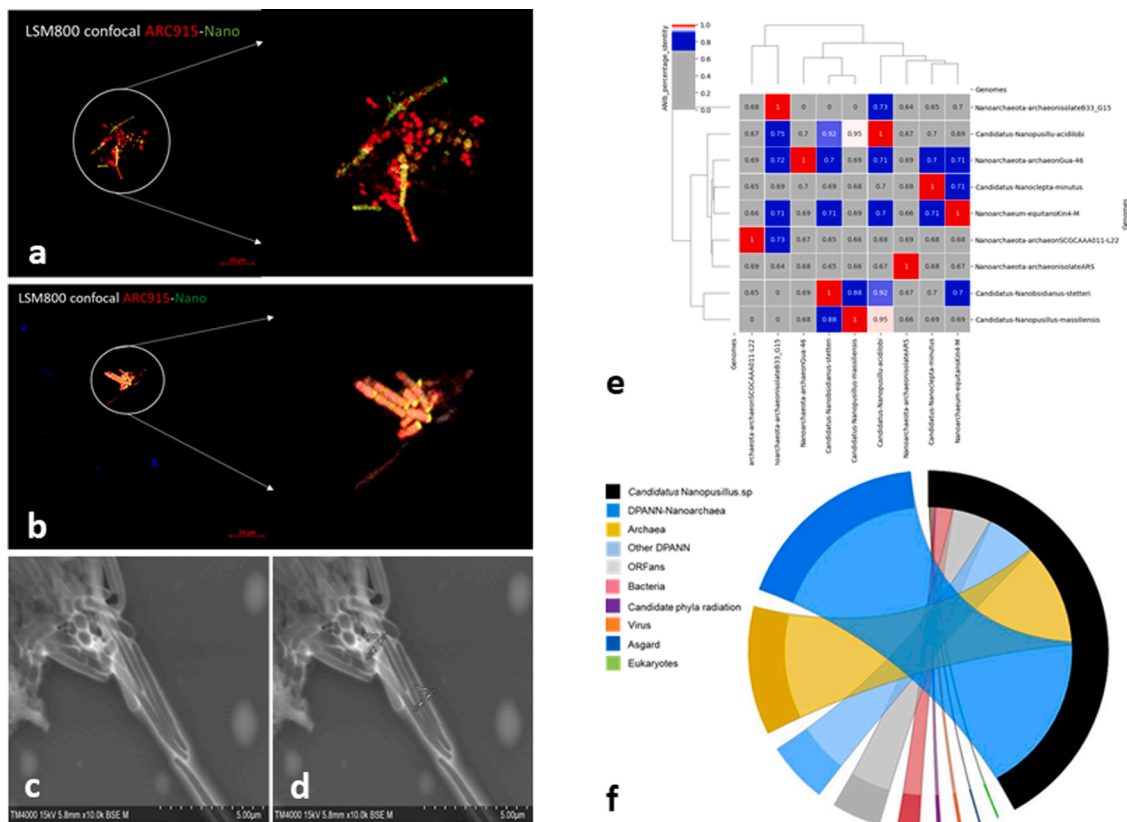
## Results

**Molecular detection of *N. massiliensis*.** A total of 102 samples collected from 102 patients were investigated for the presence of methanogens and nanoarchaea by using specifically designed archaeal (16S rRNA gene) and nanoarchaea (30S L12 gene) gene-based PCR systems, as described below. While all the negative controls introduced in every batch of PCR remained negative, 22 (21.6%) methanogen 16S rRNA gene amplicons were detected dental plate samples. Sequencing

indicated that the 15/22 dental plaque amplicons exhibited 99% sequence similarity with reference 16S rRNA gene sequence of *M. oralis* strain VD9 (accession NCBI: LN898260.1); 2 dental plaque amplicons exhibited 99.8% sequence similarity with the reference 16S rRNA gene sequence of *M. oralis* strain M2 CSUR P5920 (accession NCBI: LR590665.1); and 5 dental plaque amplicons exhibited 99% sequence similarity with the reference 16S rRNA gene sequence of *Methanobrevibacter massiliensis* N58C (accession NCBI: LN610763.1). Further, in the negativity observed in the negative controls, nanoarchaea were PCR-detected in 5 (4.90%) dental plate samples collected from 5 different patients. Sequencing indicated that these 5 amplicons exhibited 95.7% sequence similarity and 100% cover with the homologous 30S L12 gene sequence of *Candidatus* Nanopusillus acidilobi (accession NCBI CP010514.1), indicative of a novel nanoarchaea, here reported as *Nanopusillus massiliensis* sp. nov., co-detected with *M. oralis* in 4 dental plate samples and with *M. massiliensis* in 1 dental plate sample.

**Microscopic observation of *N. massiliensis*.** FISH detected nanoarchaea-forming cocci attached to the surface of a methanogen, for which bacillary morphology suggested *M. oralis*, in 4 nanoarchaea PCR-positive dental plates (Fig. 1A and B). Further scanning electron microscopy of these 4 samples showed 300–500 nm diameter cells attached to the surface of *M. oralis* cells, exhibiting a characteristic diameter of < 0.7–1.2  $\mu\text{m}$  and elongated morphology (Ferrari et al., 1994) (Fig. 1C and D), whereas such small cells were not observed in the negative controls (SI Appendix, Fig. S2).

***N. massiliensis* genome features.** After three runs of MiSeq sequencing, a dental plaque sample PCR-positive for nanoarchaea and *M. oralis*



**Fig. 1.** a, b, Fluorescent *in situ* hybridization representative detection of *Nanopusillus massiliensis* and *Methanobrevibacter oralis* in dental plaque. (A) overlay of universal DNA DAPI staining exhibiting blue microorganisms, red ARC 915 and green nanoarchaeota 515mcR2 probes exhibiting organisms with the coccis form stuck to the surface of a methanogen. Scale bar, 10  $\mu\text{m}$ . c-d, nanoarchaeota in dental plaque were observed using scanning electron microscopy with TM4000PLUS (Hitachi). e, Rhizome representation of *Candidatus Nanopusillus massiliensis* based on the protein sequences. The best hit for each coding gene was chosen following a comparison using BLASTp, according to the following criteria: minimum of identity: 30% minimum of coverage: 40% and maximum evaluate: 0.001. The presentation of rhizome was built using Power BI software. f, OrthoANI heatmaps between *Nanopusillus massiliensis* and other Nanoarchaea genomes. OrthoANI matrix was visualized using MORPHEUS software (<https://software.broadinstitute.org/morpheus/>).

yielded 3125,000 paired end reads and the generated read length is between 1 and 3000 bp, which were assembled and blasted against the nr database. After 12 *Methanobrevibacter* sp. contigs were detected, remaining reads were mapped against the *Candidatus N. acidilobi* reference genome using 0.5 length fraction and 0.3 similarity fraction as mapping parameters, to recover a 606,947-bp unique contig exhibiting a gap ratio of 19%, with N-bearing regions varying from two bp to 1200-pb, representative of a draft genomic sequence of *N. massiliensis*. Finishing yielded a unique 606,947-bp long chromosome sequence (WGS accession number: CAKLBW000000000; 16S rRNA gene sequencing accession: Q6268) with 23.6% GC encoding 16 tRNA, 3 rRNA and 942 coding DNA sequences, of which 400 were assigned to clusters of orthologous groups (COGs) (SI Appendix, Table S3). A nine-nanoarchaea pangenome, including *N. massiliensis*, incorporated 5294 genes, including 4329 cloud genes and 959 shell genes. Six genes were common to the 9 nanoarchaea genomes; they encoded the 50S ribosomal protein L18, 50S ribosomal protein L23, 30S ribosomal protein S19, 30S ribosomal protein S5, DNA-directed RNA polymerase subunit K and replication factor C small subunit (SI Appendix, Fig. S3). We observed that single nucleotide polymorphisms (SNPs) varied from 29 SNPs between the novel nanoarchaea and *Candidatus N. acidilobi* up to 615 SNPs between *N. massiliensis* and Nanoarchaeota archaeon isolate B33\_G15 (SI Appendix, Fig. S4 and Table S4). The maximum OrthoANI value was 95.21% between new nanoarchaea *N. massiliensis* and *Candidatus N. acidilobi* (SI Appendix, Fig. S6), which exhibited a maximum dDDH value of 69.4 (confidence interval = [66.4 - 72.2]) (SI Appendix Table S5). A mosaic representation of rhizome incorporating 1457 genes indicated that 618 (42.4%) novel nanoarchaea genes overlapped with DPANN-nanoarchaea, 191 (13.1%) with other members of the DPANN superphylum (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota and Nanohaloarchaeota, Micrarchaeota, Woesearchaeota), 399 (27.38%) with Archaea, 69 (4.73%) with Bacteria, 12 (0.82%) with Candidate phyla radiation, 10 (0.68%) with virus, 3 with Asgard and 3 with Eukaryota, whereas 157 (10.57%) genes were orphans (Fig. 1F). Furthermore, no antibiotic resistance determinant was detected, nor were any secretion systems identified.

***N. massiliensis* metabolism.** A Venn diagram incorporating enzymes detected by RAST annotation in *N. massiliensis*, *Candidatus N. acidilobi* and *M. oralis* genomes detected five enzymes unique to *N. massiliensis*: *i.e.*, lead, cadmium, zinc and mercury transporting ATPase, copper-translocating P-type ATPase, DNA reverse gyrase, alkaline phosphatase and cell division protein FtsH. A total of 108 enzymes were unique to *M. oralis* and 14 enzymes were common to *M. oralis*, *Candidatus N. acidilobi* and *N. massiliensis* (SI Appendix, Fig. S5 and Table S6). The *N. massiliensis* genome encoded seven enzymes participating in glycolysis. Likewise, the *N. massiliensis* genome encoded five gluconeogenesis enzymes and enzymes involved in the complete synthesis and degradation of glycogen. RAST annotation showed that *N. massiliensis* was not able to synthesize purines and pyrimidines, nor had any alternative metabolism to obtain the nucleoside monophosphates, diphosphates and triphosphates for the synthesis of nucleic acids. Instead, we detected ribonucleoside-diphosphate reductase subunit alpha (providing necessary precursors for DNA synthesis), catalyzing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides. Similarly, *N. massiliensis* was not found to be able to synthesize amino acids, whereas 19 aminoacyl-tRNA biosynthesis genes were detected. *N. massiliensis* had the potential to catabolize inorganic sulfur compounds: we detected sulfate adenylyltransferase that may catalyze the formation of adenosine 5'-phosphosulfate from ATP and inorganic sulfate. As described for other nanoarchaea, *N. massiliensis* lacked a membrane ATP synthase complex as well as all components participating in the respiratory chain, but encoding a reduced repertoire of transporters, including two ATP-binding cassette (ABC) transporters and a Major facilitator superfamily (MFS) transporter. We detected only one protein (FlaL) of the archaeum complex (archaeal flagellum), leaving unknown whether *N. massiliensis* has motility (Fig. 2).

***N. massiliensis* isolation by co-culture.** After PCR-sequencing co-detected *N. massiliensis* with *M. oralis*, suggesting that *M. oralis* could host *N. massiliensis*, we used an enrichment medium promoting *M. oralis* growth and, consequently, growth of *M. oralis*-associated nanoarchaea to inoculate one dental plaque sample. After methane was detected at day 15, PCR-sequencing targeting 30S was positive on both supernatant and pellet issuing from this culture. However, only the pellet, but not the supernatant, yielded cocci-like nanoarchaea sized 200–400 nm, either as free cells or located around *M. oralis* cells, as observed by scanning electron microscopy in parallel to fluorescent *in situ* hybridization, the observations being enhanced by deglycosylation of the pellet (Fig. 3). In parallel, colonies of hosting *M. oralis* observed at day 10 were firmly identified as *M. oralis* (strain YH) by PCR-sequencing of the 16S rRNA gene (The 16S rRNA gene sequence accession is OU484279, the GenBank access number of genome is CAJVUI000000000), exhibiting 100% sequence similarity with the homologous 16S rRNA gene sequence of *M. oralis* strain VD9 (accession NCBI: LN898260.1). *M. oralis* strain YH colonies were sub-cultured on liquid SAB medium, and viability and culture of the strain were monitored by CH<sub>4</sub> detection, as described above. *M. oralis* strain YH whole genome sequence (1.9 Mp) yielded a DDH value of 97% with reference *M. oralis* strain M2 CSUR P5920, firmly confirming the identity of strain YH, which has been made available by depository in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM875) as CSUR Q6267, while the co-culture *M. oralis*-*N. massiliensis* has been deposited under number CSUR Q6268.

#### Description of the novel species *Nanopusillus massiliensis* sp. nov

We report co-culture isolation of *Nanopusillus massiliensis* as the first co-isolated member of Nanoarchaeota of human origin, a strain representative of a new species in the previously described genus *Nanopusillus* within the phylum Nanoarchaeota. Its formal description is provided below.

#### *Nanopusillus massiliensis* sp. nov

**Etymology.** Nanus (Latin adj.) 'dwarf', referring to its size and denoting placement in the Nanoarchaeota; pu'sil.lus (Latin adj.) very small, indicating its extremely small size, at the limit of cellular life, like its closest neighbor *Nanopusillus acidilobi*; mas.si.li.en'sis. L. fem. adj. massiliensis referring to Massilia, the past Roman name of Marseille, France where this nano-organism has been discovered.

Coccioid cells, 150–400 nm in diameter, obligate ectosymbionts/parasites on the surface of the methanogen *Methanobrevibacter oralis*. Occasional free cells may be observed in the co-culture, which viability is unknown. Optimal growth occurs in co-culture with its host at 37 °C and pH 7. Initially isolated from human dental plaque. *N. massiliensis* has been deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR) under CSUR Q6268.

#### Discussion

We here report the unprecedented observation of nanoarchaea in clinical specimens, authenticated by concurring results obtained in the very same clinical samples by unrelated laboratory methods consisting of microscopic observations, molecular investigations culminating in one whole genome sequence and isolation in co-culture in the presence of negative controls introduced in every experimental step; for a group of nano-organisms that had never been previously studied in our laboratory.

This discovery was made possible after we developed nanoarchaea-oriented specific laboratory tools, capitalizing on the previous expertise that we have acquired over years in the clinical microbiology of archaea, methanogens and halophilic archaea (Khelaifia et al., 2018), including their potential role in pathological situations in dysbiosis (Grine et al., 2018), anaerobe abscesses (Nkamga et al., 2018; Nkamga et al., 2016; Drancourt et al., 2017) and archaeamia (Drancourt et al.,

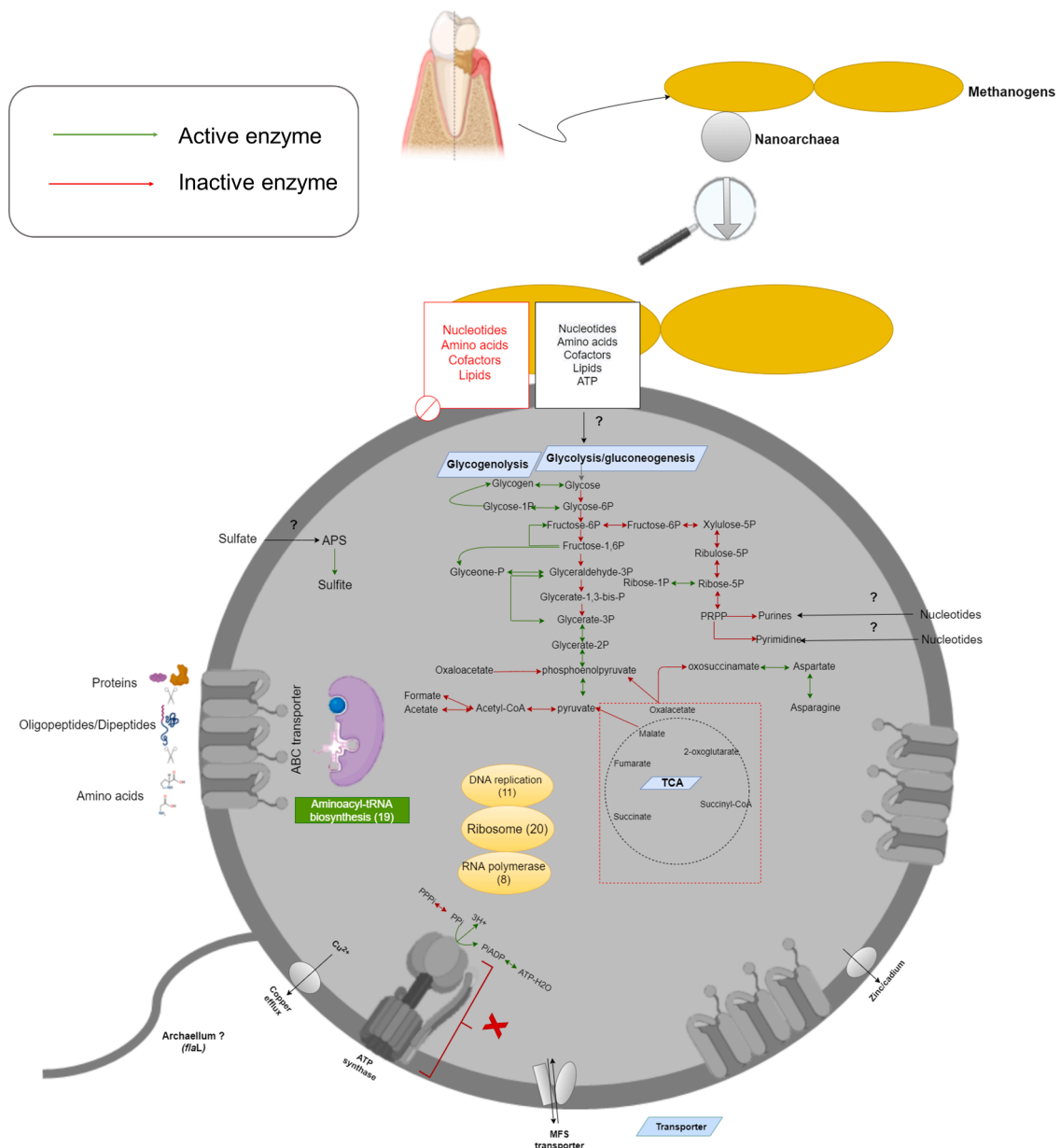


Fig. 2. Genome sequence-derived metabolic pathways of the clinical nanoarchaea *Nanopusillus massiliensis*.

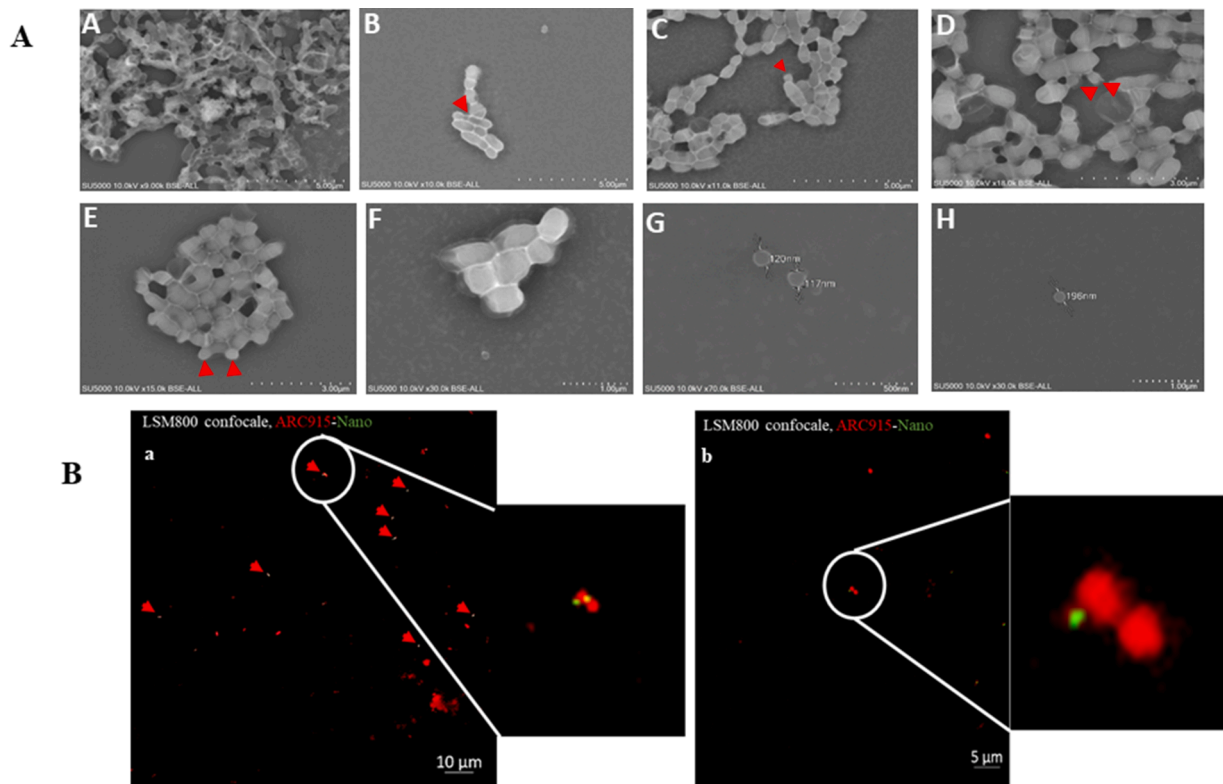
2020). Routinely available scanning electron microscopy was highly contributive to this work and discovery. Nevertheless, nanoarchaea specific molecular detection tools and isolation by culture detailed in this report can be translated to any clinical microbiology laboratory with expertise in the manipulation of oxygen-intolerant microorganisms, to set-up the prospective search for nanoarchaea in microbiota and pathological samples.

The first nanoarchaea *N. massiliensis* was co-detected by microscopy, molecular techniques and co-isolation and culture with hosting methanogen *M. oralis*. Several lines of microscopy observations indicated that *N. massiliensis* tightly attaches to *M. oralis*, an observation previously reported for the environmental *N. equitans* attaching to hosting *I. hospitalis* (Junglas et al., 2008). In the latter example, electron cryotomography disclosed either direct contacts between the two organism membranes, as we observed here, or fibrous material bridging the two organisms, an observation that we did not make here, despite *N. massiliensis* encoding one flagellum component similar to secretion type IV, previously reported to sustain physical relationships between another group of nano-organisms, the *Candidate phyla radiation* and host

bacteria (Anantharaman et al., 2016).

Nevertheless, tight connections between *M. oralis* and *N. massiliensis* may favor metabolite exchanges through ABC transporters, of which two representative families are encoded by the *N. massiliensis* genome. In the absence of a complete glycolysis pathway, *N. massiliensis* energy production may rely on gluconeogenesis and ABC transporter importation of fructose, metabolized into fructose 6-phosphate. Likewise, the inability of *N. massiliensis* to synthesize purines and pyrimidines could be overcome by a ribonucleoside diphosphate reductase providing precursors for DNA synthesis, catalyzing deoxyribonucleotide biosynthesis from the corresponding ribonucleotides. The inability to synthesize amino acids may be overcome by their recovery resulting from extracellular protein degradations, in line with detection of 19 aminoacyl t-RNA in the *N. massiliensis* genome. Finally, detection of sulfate adenylyl-transferase suggested that *N. massiliensis* incorporated organic sulfur into organic molecules involved in cofactors and amino acids synthesis, gaining an advantage for its habitat in the oral cavity hosting sulfate-reducing bacteria (Kushkevych et al., 2020).

*N. massiliensis* is the only human-cultured nanoarchaea, strikingly



**Fig. 3.** Tracking of the culture by scanning electron microscopy (SU5000). A. culture pellet before deglycosylation. B. culture pellet After deglycosylation. C—H deglycosylated pellet inoculum: culture at D15. k-o deglycosylated pellet inoculum: culture at D30. B Tracking of the culture by fluorescence *in situ* hybridization. a-b deglycosylated pellet inoculum: culture at D30.

broadening the spectrum of ecological niches when searching for intriguing nanoorganisms. Previously the only known representatives *N. equitans*, *Candidatus N. acidilobi*, *Candidatus Nanobsidianus stetteri* and *Candidatus Nanoclepta minutus* have been detected in so-called extreme environmental ecosystems (John et al., 2019) featuring thermophilic organisms growing at about 80 °C, and organisms also growing in acidic environments at pH 6 and a salinity concentration of 2% (Wurch et al., 2016). However, nanoarchaeales sequences have also been identified in ecosystems with temperatures ranging from 4 °C to 10 °C and 6 °C to 67 °C (McCliment et al., 2006).

The discovery of the novel nanoarchaea *N. massiliensis* is opening a completely new field of clinical investigations that we have named clinical nanoarchaeology, consisting of establishing the repertoire of

nanoarchaea in various human microbiota along with specifying their role in the physiology of those microbiota; and we detail in this report some laboratory keys to access this new world in clinical microbiology.

### Funding

YS benefits from PhD grants from the Fondation Méditerranée Infection, Marseille, France. This work was supported by the French Government under the «Investissements d'avenir» (Investments for the Future) program managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research), (reference: Méditerranée Infection 10- IAHU-03).

### Research in context

#### Evidence before this study

There is non evidence regarding the presence of the Nanoarchaea in human. The previous study had shown that Nanoarchaea are present in environmental biota specially in hypersaline lac.

#### Added value of this study

This study is the first to have initiated the detection of Nanoarchaea by molecular approaches and by culture-based technics in human microbiota and this is the first to have showed that human oral microbiota is colonized by viable Nanoarchaea which is *Nanopusillus massiliensis*.

#### Implications of all the available evidence

In this study, using specific laboratory tools oriented towards the detection of Nanoarchaea comprising of microscopic observations, molecular investigations culminating in one whole genome sequence and isolation in coculture, we discovered an unprecedented Nanoarchaea species *Nanopusillus massiliensis* detected and isolated in dental plate samples which showed a close physical contact with *Methanobrevibacter oralis*. This discovery extends our knowledge on the diversity of the human microbiota and open a new field of research which is the Nanoarchaea in clinical microbiology.

## CRedit authorship contribution statement

**Y. Hassani:** Conceptualization, Methodology, Writing – original draft. **J. Saad:** Data curation, Writing – original draft. **E. Terrer:** Methodology, Data curation. **G. Aboudharam:** Data curation, Investigation, Supervision. **B Giancarlo:** Data curation. **F. Silvestri:** Data curation. **D. Raoult:** Data curation, Supervision, Writing – original draft, Writing – review & editing. **M. Drancourt:** Data curation, Supervision, Methodology, Writing – original draft, Writing – review & editing, Validation. **G. Grine:** Conceptualization, Methodology, Methodology, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

All the authors declare that there are no conflicts of interest.

## Acknowledgements

The authors thank all Hitachi team members in Japan for the collaborative study with Hitachi High Technology and Institut Hospitalier Universitaire and for the installation of and service of the TM4000 microscope in our facility.

We thank Mr Louis TSAKOU NGOUAFO for his help in *Nanopusillus Massiliensis* genome finishing and Mrs. Sarah BELLALI for her help in microscopy analysis.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2021.100100](https://doi.org/10.1016/j.crmicr.2021.100100).

## References

- Casanueva, A., Tuffin, M., Cowan, D.A., 2011. 1 The Nanoarchaeota: Physiology, Genomics and Phylogeny, Archaea: Structure, Habitats and Ecological Significance Chapters. NOVA Sciences Publishers, pp. 163–174.
- John, E.S., Flores, G.E., Meneghin, J., Reysenbach, A.L., 2019. Deep-sea hydrothermal vent metagenome-assembled genomes provide insight into the phylum Nanoarchaeota. *Environ. Microbiol. Rep.* 11, 262–270.
- Nakai, R., 2020. Size matters: ultra-small and filterable microorganisms in the environment. *Microb. Environ.* 35.
- Huber, H., et al., 2002. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* 417, 63–67.
- Castelle, C.J., Banfield, J.F., 2018. Major new microbial groups expand diversity and alter our understanding of the tree of life. *Cell* 172, 1181–1197.
- Casanueva, A., et al., 2008. Nanoarchaeal 16S rRNA gene sequences are widely dispersed in hyperthermophilic and mesophilic halophilic environments. *Extremophiles* 12, 651–656.
- Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* 55, 541–555.
- Nguyen-Hieu, T., Khelaifia, S., Aboudharam, G., Drancourt, M., 2013. Methanogenic archaea in subgingival sites: a review. *APMIS* 121, 467–477.
- Grine, G., et al., 2018. Tobacco-smoking-related prevalence of methanogens in the oral fluid microbiota. *Sci. Rep.* 8, 9197.
- Nkamga, V.D., Henriessat, B., Drancourt, M., 2017. Archaea: essential inhabitants of the human digestive microbiota. *Hum. Microbiome J.* 3, 1–8.
- Huynh, H.T.T., Pignoly, M., Drancourt, M., Aboudharam, G., 2017. A new methanogen “*Methanobrevibacter massiliense*” isolated in a case of severe periodontitis. *BMC Res. Notes* 10, 657.
- Belkacemi, S., et al., 2018. Peri-implantitis-associated methanogens: a preliminary report. *Sci. Rep.* 8, 9447.
- Scanlan, P.D., Shanahan, F., Marchesi, J.R., 2008. Human methanogen diversity and incidence in healthy and diseased colonic groups using mcrA gene analysis. *BMC Microbiol.* 8, 79.
- Million, M., et al., 2016. Increased gut redox and depletion of anaerobic and methanogenic prokaryotes in severe acute malnutrition. *Sci. Rep.* 6, 26051.
- Grine, G., et al., 2019. Detection of *methanobrevibacter smithii* in vaginal samples collected from women diagnosed with bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 1643–1649.
- Nkamga, V.D., et al., 2018. *Methanobrevibacter oralis* detected along with *Aggregatibacter actinomycetemcomitans* in a series of community-acquired brain abscesses. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 24, 207–208.
- Nkamga, V.D., Lotte, R., Roger, P.M., Drancourt, M., Ruimy, R., 2016. *Methanobrevibacter smithii* and *bacteroides thetaiotaomicron* cultivated from a chronic paravertebral muscle abscess. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 22, 1008–1009.
- Drancourt, M., et al., 2020. *Methanobrevibacter smithii* archaeemia in febrile patients with bacteremia, including those with endocarditis. *Clin. Infect. Dis.* <https://doi.org/10.1093/cid/ciaa998>.
- Khelaifia, S., Raoult, D., Drancourt, M., 2013. A versatile medium for cultivating methanogenic archaea. *PLoS ONE* 8, e61563.
- Waters, E., et al., 2003. The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. *Proc. Natl. Acad. Sci.* 100, 12984–12988.
- Wurch, L., et al., 2016. Genomics-informed isolation and characterization of a symbiotic Nanoarchaeota system from a terrestrial geothermal environment. *Nat. Commun.* 7, 12115.
- Dridi, B., Henry, M., Khéchine, A.E., Raoult, D., Drancourt, M., 2009. High prevalence of *methanobrevibacter smithii* and *methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS ONE* 4, e7063.
- Bankevich, A., et al., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477.
- Tulloch, R.L., Kok, J., Carter, I., Dwyer, D.E., Eden, J.S., 2021. An amplicon-based approach for the whole-genome sequencing of human metapneumovirus. *Viruses* 13, 499.
- Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069.
- OrthoANI: An Improved Algorithm and Software for Calculating Average Nucleotide Identity | Microbiology Society. <https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijsem.0.00076crawler=true>. (2021).
- Meier-Kolthoff, J.P., Klenk, H.P., Göker, M., 2014. Taxonomic use of DNA G+C content and DNA–DNA hybridization in the genomic age. *Int. J. Syst. Evol. Microbiol.* 64, 352–356.
- Togo, A.H., et al., 2019. Culture of methanogenic archaea from human colostrum and milk. *Sci. Rep.* 9, 18653.
- Methanobrevibacter oralis* partial 16S rRNA gene, strain VD9. (2015).
- Ferrari, A., Brusa, T., Rutili, A., Canzi, E., Biavati, B., 1994. Isolation and characterization of *Methanobrevibacter oralis* sp. nov. *Curr. Microbiol.* 29, 7–12.
- Khelaifia, S., et al., 2018. Genome sequence and description of *Haloflex massiliense* sp. nov., a new halophilic archaeon isolated from the human gut. *Extremophiles* 22, 485–498.
- Drancourt, M., et al., 2017. Evidence of archaeal methanogens in brain abscess. *Clin. Infect. Dis.* 65, 1–5.
- Junglas, B., et al., 2008. *Ignicoccus hospitalis* and *Nanoarchaeum equitans*: ultrastructure, cell–cell interaction, and 3D reconstruction from serial sections of freeze-substituted cells and by electron cryotomography. *Arch. Microbiol.* 190, 395.
- Anantharaman, K., et al., 2016. Analysis of five complete genome sequences for members of the class Peribacteria in the recently recognized Peregrinibacteria bacterial phylum. *PeerJ* 4, e1607.
- Kushkevych, I., Coufalová, M., Vítězová, M., Rittmann, S.K.M.R., 2020. Sulfate-reducing bacteria of the oral cavity and their relation with periodontitis – recent Advances. *J. Clin. Med.* 9, 2347.
- McCliment, E.A., et al., 2006. Colonization of nascent, deep-sea hydrothermal vents by a novel Archaeal and Nanoarchaeal assemblage. *Environ. Microbiol.* 8, 114–125.