

Halocynthiibacter halioticoli sp. nov., isolated from the viscera of abalone *Haliotis discus hannai*

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Research Article

Keywords: Halocynthiibacter halioticoli, 16S rRNA gene, phylogenetic analysis, genome

Posted Date: February 13th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2566897/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Archives of Microbiology on October 8th, 2023. See the published version at <https://doi.org/10.1007/s00203-023-03686-7>.

Abstract

A Gram-stain-negative, rod-shaped, glide, non-flagellated, and facultatively aerobic bacterial strain, designated as Z654^T, was isolated from the gut of abalone *Haliotis discus hannai* from Rongcheng, Shandong province, China. Cells are 0.2–0.8 µm in width and 0.7–3.4 µm in length. Cells grew best at 30°C (range, 15–37°C), pH 7 (range, 6.0–8.5) and NaCl concentration of 2.0% (w/v) (range, 1–10%). According to the phylogenetic analysis of 16S rRNA gene sequence, the strain belongs to the genus *Halocynthiibacter* and the most close strain is *Halocynthiibacter arcticus* KCTC 42129^T (97.12%). The genome size of strain Z654^T was 3296250 bp and the DNA G + C content was 54.2 mol%. The average nucleotide identity (ANI) scores and digital DNA–DNA hybridization (dDDH) scores with *H. arcticus* KCTC 42129^T was 70% and 14.6–18.2% respectively. The predominant quinone was Q-10 and the major fatty acids were C_{18:0}, C_{18:1} ω7c 11-methyl and summed future 8. The polar lipids were consisted of phosphatidylcholine, phosphatidylglycerol, unidentified aminolipid and unidentified lipids. Based on the phenotypic, phylogenetic and chemotaxonomic data, strain Z654^T was considered to represent a novel species of the genus *Halocynthiibacter*, for which the name *Halocynthiibacter halioticoli* sp. nov., is proposed. The type strain is Z654^T (= MCCC 1H00503^T = KCTC 92003^T).

Introduction

The genus *Halocynthiibacter* belongs to the family *Rhodobacteraceae*. Currently, the genus *Halocynthiibacter* has three species, *Halocynthiibacter* was originally identified by Kim et al. (2014) with the type species *Halocynthiibacter namhaensis* KCTC 32362^T, followed by the effective publication of the strain of *Halocynthiibacter arcticus* KCTC 42129^T (Baek et al., 2015). In addition, 'Candidatus *Halocynthiibacter alkanivorans*', which has not been effectively published, was studied and its microbial communities were reported to have biodegradation potential (Campeão et al., 2019). The taxonomic position of the new strain Z654^T was reported in this study, based on phenotypic, chemotaxonomic and phylogenetic characters strain Z654^T is considered a new species of the genus *Halocynthiibacter* for which *Halocynthiibacter halioticoli* sp. nov is proposed.

Materials And Methods

Isolation and cultivation

The viscera samples of abalone (*Haliotis discus hannai*) from Rongcheng (122°14'E, 36°54'N), Shandong province, China, were taken and dissected with a sterile scalpel to expose the viscera, which were then placed in a glass mortar and obtained by thoroughly grinding. Take 1 g of organisms, serially dilute the sample to 10⁻⁴ with sterile seawater, and take 100 µl of each gradient dilution and spread it evenly onto marine agar 2216 medium (MA; Becton Dickinson). After incubation at 30°C for 5 d, a beige colony, designated as Z654^T, was obtained and stored at -80°C in 20.0% (v/v) sterile glycerol plus 3.0% (v/v) NaCl. The type strains *H. arcticus* KCTC 42129^T and *H. namhaensis* KCTC 32362^T were purchased from

the Korean Collection for Type Cultures (KCTC). They are the most closely related type strains to strain Z654^T and were used to compare physiological and chemometric characteristics.

16s Rrna Gene Sequence And Phylogenetic Analysis

Universal primers 27F and 1492R were employed for PCR amplification of strain Z654^T (Liu et al., 2014) and the nearly complete 16S rRNA gene fragment was obtained. The PCR products were purified and ligated into a pMD18-T vector (Takara), then cloned according to the manufacturer's instructions and sent to BGI Co. Ltd (Qingdao, China) for screening and sequencing. The almost complete 16S rRNA gene sequence of strain Z654^T was obtained by traditional Sanger sequencing and submitted to the GenBank database. The phylogenetic tree was constructed using the neighbor-joining (NJ) (Saitou *et al.*, 1987), the maximum-likelihood (ML) (Yang, 2007) and maximum-parsimony (MP) using MEGA X software (Molecular Evolutionary Genetics Analysis) (Kumer *et al.*, 2018).

Genome Sequence Analysis

The genomic DNA of strain Z654^T was extracted according to the instructions of the genomic DNA extraction kit (Takara). The genomic draft sequence of strain Z654^T was sequenced by Beijing Bioinformatics Co., Ltd. (Beijing, PR China) using the Illumina PE150 platform. The G + C content of the DNA of strain Z654^T was obtained by submitting the sequence to the GenBank database. The NCBI Prokaryotic Genome Annotation Pipeline (PAGP) was used to obtain annotated genome content and the genes included in the metabolic pathways were analyzed by KEGG Database. Using the Bacterial Pan Genome Analysis (BPGA) tool, a quick pan-genome analysis workflow, and a cutoff of 50% amino acid sequence identity, core and unique genes were analyzed (Chaudhari et al., 2016). The average nucleotide identity (ANI) was calculated by the OrthoANI algorithm (Yoon et al., 2017) between strain Z654^T and *H. arcticus* KCTC 42129^T. Digital DNA-DNA hybridization (dDDH) values were calculated by Genome to Genome Distance Calculator (GGDC) (<http://ggdc.dsmz.de/ggdc.php/>). The presence of gene clusters expressing secondary metabolites was predicted in strain Z654^T using the antiSMASH 6.0 database. Carbohydrate-active enzymes of strain Z654^T were annotated using the dbCAN2 meta server (Zhang et al., 2018). Further phylogenetic and taxonomic studies were carried out to determine its exact taxonomic status. The phylogenetic analysis was reconstructed by FastTree (Price et al., 2009) used JTT + CAT parameters and IQTree (Trifinopoulos et al., 2016) used the LG + F + I + G4 model and 1000 self-replications on the basis of 17 genomes were completed. All genomes were available from the NCBI.

Phenotypic Characterization (Dup: Abstract ?)

Strain Z654^T was placed on MA and incubated at 30°C for 3 days for morphological and physiological characteristics detection. Gram staining was performed according to the method described previously (Smibert *et al.*, 1994). The surface morphology of the colonies was observed using a light microscope

(E600; Nikon) and a scanning electron microscope (FEI Nanonova SEM450). The gliding motion of the bacteria was detected as described by Bowman (Bowman, 2000). Petri dishes were placed at different temperatures (0, 4, 15, 20, 25, 28, 30, 33, 35, 37, 40 and 45°C) and colonies were observed every 12 hours for more than 1 week to obtain the temperature range at which the strains could grow. The effect of NaCl concentration on growth of strain Z654^T was examined on MA, which was prepared with artificial seawater (0.23% MgCl₂, 0.32% MgSO₄, 0.12% CaCl₂, 0.07% KCl, and 0.02% NaHCO₃, all w/v) and supplemented with different concentrations of NaCl (0%, 0.5%, and 1–10% in 0.5% increments, w/v) at 30°C. Colony development was monitored every 12 hours. The pH range of growth was tested at pH 5.5–9.5 (interval 0.5 pH unit), and a series of buffers were added to marine broth 2216 (MB, Becton Dickinson) for adjustment. After two weeks of incubation in an anaerobic tank containing 0.1% (w/v) KNO₃ or not, the growth status was determined under anaerobic conditions. The nitrate reduction experiment was done as previously described (Cowan *et al.*, 1974). The generation of bubbles in 3% (v/v) hydrogen peroxide aqueous solution was measured to detect catalase activity. According to manufacturer instructions, a bioMérieux oxidase kit was used to determine oxidase activity. Based on the previous methods (Dong *et al.*, 2001), the hydrolysis of starch, cellulose, agar, alginate, casein and Tweens (20, 40, 60 and 80) were detected. DNase agar (HopeBio) was used to detect DNA enzyme activity. Susceptibility to antibiotics was investigated on MA at 30°C for 7 days using filter-paper discs containing various antibiotics as described previously (Du *et al.*, 2014) and according to procedures outlined by the Clinical and Laboratory Standards Institute (CLSI 2018). Other physiological and biochemical properties were assessed using API 20NE, API ZYM and API 50CHB strips (bioMérieux, Marcy-l'Étoile, France) and Biolog GEN III microplates according to the manufacturer's recommendations.

Chemotaxonomic Analysis (Dup: Abstract ?)

The cells of Strain Z654^T were harvested at exponential period and freeze-dried. It was used for the extraction of fatty acids, polar lipids, and respiratory quinones at a later stage (Kuykendall *et al.*, 1988). The Sherlock microbial identification system (MIDI) was used to determine cellular fatty acid composition. According to the description (Tindall, 1990), polar lipids were extracted, experiments were performed using two-dimensional thin-layer chromatography (TLC), and appropriate detection reagents were sprayed for dyeing (Kamekura *et al.*, 1993). The respiratory quinone was extracted according to the researchers (Collins, 1994), and analyzed by high-performance liquid chromatography (HPLC) (Kroppenstedt, 1982).

Results And Discussion

16S rRNA gene sequence and phylogenetic analysis

The almost complete 16S rRNA gene sequence of Z654^T (1488bp) was obtained by PCR and cloning. Phylogenetic analysis of 16S rRNA gene sequences showed that the 16S rRNA gene sequence of strain Z654^T was closest with *H. arcticus* KCTC 42129^T (97.1%), and *H. namhaensis* KCTC 32362^T (96.3%) (Kim

et al., 2014). The phylogenetic trees constructed with different tree generation algorithms (Fig. 1) showed that strain Z654^T was located in a phylogenetic tree composed of *H. arcticus* KCTC 42129^T, and *H. namhaensis* KCTC 32362^T. Strain Z654^T was placed on a node that was more distantly related to the other strains. Similar tree topologies were also shown in the 16S rRNA genes based phylogenetic trees with the maximum-likelihood and maximum-parsimony algorithms. (Fig. S1 and Fig. S2), available with the online Supplementary Information). The above results support that strain Z654^T was a unique new species of the genus *Halocynthiibacter*.

Draft Genome Sequence Analysis

The draft genome of strain Z654^T was 3296250 bp and the N50 length was 1154162 bp. The longest scaffold was 1576913 bp. An average genome depth of 467× was achieved. The output report of the genomic information annotated by PGAP showed that there were 3227 genes, 3168 protein-coding genes, 39 tRNA, 3 rRNA, 3 ncRNAs, and 1 full sequence of 16S rRNA genes in the inclusions. The ANI and dDDH values for strain Z654^T and its closest relative species *H. arcticus* KCTC 42129^T were 70% and 14.6–18.2%, respectively. 95–96% of the ANI and 70% of the dDDH values are generally considered as species boundaries (Chun et al., 2018). After these calculations, strain Z654^T can be classified as a new species within the genus *Halocynthiibacter*. Phylogenetic analysis of the genome amino acid sequences using the FastTree and IQTree method showed that strain Z654^T formed a unique lineage in the *Halocynthiibacter* genus (Fig. 2 and Fig. S3).

Due to the emission of large amounts of organic or inorganic pollutants from human activities [Gao *et al.*, 2017; Luo et al., 2010], mollusks such as abalone living in sediments may accumulate many pollutants from long-term exposure to fairly high levels of chemical stress (Xing *et al.*, 1997). In particular, cadmium, arsenic, lead and mercury are heavy metals with known biological toxicity (Jiang et al., 2015). We identified certain key genes: *ACR3* and *ArsH* associated with arsenic resistance in strain Z654^T. The cluster of gene *ACR3* previously was shown to confer arsenical resistance in *Saccharomyces cerevisiae*. The overexpression of *ACR3* induced high level arsenite resistance (Wysocki et al., 1997). *ArsH* contributes to the mitigation of toxicity in As by mediating the reduction of ROS generated in vivo upon exposure to oxygen anions, for example by generating FMNH₂ to facilitate ROS quenching activity (Páez-Espino et al., 2020).

After detailed analysis of the metabolic pathways of Z654^T by the KEGG database, the results showed that the most genes were on environmental information processing, followed by genetic information processing and carbohydrate metabolism. In addition, we have comparatively analyzed the metabolic pathways of the three species, including carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, amino acid metabolism, and metabolism of cofactors and vitamins. Three *Halocynthiibacter* strains' genomic analyses revealed that they had a large number of genes involved in carbohydrate metabolism, all strains have complete pathways of glycolysis (M00002), gluconeogenesis (M00003), pyruvate oxidation (M00307), citrate cycle (M00009, M00010, M00011), pentose phosphate

pathway (M00004, M00006, M00007), PRPP biosynthesis (M00005), Entner-Doudoroff pathway (M00008) and several other carbohydrate metabolism (Fig. 3). Also, only strains Z654^T and *H. arcticus* KCTC 42129^T, which possess the complete d-galacturonate degradation (M00631), d-Glucuronate degradation (M00061), d-galactonate degradation (M00552) pathways, and strain *H. namhaensis* KCTC 32362^T, which does not (Fig. 3). This gives them a strong carbohydrate utilization capacity and this property plays an important role in their gastrointestinal colonization and probiotic action (Cui et al., 2021). At the same time, Z654^T has some strain specificity. Only strains Z654^T possess a complete dissimilatory nitrate reduction (M00530), denitrification (M00529), ectoine biosynthesis (M00033), polyamine biosynthesis (M00134), whereas the pathway was not complete in the other two strains (Fig. 3).

BPGA was used to conduct a pan-genome analysis to find orthologous groupings among strains Z654^T, *H. arcticus* KCTC 42129^T, and *H. namhaensis* KCTC 32362^T. A comparative analysis based on the orthologous groups of protein revealed a total of 1439 core genes shared in the three species. The percentage of core genomes per genome ranged from 35.0–45.5%. In the three genomes, the ratio of accessory genes to unique genes differed significantly (Fig. 4). Core genes contribute more to energy metabolism, folding sorting and degradation, neurodegenerative diseases, nucleotide metabolism, replication and repair, Transcription, and translation than unique and accessory genes (Fig. S7). The accessory genes and unique genes content in strain *H. arcticus* KCTC 42129^T were higher than in several other strains (Fig. 4). Accessory and unique genes play a larger role than core genes in carbohydrate metabolism, lipid metabolism, membrane transport and xenobiotic degradation metabolism, especially cell motility (Fig. S7). It was verified that the bacterium has gliding properties, which may confer a certain nutrient-seeking behavior (Sato et al., 2021) and increase the environmental adaptability of the bacterium in the abalone viscera.

Four probable biosynthetic gene clusters were discovered in the genome of strain Z654^T based on secondary metabolite analyses predicted by antiSMASH. These clusters include one RiPP-like cluster, one hserlactone cluster, one ectoine and one terpene cluster region. These synthesized secondary metabolites are considered a very promising source of novel pharmaceutical compounds (Chiriac et al., 2018).

According to reports, seaweed-associated species including marine invertebrates and bacteria are the primary hosts of enzymes that could catabolize seaweed polysaccharides. The most significant manufacturers of these enzymes are phytophagous marine gastropods, which include sea hares and abalone (Ojima et al., 2018). Different polysaccharide-degrading enzymes have so far been identified from these animals' hepatopancreas and digestive fluids, such as alginate lyase, 1,3-glucanase, mannanase, and cellulase (Ojima et al., 2018). Subsequently, we analyzed the carbohydrate activity of strain Z654^T. Strain Z654^T contained 108 carbohydrate-active enzymes (Fig. S4). Among these carbohydrate-active enzymes, glycosyltransferase (GTs) were the most numerous enzymes, with 65 in total. This is followed by the glycoside hydrolases (GHs) family, with 30 in total, which proves that the strain has a strong ability for polysaccharide degradation. Besides, there were two polysaccharide lyases

(PLs) in strain Z654^T, which were not annotated in *H. arcticus* KCTC 42129^T and *H. namhaensis* KCTC 32362^T. Considering that the abalone *Haliotis discus hannai* feeds mainly on brown algae, therefore its gut microorganisms may be involved in polysaccharide degradation processes (Nam et al., 2018). The strong polysaccharide degradation ability of this strain helps abalone to extract energy from brown algae polysaccharides and survive better.

Phenotypic Characterization

Cells of strains Z654^T were Gram-stain-negative, rod-shaped and facultatively aerobic. Colonies grown on MA were round, convex, flaxen with smooth, moist edges, besides the SEM (Fig. S5) showed Z654^T cells were approximately 0.2–0.8 µm in width and 0.7–3.4 µm in length. The cells were gliding on the semisolid agar. Optimal growth occurred at 30°C (range 15–37°C) and pH 7.0 (range 6.0–8.5) with 2% (w/v) NaCl (range 1.0–10.0%). No growth was observed under anaerobic conditions. The cells were positive for nitrate reduction and negative for catalase and oxidase activity. The hydrolysis of Tween 40 and starch was detected, but casein, cellulose, alginate and Tweens 20, 60, 80 were not hydrolyzed. Despite strain Z654^T showed many common traits with *H. arcticus* KCTC 42129^T and *H. namhaensis* KCTC 32362^T including the positive reaction of esterase (C4), naphthol-AS-BI-phosphohydrolase, negative for trypsin, α-galactosidase, β-glucuronidase, α-mannosidase, gelatinase, glucose, mannitol. However, there were some differences between the strains, such as that Z654^T showed positive for the esterase lipase (C8) and lipase (C14), while *H. arcticus* KCTC 42129^T and *H. namhaensis* KCTC 32362^T were negative for those. Further detailed characteristics determined by the API ZYM, API 20NE, API 50CHB identification systems (BioMérieux) and Biolog GEN III MicroPlates of strains are given in Table S2. Strain Z654^T showed resistance to neomycin (30 µg), streptomycin (10 µg), gentamycin (10 µg), carbenicillin (100 µg), tetracycline (30 µg), kanamycin (30 µg), polymyxin B, ampicillin (10 µg), tobramycin (10 µg), penicillin (30 µg) and norfloxacin (10 µg).

Chemotaxonomic Analysis

The only isoprenoid quinone detected in strain Z654^T is ubiquitin-10 (Q-10), which is the same as the description of all members of the genus *Halocynthiibacter*. The major cellular fatty acid of strain Z654^T were C_{18:0} (17.0%), C_{18:1} ω7c 11-methyl (13.3%) and summed future 8 (C_{18:1} ω7c and/or C_{18:1} ω7c) (58.0%) (Table S1). The summed future 8 was also the main fatty acid for the other two reference strains. The main polar lipids of strain Z654^T were found to include phosphatidylcholine (PC), phosphatidylglycerol (PG), two unidentified aminolipid (AL1, AL2) and five unidentified lipids (L1, L2, L3, L4, L5). PC and PG were also present in the type strains *H. arcticus* KCTC 42129^T and *H. namhaensis* KCTC 32362^T, and these strains both contained AL. Three phospholipids (PL1, PL2, PL3) and aminophospholipid (APL) were identified in *H. namhaensis* KCTC 32362^T. Z654^T contained a higher amount of unidentified lipids. The further detailed polar lipids images were shown in Fig. S6.

According to all these results of phenotypic, biochemical and physiological analyses, together with the phylogenetic differences, strain Z654^T can be assigned to the genus *Halocynthiibacter* within the family *Rhodobacteraceae*, as representing a novel species, for which the name *Halocynthiibacte halioticoli* sp. nov. is proposed.

Description of *Halocynthiibacte halioticoli* sp. nov.

Halocynthiibacte halioticoli (ha.li.o.ti.co.li. N.L. fem. n. Haliotis, the genus name for abalone, a rock-climbing gastropod mollusk; L. neut. n. colon, colum, great gut; N.L. gen. neut. n. halioticoli, of the gut of Haliotis).

Cells are Gram-stain-negative, rod-shaped and facultatively aerobic. Colonies grown on MA were round, convex, and flaxen with smooth, moist edges. Cells are 0.2–0.8 µm in width and 0.7–3.4 µm in length. The cells can grow in 15–37°C (optimum at 30°C), pH 6.0–8.5 (optimum at pH 7.0), and NaCl concentration of 1–10% (w/v) (optimum at 2%). Starch and Tween 40 are hydrolyzed, but agar, DNA, CM-cellulose, alginate and Tweens 20, 60 and 80 are not. Cells are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, β -galactosidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, β -fucosidase, Voges–Proskauer reaction. Acids are produced from d-arabinose, l-arabinose, d-xylose, potassium 5-keto-gluconate. The predominant quinone was Q-10 and the major fatty acids were C_{18:0}, C_{18:1} ω 7c 11-methyl and summed future 8. The polar lipids were consisted of PC, PG, three unidentified aminolipids and five unidentified lipids. The DNA G + C content of strain Z654^T is 54.2 mol%.

The type strain, Z654^T (= MCCC 1H00503^T = KCTC 92003^T), was obtained from the entrail of abalone in Rongcheng, Shandong province, China. The Whole Genome Shotgun project has been deposited at GenBank under the accession JAOYFC000000000. The accession number of the 16S rRNA gene sequence is OP218735.

Declarations

Acknowledgements

The implementation of scanning electron microscope was supported by the Physical-Chemical Materials Analytical and Testing Center of Shandong University at Weihai.

Funding information

This work was supported by Science & Technology Fundamental Resources Investigation Program (Grant No. 2022FY101100, 2019FY100700) and the National Natural Science Foundation of China (32070002).

Conflicts of interests and ethical statements

The authors declare that they have conflict of interest.

This article does not contain any studies with animals performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

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Tables

Table 1. Differential characteristics between strain Z654^T and the reference strains. Strains: 1, Z654^T; 2, *H. arcticus* KCTC 42129^T; 3, *H. namhaensis* KCTC 32362^T. All data are from this study. +, Positive; –, negative. NA, no data.

Characteristic	1	2	3
Temperature (°C)	30	25 ^a	21 ^b
Nitrate reduction	+	_a	_b
Catalase activity	-	+ ^a	+ ^b
oxidase activity	-	+ ^a	+ ^b
DNA G+C content (mol%)	54.2	53.2 ^a	52.9 ^b
Enzyme activities(API ZYM and 20NE)			
alkaline phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, cystine arylamidase, valine arylamidase, β-fucosidase, o-nitrophenyl-β-d-galactopyranoside, Voges-Proskauer reaction	+	-	-
leucine arylamidase	+	+	-
acid phosphatase	+	-	+
Hydrolysis of			
Tween 60	-	+ ^a	NA
Tween 80	-	+ ^a	_b
starch	+	_a	_b
gliding motion	+	_a	NA

Data from: ^aKim et al (2015); ^bBaek et al (2014).

Figures

Image not available with this version

Figure 1

The Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strains Z654^T, and other closely related species is shown. Filled circles indicate branches that were also recovered in maximum-parsimony and with the maximum-likelihood algorithm. Percentage bootstrap values expressed as percentages of 1000 replications above 70% are shown at branch nodes. *Stappia stellulata* IAM_12621^T was used as the out group. Bar, 0.01 substitutions per nucleotide position.

Image not available with this version

Figure 2

Phylogenetic analysis of the genomic amino acid sequences performed with the FastTree showing the phylogenetic position of strain Z654^T among closely related taxa. *Stappia stellulata* IAM_12621^T was used as the out group. Bar, 0.05 substitutions per nucleotide position.



Figure 3

Heat maps of complete and incomplete metabolic pathways in the genomes of strains Z654^T and related strains. Strains: 1, Z654^T; 2, *H. arcticus* KCTC 42129^T; 3, *H. namhaensis* KCTC 32362^T.

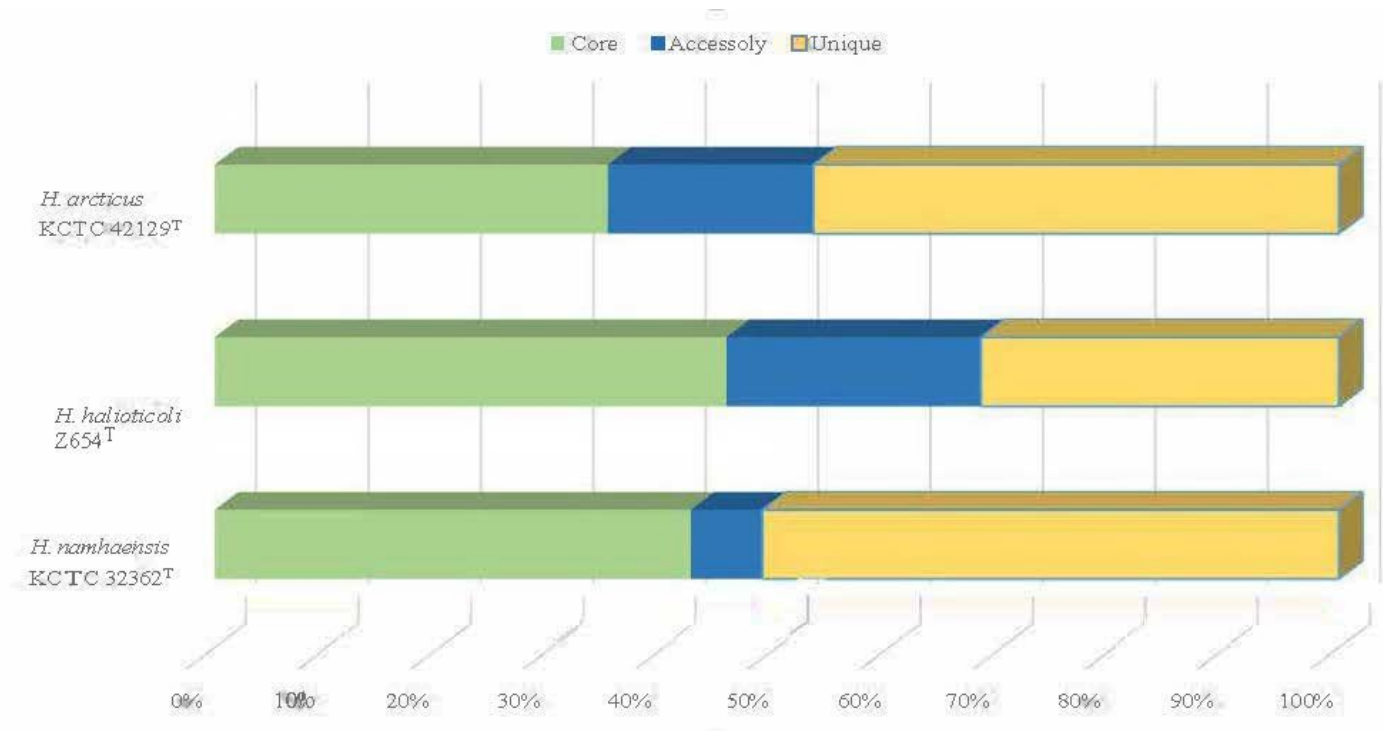


Figure 4

Comparison of percentages of orthologous protein groups regarding core, accessory, and unique genes for three *Halocynthiibacter* genomes.

Supplementary Files

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