

Characterization of *Winogradskyella luteola* sp.nov., *Erythrobacter ani* sp.nov., and *Erythrobacter crassostrea* sp.nov., three novel species of two different genus *Winogradskyella* and *Erythrobacter*, from the hemolymph of Pacific Oyster *Crassostrea gigas*. Prediction of bioremediation

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Research Article

Keywords: Bioremediation, Erythrobacter, Polyphasic taxonomy, Winogradskyella, Genome mining

Posted Date: May 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1676633/v1>

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Abstract

Three new bacterial strains, WHY3^T, WH131^T, and WH158^T, were isolated and described from the hemolymph of the Pacific oyster *Crassostrea gigas* utilizing polyphasic taxonomic techniques. The 16S rRNA gene sequence analysis revealed that WHY3^T was a member of the genus *Winogradskyella*, whereas WH131^T and WH158^T were members of the genus *Erythrobacter*. According to the polygenomic study, three strains generated three distinct and strong branches from all reference strains. The comparison of dDDH values, ANIs, percentage of conserved proteins (POCP), and average amino acid identity (AAI) between the three strains and their relatives established that the three strains constituted an appropriate taxonomy representing three distinct species of two separate genera. MK-6 was reported as the major respiratory quinone in WHY3^T and Q-10 for WH131^T and WH158^T, respectively. The major cellular fatty acids for WHA3^T were C_{15:0}, anteiso-C_{15:1} ω7c, iso-C_{15:0}, C_{16:1} ω7c. The major cellular fatty acids for WH131^T and WH158^T were C_{14:0} 2-OH and t_{18:1} ω12 for WH131^T and C_{17:0}, and C_{18:1} ω7c for WH158^T. Sudan Black B indicated a preference for PHAs granules for WH131^T and WH158^T but not for WHY3^T. The DNA G + C contents of strains WHY3^T, WH131^T and WH158^T were 34.40, 59.7 and 56.64%, respectively. Gene clusters predicted some important genes involved in the bioremediation process. Due to the accomplishment of polyphasic taxonomy, we propose three novel species *Winogradskyella luteola* sp.nov. (type strain WHY3^T = DSM 111804^T = NCCB 100833^T), *Erythrobacter ani* sp.nov. (WH131^T = DSM 112099^T = NCCB 100824^T) and *Erythrobacter crassostrea* sp.nov. (WH158^T = DSM 112102^T = NCCB 100877^T).

1. Introduction

Pacific oyster *Crassostrea gigas* is the most globally diverse of all oyster species. It was deliberately introduced into dozens of nations for aquaculture reasons and has now surpassed all other shellfish aquaculture species in terms of worldwide output. The Pacific oyster has the ability to significantly modify its habitat, benefiting and harming native species and ecosystems alike. It is an important non-native aquaculture species that has the potential to outcompete native species and as a habitat-forming species to function as a stepping stone for other non-native species (e.g., the brown algae *Sargassum muticum* in the North Sea) (Harris 2008).

Pacific oysters are ecosystem engineers, having a significant physical influence on their habitats (Dumbauld et al. 2009). *C. gigas* forms thick mats known as oyster reefs, and when they reach a certain density, they trigger physical changes in the surrounding environment (Escapa et al. 2004). Oysters are well-known for their capacity to transform soft substrates such as mud and silt into hard substrates. Numerous studies of oyster beds in various places have shown larger concentrations of benthic invertebrates, such as crabs, bivalves, and worms, living on the oyster beds' hard substrate compared to the surrounding soft substrate (Escapa et al. 2004; Dumbauld et al. 2009). They also provide a hard substrate for various macroalgae to grow on. Besides altering the benthic environment, oysters are filter feeders, consuming suspended plankton and organic debris. It has been a source of great interest for the study of associated marine bacteria due to their accumulation in various tissues such as the gills, gastrointestinal tissues, and mantle (Li et al. 2017), as well as in the circulatory fluid system, the hemolymph (Olafsen et al. 1993; Lokmer and Mathias Wegner 2015; Dupont et al. 2020), which has been targeted as the most promising component to study oyster microbiota due to its role in the immune system (Li et al. 2017).

Nedashkovskaya et al. identified the genus *Winogradskyella*, a member of the family *Flavobacteriaceae* in the phylum Bacteroidetes (He et al. 2019). The genus *Winogradskyella* now has 44 species with validly published names. Following several revisions to the genus description, the genus is now defined as catalase-positive, strictly aerobic or facultatively anaerobic, motile by gliding, yellowish to orange-colored, rod- or coccoid-shaped bacteria that contain phosphatidylethanolamine as a major polar lipid and menaquinone-6 (MK-6) as a major respiratory quinone (Song et al. 2018).

Shiba and Simidu erected the bacterium genus *Erythrobacter* under the family *Erythrobacteraceae* (Lee et al. 2005) with the identification of a single type species of *Erythrobacter longus* (Shiba et al. 1982). *Erythrobacter* is currently comprised of 34 species with validly published names. They are abundant in marine habitats and have been isolated and identified largely from tidal fat deposits, marine cyanobacterial mats, and marine plants (Shiba et al. 1982; Yurkov et al. 1994; Park et al. 2020). Significant features of *Erythrobacter* species include negative Gram staining, aerobic or facultatively anaerobic chemoorganotrophic metabolisms, and the synthesis of ubiquinone-10 as their predominant isoprenoid quinone (Tonon et al. 2014).

The bioremediation technique makes use of microorganisms' metabolic ability to clean up polluted environments. It refers to microorganisms' metabolic capacity to mineralize or change organic pollutants into less hazardous chemicals that may be incorporated into natural biogeochemical cycles. Bioremediation is an effort to speed up naturally occurring degradation by optimizing the limiting conditions in a non-destructive, treatment, and cost-effective method, by incorporating these processes into a logistically advantageous clean-up technique (Margesin and Schinner 2001). The major obstacle with in *situ* bioremediation are adverse environmental conditions. Elevated or low temperature, alkaline or acidic pH, high pressure, or high salt content are some of these challenging conditions. Marine bacteria are a kind of bacterium that is naturally exposed to harsh environments. Therefore, marine bacteria with bioremediation capacity might be great candidates for the biological treatment of harsh contaminated ecosystems. For instance, copper becomes toxic in higher concentrations (for example, >

0.08 μM Cu), impairing the metabolic activities of marine organisms. Bioremediation is a cost-effective method of treating polluted environments. Copper sorption by highly efficient bacteria may be employed to remove copper from polluted locations (Leal et al. 2018).

In this study, we isolated and completed the polyphasic taxonomy of three novel species belonging to the *Erythrobacter*, and *Winogradskyella* genus (Strains WHY3^T, WH131^T and WH158^T) isolated from the hemolymph of the Pacific oyster *Crassostrea gigas* and predicted based on genome data gene clusters for bioremediation and other important processes like Polyhydroxyalkanoic acid.

2. Materials And Methods

2.1 Isolation

Following collection of wild oysters from the Wadden Sea near Wilhelmshaven, Germany (Latitude: 53.5131, Longitude: 08.14714) in December 2019, were prepared, both valves were cleaned externally with a brush and sterile water to eliminate any dirt or debris that may contaminate the extraction process. The adductor muscle was entirely excised with a scalpel blade, and the remainder of the tissues were pooled together. The adductor muscle can be used to capture the hemolymph contained within (King et al. 2019). Serial dilutions of samples were performed (1:10) to a 10^{-5} dilution using 100 μL of sample and 900 μL sterile water in 1.5mL Eppendorf tubes. The artificial saltwater medium (ASW) supplemented with vitamin, and antifungal agent (ATI Coral Ocean salt (39 g/L), agar (15 g/L), nicotinic acid (20 mg/L), thiamine (vitamin B1, 10 mg/L), biotin (vitamin B7, 2 mg/L), 4-aminobenzoic acid (10 mg/L), pantothenic acid (5 mg/L), pyridoxamine (vitamin B6, 50 mg/L), cyanocobalamin (vitamin B12, 20 mg/L), and cycloheximide (100 mg/L), pH 7.3) was used to perform preliminary isolation. The incubation was performed for 6 days at 30 °C.

The orange and yellow colonies (WHY3^T, WH131^T, and WH158^T) were selected and transferred to Bacto marine agar (MA, Difco 2216), where they were purified successively by streaking over the same medium. The strains were held at -80 °C in a bacterial solution for long-term preservation.

2.2. Morphological, physiological and biochemical studies

Cells were grown on MB (marine broth) media for 3 days at 30°C followed by morphological observations, including motility, using a light microscope (Zeiss Axio Sc pie. A1 microscope). Cells grown in MB media for 2 days at 30°C were fixed with aldehydes (final concentrations: 5% formaldehyde and 2% glutaraldehyde), dehydrated in a gradient series of acetone, critical point dried, and coated with gold-palladium according to a previously published protocol (Landwehr et al. 2018). Images were acquired at various magnifications using a Zeiss Merlin field emission scanning electron microscope (FESEM) equipped with a 25:75% ratio of Everhart-Thornley SE and InLens SEM detectors. The sodium chloride tolerance of WHY3^T, WH131^T, and WH158^T was determined using the following NaCl (w/v) concentrations: 0%, 2.5%, 5.0%, 7.5%, 10%, 15%, 25%, and 30% using the method of Kutzner (Kutzner 1981). The growth of bacteria on solitary carbon and nitrogen sources was evaluated using R uger's technique on Microlog GEN III plates (Biolog). Sudan black B staining was employed at a concentration of 3% (w/v in 70% ethanol) to identify polyhydroxyalkanoate granules (PHAs) (Legat et al. 2010). MA medium was used to assess growth over a variety of temperatures (4, 15, 20, 25, 30, 35, 40, and 45°C) and pH values (pH 5, 6, 7, 8, 9, 10, and 11). Antibiotic susceptibility tests were performed on MA medium over 48 hours with the following antibiotics: gentamycin (10 $\mu\text{g}/\text{mL}$), oxytetracycline (10 $\mu\text{g}/\text{mL}$), thiostrepton (50 $\mu\text{g}/\text{mL}$), trimethoprim (50 $\mu\text{g}/\text{mL}$), ampicillin (10 $\mu\text{g}/\text{mL}$), chloramphenicol (30 $\mu\text{g}/\text{mL}$), polymyxin (50 $\mu\text{g}/\text{mL}$), spectinomycin (50 $\mu\text{g}/\text{mL}$), kanamycin (30 $\mu\text{g}/\text{mL}$), cephalosporin (50 $\mu\text{g}/\text{mL}$), fusidic acid (50 $\mu\text{g}/\text{mL}$), bacitracin (50 $\mu\text{g}/\text{mL}$), erythromycin (15 $\mu\text{g}/\text{mL}$), and tetracycline (50 $\mu\text{g}/\text{mL}$). Table S1. Api Zym (Humble et al. 1977), Api 20NE, Api 20E (bioMe'rieux) (O'Hara et al. 1992), and GEN III microplate (Biolog) were used in the biochemical investigation.

2.3. 16S rRNA gene analysis

The Invisorb Spin Plant Mini Kit was used to extract genomic DNA in accordance with the manufacturer's instructions (Strattec Molecular, Germany). PCR amplification of the 16S rRNA gene was applied according to Primahana et al. (Primahana et al. 2020) with the primer F27 (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTACGACTT-3') (Chaiya et al. 2019). The 16S rRNA gene was sequenced employing an Applied Biosystems 3730XL automated sequencer (ABI). BioEdit software was used to modify and assemble the sequence (version 7.0.5.3) (Hall 1999). The 16S rRNA gene sequence of strains WHY3^T (1,526 bp), WH131^T (1,486 bp), and WH158^T (1,486 bp) were almost completely sequenced and submitted to GenBank under the accession number MW888983, MW888981, and MW888982, respectively. The phylogenetically closest strains of WHY3^T, WH131^T, and WH158^T were determined based on 16S rRNA gene sequence similarity using the EZBioCloud system (<https://www.ezbiocloud.net/>) (Yoon et al. 2017a). Phylogenetic analysis of the 16S rRNA gene of strains WHY3^T, WH131^T, and WH158^T were inferred using the GGDC web server for closely similar type strains (<http://ggdc.dsmz.de/>) (Meier-Kolthoff et al. 2013). The sequence was analysed using a single-gene adaptation of the DSMZ phylogenomics program (Meier-Kolthoff et al. 2014). Multiple sequence alignment was performed using MUSCLE (Edgar 2004). Randomised Axelerated Maximum Likelihood (RAxML) (Stamatakis 2014) and TNT (Tree analysis using New Technology) (Goloboff et al. 2008) programs were applied to estimate Maximum likelihood (ML) and Maximum parsimony (MP) trees, respectively. We employed rapid bootstrapping using the autoMRE (extended majority rule) bootstrapping

parameters (Pattengale et al. 2010). 1000 bootstrapping replicates, tree bisection, reconnection branch switching, and ten random sequence addition repetitions were used in the case of MP. The X2 tests used in PAUP* (Phylogenetic Analysis using Parsimony*) were used to analyse the sequences (Swofford et al. 2003).

2.4. Chemotaxonomy

Bacterial biomass was produced and collected for 7 days at 30°C in a 250 mL flask containing 100 mL MB medium on a rotary shaker (160 revolutions per minute). The chemotaxonomic study was performed from freeze-dried biomass. Isoprenoid quinone production and analysis were conducted according to Minnikin (Minnikin et al. 1984). Compounds were evaluated using high-performance liquid chromatography coupled with the diode array detector and mass spectrometer (HPLC-DAD-MS), as reported by Risidian et al. (Risidian et al. 2021). The mobile phase consisted of 35% isopropanol + 1% water + 0.1% formic (solvent A) and 65% acetonitrile + 1% water + 0.1% formic acid (solvent B) and were used at a flow rate of 0.3 mL/min under isocratic conditions. Isoprenoid quinones were separated using a Waters ACQUITY UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm). Fatty acid extraction and methylation were carried out according to Sasser's procedure (Sasser 1990). Fatty acid methyl esters (FAME) were analyzed utilizing a gas chromatograph equipped with a flame ionization detector from Agilent (FID). Using a Macherey Nagel Optima 5 column, the methyl esters of fatty acids were extracted (5% phenyl, 95% dimethylpolysiloxane; 50 m length; 0.32 mm inner diameter; 0.25 µm film thickness). Their retention periods were compared to standards (in-house reference standard) to identify specific fatty acid methyl esters.

2.5. Whole-genome analysis

Illumina's next-generation sequencing technology using MiSeq 600 cycle v3 was employed for whole-genome sequencing, and Unicycler was applied for genome de novo assembly (Wick et al. 2017). To estimate the purity of the 16S rRNA gene in whole-genome data, the ContEst16S technique was utilized (<https://www.ezbiocloud.net/tools/contest16s>) (Lee et al. 2017a). To accomplish automated genome annotation, the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was employed (Tatusova et al. 2016). In addition, the draft genome assembly was submitted to the RAST (Rapid Annotation Using Subsystem Technology) database for metabolic reconstruction study (<https://rast.nmpdr.org/>) (Aziz et al. 2008). A phylogenomic tree was generated using the whole-genome sequence of strains WHY3^T, WH131^T, and WH158^T and their closest phylogenetic relatives using the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff et al. 2022). The Ezbiocloud and NCBI databases were used to extract whole-genome sequences of *Winogradskyella flava* KCTC 52348^T, *Winogradskyella ouciana* ZXX205^T, *Winogradskyella echinorum* KCTC 22026^T, *Erythrobacter insulae* JBTF-M21^T, *Erythrobacter rubeus* KMU-140^T, *Erythrobacter longus* DSM 6997^T, *Erythrobacter litoralis* DSM8509^T, *Pseudopontixanthobacter vadosimaris* JL3514^T and *Parerythrobacter jejuensis* JCM 16677^T. Strains WHY3^T, WH131^T, and WH158^T genome sequences were submitted to the Type (Strain) Genome Server (TYGS) (<https://tygs.dsmz.de/>; accessed on 05 March 2022). All pairwise comparisons for phylogenomic inference were performed using Genome BLAST Distance Phylogeny (GBDP), and precise intergenomic distances were obtained using the 'trimming' process and distance formula d5 (Meier-Kolthoff et al. 2013). One hundred distance replicates were considered each. Using the specified settings, we created digital DDH (dDDH) values and confidence intervals using the Genome-to-Genome Distance Calculator (GGDC 3.0) (Meier-Kolthoff et al. 2022). Intergenomic distances and branch support from FASTME 2.1.6.1 were used to build a balanced minimal evolution tree, which included subtree pruning and regrafting postprocessing (SPR) (Lefort et al. 2015). The tree's branch support was calculated using 100 pseudobootstrap replications. The average nucleotide identity (ANI), genome size, and guanine and cytosine (G + C) content were determined using the OrthoANlu method (Yoon et al. 2017b) (<https://www.ezbiocloud.net/tools/ani>). To prove the classification of strain WH131^T and WH158^T in the appropriate genus, the average amino acid identity (AAI) value (<http://enve-omics.ce.gatech.edu/aaai/index>) and the percentage of conserved proteins (POCP) value (<https://github.com/2015qyliang/POCP>) were calculated (Qin et al. 2014). The RAST algorithm v1.073 from the KBase database <https://narrative.kbase.us/> genes (The following algorithms were used to functionally annotate the genome's structural features: Kmers V2; Kmers V1; protein similarity (Ateba et al. 2020) was used in the field of genome mining. The most important genes involving bioremediation from isolated strains of the genus *Winogradskyella* and *Erythrobacter* were annotated as follows: the gene responsible for converting the toxic form of mercury to the nontoxic form and uptake of mercury *via* membranes which carried the Hg(II) into the cytoplasm (*merA*, *merB*, *MerC*, *MerE*, *MerF*, *MerT*, *MerP*) (De Rore et al. 1994; Sone et al. 2013; Zhang et al. 2020); polyaromatic hydrocarbons (PAHs) are abundant in nature and are a significant source of environmental concern owing to their persistence, toxicity, mutagenicity, and carcinogenicity (Cerniglia 1993); anthracene and phenanthrene may be oxidized by the *PhnA* dioxygenase as carbon and energy source (Kasai et al. 2003); polyhydroxyalkanoic acid (PHAs) are stored inside cells as carbon and energy reserves (Takahashi et al. 2017); degradation of phenol (*dmpN*) (Selvaratnam et al. 1997); chromate resistance genes (*chrB*) (Aguilar-Barajas et al. 2008); heavy metal resistance protein cobalt–zinc–cadmium (*czcA*, *czcD*) (Abdelatey et al. 2011); nickel–cobalt–cadmium resistance protein (*nccA*, *nccX*) (Abou-Shanab et al. 2003; Lee et al. 2019); copper resistance (*cusA*, *cusB*, *cusC*, *cusF*, Copper resistance protein B) (see Table 6 for details) (Bondarczuk and Piotrowska-Seget 2013).

The draft genome of strains WHY3^T, WH131^T, and WH158^T was submitted to NCBI/GenBank with the accession number (JAGSPD000000000), (JAGSPB000000000), and (JAGSPC000000000), respectively.

2.6. Antimicrobial activity

Strains WHY3^T, WH131^T, and WH158^T were grown for 5 days at 30° C on a shaker in 250-milliliter Erlenmeyer flasks that contained 100 mL of MB medium with 2% (v/v) XAD-2 polymeric resin (160 revolutions per minute). The separation of XAD-2 was accomplished by separating the resin with a paper filter from the media. Acetone was used to extract the secondary metabolites from the XAD-2. A rotary evaporator was used to dry the extract at a temperature of 40°C. The dried extract was diluted in 1 mL methanol and evaluated for antimicrobial activity against a variety of the following bacteria: *Citrobacter freundii* DSM 30039, *Staphylococcus aureus* Newman, *Escherichia coli* wild type BW25113, *Escherichia coli* acrB JW25113, *Mycobacterium smegmatis* ATCC 700084, *Pseudomonas aeruginosa* DSM 19882, *Acinetobacter baumannii* DSM 30008, *Bacillus subtilis* DSM 10, *Mucor hiemalis* DSM 2656, *Wickerhamomyces anomalus* DSM 6766, and *Candida albicans* DSM 1665. The serial dilution procedure was applied using 96-well microplates in accordance with Khosravi Babadi et al. (Khosravi Babadi et al. 2021).

3. Result And Discussion

3.1. Morphological, physiological and biochemical results

Strain WHY3^T was Gram-negative, motile by gliding, rod-shaped, aerobic, no-spore-form, devoid of flagella; cell size measurements showed a diameter of 0.3–0.4 µm in width and 0.8–2.1 µm in length (Fig. 1a). Strains WH131^T (Fig. 1b) and WH158^T (Fig. 1c) were Gram-negative, no-spore-form, non-flagellated and coccoid, ovoid or rod-shaped, with cell size measurements indicating a diameter of 0.2–0.3 µm in width and 0.6–3.1 µm in length for WH131^T and 0.2–0.4 µm in width and 0.5–2.4 µm in length for WH158^T.

The ideal temperature for growth for all three strains was 30°C. Besides pH ranges, the tolerance of sodium chloride was recorded; the results of the biochemical property-based Api ZYM, Api 20NE, and Api 20E tests indicated positive activity for all strains WHY3^T, WH131^T, and WH158^T and related closes type strains for catalase, oxidase, phosphatase alkaline, leucin arylamidase, valine arylamidase; and negative for α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Other comparisons for phenotypic characteristics and also Biolog Gen III system results between the isolated strains and closet-related strains are observable in Table 1 and Table 8. Sudan Black B indicates a preference for PHAs granules for WH131^T and WH158^T but not for WHY3^T.

Table 1

Comparison of phenotypic characteristics that distinguish strains WHY3^T, WH131^T, and WH158^T from the most closely related type strains. Strains: 1, *Winogradskyella luteola* WHY3^T; 2, *Winogradskyella flava* KCTC 52348^T; 3, *Winogradskyella ouciana* ZXX205^T; 4, *Winogradskyella echinorum* KCTC 22026^T; 5, *Erythrobacter ani* WH131^T; 6, *Erythrobacter crassostrea* WH158^T; 7, *Erythrobacter insulae* JBTF-M21^T; 8, *Erythrobacter rubeus* KMU-140^T; 9, *Erythrobacter longus* DSM 6997^T; 10, *Erythrobacter litoralis* DSM8509^T.

Genus name	<i>Winogradskyella</i>					<i>Erythrobacter</i>				
	1	2	3	4	5	6	7	8	9	10
Color of colony	Yellow	Yellow	Yellow	Yellow	Yellowish orange	Orange	strong orange	Reddish orange	Orange	Orange Or Red
Temperature range for growth (°C)	5–40	10–45*	4–45†	4–37‡	20–35	20–35	30(opt) ‡	10–45**	15–40	10–40
pH spectrum for growth	6–9	7–9*	6–9†	ND	6–9	6–8	6–8‡	6-9.5**	6–9	6–8
NaCl (optimum) for growth (%)	2.5	2*	3†	1–6‡	2.5	2.5	1–2‡	2**	2.5	0
ApiZym										
Esterase (C4)	+	+	+	+	+	+	+	-**	+	+
Esterase lipase (C8)	+	+	+	+	+	+	+	-**	+	+
Lipase (C14)	-	+	-†	-‡	-	+	-‡	-**	W	-
Cystine arylamidase	-	-*	+	+	+	+	-‡	-**	+	W
Trypsin	-	+	+	-‡	+	+	+	+	+	-
α-chymotrypsin	-	+	-†	+	+	+	+	+	+	-
Phosphatase acid	+	+	+	+	+	+	-‡	+	+	-
Naphtol-AS-BI-phosphohydrolase	+	+	+	+	W	+	+	+	+	W
β-galactosidase	-	-*	-†	+	-	-	+	+	-	-
α-glucosidase	-	-*	-†	-‡	-	-	+	-**	-	-
β-glucosidase	-	W*	-†	-‡	-	-	-‡	-**	-	-
Api 20NE- Api 20E										
Nitrate reduction	-	-*	-†	-‡	+	-	-‡	+	+	-
Indole	-	-*	ND	-‡	-	-	ND	-**	+	ND
Arginine dihydrolase	-	-*	ND	ND	+	+	ND	-**	+	ND

+, positive; (w), weakly positive; -, negative; ND, no data

* Data from Lee et al.(Lee et al. 2017b)

† Data from He et al.(He et al. 2019)

‡ Data from Nedashkovskaya et al.(Nedashkovskaya et al. 2009)

‡ Data from Park et al.(Park et al. 2020)

** Data from Yoon et al.(Yoon et al. 2022)

Diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingoglycolipid (SGL), unidentified phospholipids (PL), unidentified glycolipid (GL), unidentified aminolipid (AL) and unidentified polar lipid (L).

Genus name	<i>Winogradskyella</i>				<i>Erythrobacter</i>					
Species name	1	2	3	4	5	6	7	8	9	10
Lysin decarboxylase	-	ND	ND	ND	-	-	ND	-**	-	ND
Urease	-	-*	ND	-⊠	-	-	‡	-**	+	-
Aesculin hydrolysis	-	+	ND	W⊠	+	+	‡	ND**	+	+
Gelatin hydrolysis	-	+	†	+⊠	-	-	‡	+++	+	
Tween 80 hydrolysis	-	+	†	-⊠	+	-	‡	+++	+	+
Tween 40 hydrolysis	-	ND	†	-⊠	+	+	ND	ND**	ND	ND
Tween 20 hydrolysis	ND	-*	†	-⊠	ND	ND	ND	ND**	ND	ND
Ornithine decarboxylase	-	ND	ND	ND	-	-	ND	-**	-	-
H2S production	-	-*	ND	-⊠	-	-	ND	-**	-	-
Acetoin	+	ND	ND	-⊠	-	-	ND	ND**	-	-
Assimilation of :										
D-glucose	+	-*	ND	+⊠	+	+	‡	+++	+	+
L-arabinose	-	-*	ND	-⊠	-	-	‡	ND**	+	-
D-mannose	-	-*	ND	+⊠	+	+	‡	+++	-	-
D-mannitol	-	-*	ND	-⊠	+	+	ND	+++	+	-
N-acetyl-glucosamine	-	-*	ND	-⊠	-	-	ND	+++	-	-

+, positive; (w), weakly positive; -, negative; ND, no data

* Data from Lee et al.(Lee et al. 2017b)

† Data from He et al.(He et al. 2019)

⊠ Data from Nedashkovskaya et al.(Nedashkovskaya et al. 2009)

‡ Data from Park et al.(Park et al. 2020)

** Data from Yoon et al.(Yoon et al. 2022)

Diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingoglycolipid (SGL), unidentified phospholipids (PL), unidentified glycolipid (GL), unidentified aminolipid (AL) and unidentified polar lipid (L).

Genus name	<i>Winogradskyella</i>					<i>Erythrobacter</i>				
	1	2	3	4	5	6	7	8	9	10
D-maltose	-	-*	ND	-⊠	+	+	‡	+**	+	-
Potassium gluconate	-	-*	ND	-⊠	-	-	ND	ND**	-	-
Capric acid	-	-*	ND	-⊠	-	-	ND	ND**	-	-
Adipic acid	-	-*	ND	-⊠	-	-	ND	ND**	-	-
Malic acid	+	-*	ND	-⊠	+	-	ND	+**	-	-
Trisodium citrate	-	-*	ND	-⊠	-	-	‡	ND**	+	-
Phenylacetic acid	-	-*	ND	ND	+	+	ND	ND**	-	-
Citrate	-	ND	ND	-	-	-	‡	-**	+	-
Fermentation of :										
D-glucose	-	-*	ND	-⊠	-	-	ND	ND**	+	+
D-mannitol	-	-*	+†	-⊠	-	-	ND	ND**	+	-
Inositol	-	ND	ND	-⊠	-	-	ND	ND**	-	-
D-sorbitol	-	ND	ND	-⊠	-	-	ND	ND**	+	-
L-rhamnose	-	-*	+†	-⊠	-	-	ND	ND**	-	-
D-sucrose	-	-*	+†	-⊠	-	-	ND	ND**	+	-
D-melibiose	-	ND	ND	-⊠	-	-	ND	ND**	+	-
Amygdalin	-	ND	+†	ND	-	-	ND	ND**	-	-
L-arabinose	-	ND	+†	-⊠	-	-	ND	ND**	+	-
Susceptibility to:										
Gentamycin	-	-*	ND	-⊠	+	+	‡	ND	+	-
Ampicillin	-	+*	ND	-⊠	-	-	‡	ND	-	-
Kanamycin	-	-*	ND	-⊠	+	+	‡	ND	+	+
Vancomycin	+	+*	-†	ND	-	-	ND	-**	-	-
Lincomycin	+	ND	-†	+⊠	-	-	‡	-**	-	-
Polar lipids	PE, GL 6AL, 4L	PE, 4AL 2L	PE, 4GL 4AL, 3L	PE⊠	DPG, PE PG, SGL 2PC,4L 2AL,2GL	DPG, PE PG, SGL PC,4L 2AL,GL	DPG,PE PG,SGL PC, 4L 1GL‡	PE, PG PL, 2L**	DPG, PE PG, SGL PC,2L AL,GL	DPG, PE PG, SGL PC,5L 2AL,

+, positive; (w), weakly positive; -, negative; ND, no data

* Data from Lee et al.(Lee et al. 2017b)

† Data from He et al.(He et al. 2019)

⊠ Data from Nedashkovskaya et al.(Nedashkovskaya et al. 2009)

‡ Data from Park et al.(Park et al. 2020)

** Data from Yoon et al.(Yoon et al. 2022)

Diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingoglycolipid (SGL), unidentified phospholipids (PL), unidentified glycolipid (GL), unidentified aminolipid (AL) and unidentified polar lipid (L).

Genus name	<i>Winogradskyella</i>				<i>Erythrobacter</i>					
Species name	1	2	3	4	5	6	7	8	9	10
Major fatty acid	C _{15:0} , anteiso-C _{15:1} ω7c, iso-C _{15:0} , C _{16:1} ω7c, unknown	iso-C _{15:0} , iso-C _{15:1} G, unknown	iso-C _{15:0} , iso-C _{15:1}	iso-C _{15:1} , iso-C _{15:0} , C _{15:0} , iso-C _{15:} 0 3- OH, iso-C _{17:} 0 3-OH	C _{14:0} 2- OH, t _{18:1} ω12 Unknown	C _{17:0} , C _{18:1} ω7c, Unknown	C _{17:1} ω6c, C _{18:1} ω7c, Unknown	C _{16:0} , iso-C _{18:0} , C _{18:1} ω7c	anteiso-C _{17:0} , t _{18:1} ω12 C _{14:0} 2- OH Unknown	C _{16:0} , C _{16:1} ω7c, t _{18:1} ω12
G + C content (%)	34.40	34.79	35.48	32.3	59.7	56.64	57.0	60.58	57.37	65.18
Contigs	51	11	341	10	7	7	3	4	14	22
No. of protein	3194	3362	3179	3284	3011	2447	2770	2901	3250	3050
+, positive; (w), weakly positive; -, negative; ND, no data										
* Data from Lee et al.(Lee et al. 2017b)										
† Data from He et al.(He et al. 2019)										
⊠ Data from Nedashkovskaya et al.(Nedashkovskaya et al. 2009)										
‡ Data from Park et al.(Park et al. 2020)										
** Data from Yoon et al.(Yoon et al. 2022)										
Diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingoglycolipid (SGL), unidentified phospholipids (PL), unidentified glycolipid (GL), unidentified aminolipid (AL) and unidentified polar lipid (L).										

Genus name	<i>Winogradskyella</i>					<i>Erythrobacter</i>				
Species name	1	2	3	4	5	6	7	8	9	10
rRNA	3	3	10	3	3	3	3	3	3	3
tRNA	48	41	36	40	44	41	41	43	42	46
No. of Gene	3276	3453	3349	3375	3077	2506	2823	2987	3325	3142
Other RNA	4	4	5	4	4	2	3	4	3	3
Pseudogene	27	43	119	44	15	13	6	36	27	40
+, positive; (w), weakly positive; -, negative; ND, no data										
* Data from Lee et al.(Lee et al. 2017b)										
† Data from He et al.(He et al. 2019)										
‡ Data from Nedashkovskaya et al.(Nedashkovskaya et al. 2009)										
‡ Data from Park et al.(Park et al. 2020)										
** Data from Yoon et al.(Yoon et al. 2022)										
Diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingoglycolipid (SGL), unidentified phospholipids (PL), unidentified glycolipid (GL), unidentified aminolipid (AL) and unidentified polar lipid (L).										

3.2. 16S rRNA gene analysis

According to the EZBioCloud server's results, strains WHY3^T, WH131^T and WH158^T were most closely related to the following strain types: 97.58% to *Winogradskyella flava* SFD31^T, 96.69% to *Winogradskyella echinorum* KMM 6211^T for WHY3^T; 99.15% *Erythrobacter longus* OCh101^T, 98.51% *Erythrobacter insulae* JBTF-M21^T for WH131^T; 99.08% *Erythrobacter insulae* JBTF-M21^T, 98.58% *Erythrobacter longus* OCh101^T for WH158^T. 16S rRNA gene results of WHY3^T from phylogenetic pattern demonstrated proximity of 96.55% to *Winogradskyella ouciana* ZXX205^T. Phylogenetic trees based on 16S rRNA gene sequences of strains WHY3^T, WH131^T, and WH158^T and its closely related type strains are shown in Fig. 2 and Fig. 3. It shows that strain WHY3^T formed a very high supported cluster with *Winogradskyella* species (*W. ouciana* ZXX205^T and *W. flava* SFD31^T). Furthermore, strain WHY3^T formed a well-supported branch alongside *W. ouciana* ZXX205^T; and also strains WH131^T and WH158^T formed a highly supported cluster with *Erythrobacter* species (*E. rubeus* KMU-140^T, *E. longus* OCh101^T and *E. insulae* JBTF-M21^T); strains WH131^T and WH158^T formed a well-supported branch alongside *E. rubeus* KMU-140^T and *E. insulae* JBTF-M21^T, respectively.

3.3. Chemotaxonomic Characterization

The major cellular fatty acids for WHA3^T were C_{15:0}, anteiso-C_{15:1} ω7c, iso-C_{15:0}, C_{16:1}ω7c. The major cellular fatty acids for WH131^T and WH158^T were C_{14:0}:2-OH and t_{18:1}ω12 for WH131^T and C_{17:0}, and C_{18:1}ω7c for WH158^T. In all three, unknown fatty acids were the dominant constituents (Table 2). The fatty acid iso-C_{15:1} G was detected as one of the dominant ones in *W. flava* KCTC 52348^T and *W. ouciana* ZXX205^T, but it was absent in strain WHY3^T as well as anteiso-C_{15:1} ω7c and C_{16:1}ω7c which are present in WHY3^T but not in *W. flava* KCTC 52348^T and *W. ouciana* ZXX205^T. The fatty acid iso-C_{18:0} was only detected in *Erythrobacter rubeus* KMU-140^T. C_{18:1}ω7c constituted the largest amount in strain WH158^T, *E. insulae* JBTF-M21^T, and *E. rubeus* KMU-140^T, but was absent in strains WH131^T, *E. longus* DSM 6997^T, and *E. litoralis* DSM8509^T. The fatty acid fatty t_{18:1}ω12 represented the largest amount in WH131^T, *E. longus* DSM 6997^T and *E. litoralis* DSM8509^T. C_{17:1}ω6c was detected as the major fatty acid in just *E. insulae* JBTF-M21^T.

Table 2

The fatty acid content of strains WHY3^T, WH131^T, and WH158^T and their most closely related type strains. Strains: 1, *Winogradskyella luteola* WHY3^T; 2, *Winogradskyella flava* KCTC 52348^T; 3, *Winogradskyella ouciana* ZXX205^T; 4, *Erythrobacter ani* WH131^T; 5, *Erythrobacter crassostrea* WH158^T; 6, *Erythrobacter insulae* JBTF-M21^T; 7, *Erythrobacter rubeus* KMU-140^T; 8, *Erythrobacter longus* DSM 6997^T; 9, *Erythrobacter litoralis* DSM8509^T.

Genus name	<i>Winogradskyella</i>			<i>Erythrobacter</i>					
	1	2	3	4	5	6	7	8	9
Fatty acid									
C _{14:0}	-	-	-	-	-	-	-	-	5.3
C _{15:0}	11.5	4.2	-	-	-	-	-	-	-
C _{16:0}	4.4	TR	3.8	2.5	3.2	6.6	12.5	4.1	12.5
C _{17:0}	-	-	-	2.3	8.8	-	3.9	2.1	RT
C _{18:0}	2.9	-	1.2	1.6	-	-	-	RT	-
C _{19:0}	-	-	-	TR	-	-	-	-	-
anteiso-C _{15:0}	1.3	2.0	4.7	-	-	-	-	4.2	-
anteiso-C _{15:1} A	-	1.4	1.0	-	-	-	-	-	-
anteiso-C _{17:0}	-	-	-	2.3	2.6	-	-	10.0	1.2
anteiso-C _{15:1} ω7c	17.7	-	-	-	-	-	-	-	-
iso-C _{14:0}	-	2.6	3.9	-	-	-	-	-	-
iso-C _{15:0}	17.0	20.0	23.4	-	-	-	-	-	-
iso-C _{15:0} 3-OH	-	5.5	3.6	-	-	-	-	-	-
iso-C _{15:1} G	-	26.6	13.7	-	-	-	-	-	-
iso-C _{16:0}	1.7	1.7	10.6	-	-	-	-	-	-
iso-C _{16:0} 3-OH	-	2.6	10.7	-	-	-	-	-	-
iso-C _{16:1} G	-	-	1.6	-	-	-	-	-	-
iso-C _{16:1} ω6c	-	-	-	TR	-	-	-	-	-
iso-C _{17:0}	-	-	-	-	-	4.0	-	RT	-
iso-C _{17:0} 3-OH	-	4.2	8.8	-	-	-	-	-	-
iso-t _{17:1} ω7	2.8	-	-	-	-	-	-	-	-
iso-C _{18:0}	-	-	-	-	-	-	13.4	-	-
iso-C _{18:1} ω6c	1.3	-	-	-	-	-	-	-	-
C _{16:1} ω7c	13.2	-	-	3.4	-	-	-	RT	7.2
C _{17:1} ω6c	-	-	1.2	TR	2.3	23.9	2.0	-	1.9
C _{17:1} ω8c	-	-	-	-	3.4	2.7	3.4	2.4	-
C _{18:1} ω5c	-	-	-	-	-	TR	-	-	-

TR, trace amount (< 1%); -: Not detecte

Genus name	<i>Winogradskyella</i>			<i>Erythrobacter</i>					
	1	2	3	4	5	6	7	8	9
C _{18:1} ω7c	-	-	-	-	40.0	44.0	40.9	-	-
C _{19:1} ω8c	-	-	-	-	-	-	-	1.9	-
C _{12:0} 3-OH	-	-	1.2	-	-	-	-	-	-
C _{14:0} 2-OH	-	TR	-	8.1	2.4	6.1	6.6	9.6	4.7
C _{15:0} 2-OH	-	-	TR	-	-	5.1	5.8		
C _{16:0} 2-OH	-	-	-	-	-	TR	-	1.1	RT
t _{18:1} ω12	-	-	-	52.6	-	-	-	53.79	65.93
t _{16:1} ω7	-	-	-	-	-	-	-	-	RT
cyclo-C _{19:0} d8,9	-	-	-	TR	-	-	-	-	-
11-methyl C _{18:1} ω7c	-	-	-	-	-	5.9	6.9	-	-
Unknown fatty acid	26.4	17.1	-	24.0	37.7	-	-	17.4	4.3
TR, trace amount (< 1%); -: Not detecte									

The dominant polar lipids of strain WHY3^T were phosphatidylethanolamine, an unknown glycolipid, six unidentified aminolipids, and four unidentified lipids. The dominant polar lipids of strain WH131^T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, two unknown glycolipids, sphingoglycolipids, two phosphatidylcholines, four unidentified lipids, and two unidentified aminolipids. The dominant polar lipids of strain WH158^T were diphosphatidylglycerols, phosphatidylethanolamine, phosphatidylglycerol, unknown glycolipids, sphingoglycolipids, phosphatidylcholine, four unidentified lipids, two unidentified aminolipids (see Fig. S2). Menaquinone-6 (MK-6) was found to be the major respiratory quinone in WHY3^T. Ubiquinone-10 (Q-10) was detected in WH131^T and WH158^T (Table 1).

3.4. Genomic Characteristics and Phylogenomic Analysis

Only one 16S rRNA gene sequence was discovered in the whole-genome data of strains WHY3^T, WH131^T, and WH158^T, showing that the genomic data were not contaminated by other species. The draft assembled genome sequence of strain WHY3^T comprised of the following: 3,532,486 bp with a G + C content of 34.50% (GenBank accession No. JAGSPD000000000). The genome included 3276 genes comprising 3194 protein-coding genes, 48 tRNA genes, 3 rRNA genes, and 4 non-coding RNA. In parallel, WH131^T and WH158^T had 3,153,164 bp and 2,586,581 bp with a G + C content of 59.70% and 56.64%, respectively. (GenBank accession No. JAGSPB000000000 and JAGSPC000000000). The genome included 3077 genes comprising 3011 protein-coding genes, 44 tRNA genes, 3 rRNA genes, and 4 non-coding RNA for WH131^T and 2506 genes comprising 2447 protein-coding genes, 41 tRNA genes, 3 rRNA genes, and 2 non-coding RNA for WH158^T. The phylogenomic tree (Fig. 4) shows that strain WHY3^T is well supported by *Winogradskyella* species with pseudo-bootstrap support values > 60% from 100 replications, with average branch support of 65.5%.

The phylogenomic tree (Fig. S1) with average branch support of 40.3% shows that strain WH131^T is located in a cluster, although it was not well supported, with *Pseudopontixanthobacter vadosimaris* JL3514^T instead of the other *Erythrobacter* species. However, the strain WH158^T formed a well-defined tight cluster with *Erythrobacter insulae* JBTF-M21^T, but not the far-distance vicinity of *Parerythrobacter jejuensis* JCM16677^T. Therefore, another investigation was undertaken using whole-proteome-based GBDP distances. As a consequence, a phylogenetic tree with average branch support of 94.5% was produced, which was more trustworthy than the nucleotide-based phylogenomic tree result (Fig. 5) since strains WH131^T and WH158^T were classified in the same clade as other *Erythrobacter* species with a very high support score. In the whole-proteome-based phylogenetic tree, strains WH131^T and WH158^T were located in a very high-supported clade together with *E. rubeus* KMU-140^T and *E. insulae* JBTF-M21^T, respectively.

An additional study was conducted using genomic data to identify the genus classification, using the average amino acid identity (AAI) (Rodriguez-R and Konstantinidis, 2014) and the percentage of conserved protein (POCP) values. The POCP values and AAI values between the genomes of strains WH131^T and WH158^T against *Erythrobacter insulae* JBTF-M21^T, *Erythrobacter rubeus* KMU-140^T, *Erythrobacter longus* DSM 6997^T, *Erythrobacter litoralis* DSM8509^T, *Pseudopontixanthobacter vadosimaris* JL3514^T, *Parerythrobacter jejuensis* JCM 16677^T are shown in Table 3 with 81.94% (POCP) and 87.65% (AAI) as the highest percentages against *Erythrobacter rubeus* KMU-140^T for WH131^T and 72.99%

(POCP) and 81.22% (AAI) as the highest percentages value against *Erythrobacter insulae* JBTF-M21^T for WH158^T (Qin et al. 2014; Rodriguez-R and Konstantinidis 2014). Thus, isolates WH131^T and WH158^T could be classified as *Erythrobacter* rather than *Pseudopontixanthobacter* or *Parerythrobacter*.

Table 3

Genome relatedness between the strains WH131^T and WH158^T, genus *Erythrobacter*, and other closet type strains according to the average amino acid identity (AAI) value and the percentage of conserved protein (POCP) values. 1, *Erythrobacter insulae* JBTF-M21^T; 2, *Erythrobacter rubeus* KMU-140^T; 3, *Erythrobacter longus* DSM 6997^T; 4, *Erythrobacter litoralis* DSM8509^T; 5, *Pseudopontixanthobacter vadosimaris* JL3514^T; 6, *Parerythrobacter jejuensis* JCM 16677^T.

Strain	1		2		3		4		5		6	
	POCP %	AAI %	POCP %	AAI %	POCP %	AAI %	POCP %	AAI %	POCP %	AAI %	POCP %	AAI %
<i>E. ani</i> WH131 ^T (JAGSPB000000000)	71.55	77.75	81.94	87.65	65.59	72.32	68.27	73.23	60.2	67.01	65.79	68.33
<i>E. crassostrea</i> WH158 ^T (JAGSPC000000000)	72.99	81.22	64.35	76.75	62.72	71.32	61.28	71.50	54.04	63.69	64.12	66.91
<i>E. insulae</i> JBTF-M21 ^T (GCA_007004095)	100	100	71.34	77.65	69.39	71.82	69.24	72.70	59.55	64.81	67.34	67.85
<i>E. rubeus</i> KMU-140 ^T (GCA_014705715)	71.34	77.65	100	100	64.3	72.02	68.58	73.40	57.19	64.86	66.55	68.43
<i>P. vadosimaris</i> JL3514 ^T (GCA_012979275)	59.55	64.81	57.19	64.86	56.05	64.31	58.14	65.39	100	100	60.97	67.64
<i>P. jejuensis</i> JCM 16677 ^T (GCA_009827995)	67.34	67.85	66.55	68.43	62.46	66.08	64.51	67.67	60.97	67.64	100	100
POCP (percentage of conserved proteins)												
AAI (average amino acid identity)												

Additionally, as shown in Table 4 and Table 5, all of the type strains had an ANI value less than the species cut-off value of 95% and dDDH scores less than the threshold value of 70%, indicating that strains WHY3^T, WH131^T, and WH158^T can be distinguished from the other known available *Winogradskyella* and *Erythrobacter* species (Chun et al. 2018).

Table 4

ANI values of the strains WHY3^T, WH131^T and WH158^T and its closely related type strains. 1, *Winogradskyella luteola* WHY3^T; 2, *Winogradskyella flava* KCTC 52348^T; 3, *Winogradskyella ouciana* ZXX205^T; 4, *Winogradskyella echinorum* KCTC 22026^T; 5, *Erythrobacter ani* WH131^T; 6, *Erythrobacter crassostrea* WH158^T; 7, *Erythrobacter insulae* JBTF-M21^T; 8, *Erythrobacter rubeus* KMU-140^T; 9, *Erythrobacter longus* DSM 6997^T; 10, *Erythrobacter litoralis* DSM8509^T; 11, *Pseudopontixanthobacter vadosimaris* JL3514^T; 12, *Parerythrobacter jejuensis* JCM 16677^T.

Genus name	<i>Winogradskyella</i>				<i>Erythrobacter</i>							
	1	2	3	4	5	6	7	8	9	10	11	12
Species name	ANI	ANI	ANI	ANI	ANI	ANI	ANI	ANI	ANI	ANI	ANI	ANI
<i>W. luteola</i> WHY3 ^T (JAGSPD000000000)	100	77.53	77.90	77.39	-	-	-	-	-	-	-	-
<i>W. flava</i> KCTC 52348 ^T (GCA_014243395)	77.53	100	77.68	76.74	-	-	-	-	-	-	-	-
<i>W. ouciana</i> ZXX205 ^T (GCA_009709615)	77.90	77.68	100	77.07	-	-	-	-	-	-	-	-
<i>W. echinorum</i> KCTC 22026 ^T (GCA_014297365)	77.39	76.74	77.07	100	-	-	-	-	-	-	-	-
<i>E. ani</i> WH131 ^T (JAGSPB000000000)	-	-	-	-	100	74.90	74.98	81.72	72.87	74.61	73.58	71.74
<i>E. crassostrea</i> WH158 ^T (JAGSPC000000000)	-	-	-	-	74.90	100	77.45	74.89	72.41	72.80	70.09	70.86
<i>E. insulae</i> JBTF-M21 ^T (GCA_007004095)	-	-	-	-	74.98	77.45	100	75.23	72.47	72.93	70.35	71.09
<i>E. rubeus</i> KMU-140 ^T (GCA_014705715)	-	-	-	-	81.72	74.89	75.23	100	73.02	75.54	71.54	71.96
<i>E. longus</i> DSM 6997 ^T (GCA_000715015)	-	-	-	-	72.87	72.41	72.47	73.02	100	72.47	70.71	70.24
<i>E. litoralis</i> DSM8509 ^T (GCA_000714795)	-	-	-	-	74.61	72.80	72.93	75.54	72.47	100	72.38	72.07
OrthoANIu (ANI) values ≥ 95% demonstrated in bold.												

Table 5

dDDH values of the strains *Winogradskyella* sp WHY3^T, *Erythrobacter* sp WH131^T, *Erythrobacter* sp WH158^T, and its closely related type strains. 1, *Winogradskyella luteola* WHY3^T; 2, *Winogradskyella flava* KCTC 52348^T; 3, *Winogradskyella ouciana* ZXX205^T; 4, *Winogradskyella echinorum* KCTC 22026^T; 5, *Erythrobacter ani* WH131^T; 6, *Erythrobacter crassostrea* WH158^T; 7, *Erythrobacter insulae* JBTF-M21^T; 8, *Erythrobacter rubeus* KMU-140^T; 9, *Erythrobacter longus* DSM 6997^T; 10, *Erythrobacter litoralis* DSM8509^T; 11, *Pseudopontixanthobacter vadosimaris* JL3514^T; 12, *Parerythrobacter jejuensis* JCM 16677^T.

Genus name	<i>Winogradskyella</i>				<i>Erythrobacter</i>							
	1	2	3	4	5	6	7	8	9	10	11	12
Species name	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH	dDDH
<i>W. luteola</i> WHY3 ^T (JAGSPD000000000)	100	20.90	21.00	20.50	--	--	--	--	--	--	--	--
<i>W. flava</i> KCTC 52348 ^T (GCA_014243395)	20.90	100	20.70	20.10	--	--	--	--	--	--	--	--
<i>W. ouciana</i> ZXX205 ^T (GCA_009709615)	21.00	20.70	100	20.40	--	--	--	--	--	--	--	--
<i>W. echinorum</i> KCTC 22026 ^T (GCA_014297365)	20.50	20.10	20.40	100	--	--	--	--	--	--	--	--
<i>E. ani</i> WH131 ^T (JAGSPB000000000)	--	--	--	--	100	18.30	18.50	23.50	19.20	18.90	31.80	19.00
<i>E. crassostrea</i> WH158 ^T (JAGSPC000000000)	--	--	--	--	18.30	100	19.80	18.70	17.50	17.90	18.80	17.90
<i>E. insulae</i> JBTF-M21 ^T (GCA_007004095)	--	--	--	--	18.50	19.80	100	18.50	18.80	18.00	18.70	18.60
<i>E. rubeus</i> KMU-140 ^T (GCA_014705715)	--	--	--	--	23.50	18.70	18.50	100	18.90	19.10	19.40	18.80
<i>E. longus</i> DSM 6997 ^T (GCA_000715015)	--	--	--	--	19.20	17.50	18.80	18.90	100	18.60	20.80	19.00
<i>E. litoralis</i> DSM8509 ^T (GCA_000714795)	--	--	--	--	18.90	17.90	18.00	19.10	18.60	100	18.40	18.60
dDDH values > 70% demonstrated in bold.												

The genes related to bioremediation using <https://narrative.kbase.us/> for *Winogradskyella luteol* WHY3^T, *Erythrobacter ani* WH131^T, *Erythrobacter crassostrea* WH158^T, and closet type strains are reported in Table 6.

Table 6

Distribution of genes involved in bioremediation in 1, *Winogradskyella luteola* WHY3^T; 2, *Winogradskyella flava* KCTC 52348^T 3, *Winogradskyella ouciana* ZXX205^T; 4, *Winogradskyella echinorum* KCTC 22026^T; 5, *Erythrobacter ani* WH131^T; 6, *Erythrobacter crassostrea* WH158^T; 7, *Erythrobacter insulae* JBTF-M21^T; 8, *Erythrobacter rubeus* KMU-140^T; 9, *Erythrobacter longus* DSM 6997^T; 10, *Erythrobacter litoralis* DSM8509^T using <https://narrative.kbase.us/> accessed on 04 April 2022.

Genus name	<i>Winogradskyella</i>					<i>Erythrobacter</i>				
	1	2	3	4	5	6	7	8	9	10
Target substance and genes										
Inorganic mercury (<i>merA, merB, MerC, MerE, MerF, MerT, MerP</i>)	<i>merT</i> <i>merP</i>	-	<i>merT</i> <i>merP</i>	-	-	-	-	-	-	-
PAH (<i>phnA</i>)	<i>phnA</i>	<i>phnA</i>	<i>phnA</i>	<i>phnA</i>	-	-	-	-	-	-
Polyhydroxyalkanoic acid	-	-	-	-	+	+	+	+	+	+
Phenol degradation (<i>dmpN</i>)	-	-	-	-	-	-	-	-	-	-
Chromate resistance (<i>chrB</i>)	-	-	-	-	-	-	-	-	-	-
Cobalt–zinc–cadmium resistance protein (<i>czcA, czcD</i>)	<i>czcD</i>	<i>czcD</i> <i>czcA</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>	<i>czcD</i>
Nickel–cobalt–cadmium resistance protein (<i>nccA, nccX</i>)	-	-	-	-	<i>nccX</i>	-	-	-	<i>nccX</i>	-
Copper resistance (<i>cusA, cusB, cusC, cusF, protein B</i>)	-	<i>cusA</i>	-	-	<i>cusA</i> <i>cusB</i> <i>cusC</i> protein B	-	protein B	protein B	<i>cusA</i> <i>cusB</i>	protein B
+, presence; -, absence of gene										

Mercury resistance in the environment might be due to the presence of *merT* and *merP* genes in *Winogradskyella luteol* WHY3^T and *Winogradskyella ouciana* ZXX205^T, as described as a different approach to mercury resistance and bioaccumulation by marine bacteria (Zhang et al. 2020). The presence of *phnA* gene in strain *Winogradskyella luteol* WHY3^T and other nearest type strains emphasized the role of *Winogradskyella* for oxidation of anthracene and phenanthrene, although these genes were not detected in *Erythrobacter ani* WH131^T and *Erythrobacter crassostrea* WH158^T. The heavy metal resistance protein cobalt–zinc–cadmium *czcD* gene was found in *Winogradskyella luteol* WHY3^T, *Erythrobacter ani* WH131^T and *Erythrobacter crassostrea* WH158^T. A related gene for granulate polyhydroxyalkanoates (PHAs) was present in *Erythrobacter ani* WH131^T and *Erythrobacter crassostrea* WH158^T. The genes *cusA*, *cusB*, *cusC*, and protein B (related to Copper resistance genes) and the Nickel–cobalt–cadmium resistance protein genes *nccX* were reported just for *Erythrobacter ani* WH131^T, but not for *Erythrobacter crassostrea* WH158^T (Table 6).

Additionally, gene annotation using RAST analysis (<https://rast.nmpdr.org>) predicted 3319, 3147, and 2564 coding sequences in the genome of WHY3^T, WH131^T and WH158^T, respectively. The dominant fraction of subsystem features for WHY3^T were amino acids and derivatives (169), Cofactors – Vitamins - Prosthetic Groups - Pigments (123), protein metabolism (139), carbohydrates (93), Fatty Acids - Lipids, and Isoprenoids (50). Other genes which were detected that have a role in the development process, were present as follows: virulence, disease, and defense (24), stress Response (20), and metabolism of Aromatic Compounds (9). For the protein metabolism genes, a significant percentage was for Protein biosynthesis. The dominant fraction of subsystem features for WH131^T and WH158^T were amino acids and derivatives (214,158), protein metabolism (164, 84), carbohydrates (136,91), Cofactors – Vitamins - Prosthetic Groups – Pigments (110,94), Membrane Transport (100,42), Fatty Acids - Lipids, and Isoprenoids (80,65), Respiration (83,63) and Stress Response (51,31), respectively. The presence of protein and nucleoprotein secretion system gene (Type IV) was remarkable in WH131^T as it is absent in WH158^T. (Fig. S3) (Table S2).

The extract of strain WHY3^T could moderately inhibit the growth of *Staphylococcus aureus* Newman, *Candida albicans* DSM 1665 and weak inhibited *Bacillus subtilis* DSM 10 and *Wickerhamomyces anomalus* DSM 6766; The extract of strain WH131^T and WH158^T showed no remarkable inhibition against the most tested microbes (Table 7).

Table 7. MIC values (%) of inhibition test with extracts from strains WHY3^T, WH131^T, and WH158^T. Test strains were: 1, *E. coli* wild type BW25113; 2, *E. coli* acrB JW25113; 3, *P. aeruginosa* DSM 19882; 4, *S. aureus* Newman; 5, *C. freundii* DSM 30039; 6, *A. baumannii* DSM 30008; 7, *B. subtilis* DSM 10; 8, *M. smegmatis* ATCC 700084; 9, *M. hiemalis* DSM 2656; 10, *W. anomalus* DSM 6766; 11, *C. albicans* DSM 1665.

Microorganisms	1	2	3	4	5	6	7	8	9	10	11
WHY3 ^T	-	-	-	0.84	-	-	0.84	6.67	-	3.37	0.84
WH131 ^T	-	-	-	-	-	-	-	-	-	-	-
WH158 ^T	-	-	-	-	-	-	6.67	-	-	6.67	-

MIC value = 6.67- 3.34% (low activity); 1.67 - 0.42% (moderate activity); and 0.21 - 0.05% (strong activity).

4. Conclusion

This polyphasic study indicates that isolates WHY3^T, WH131^T, and WH158^T are new species belonging to the genus *Winogradskyella* and *Erythrobacter*. Based on our results, we propose the name *Winogradskyella luteola* sp.nov. for strain WHY3^T; and *Erythrobacter ani* sp.nov. and *Erythrobacter crassostrea* sp.nov. for strains WH131^T and WH158^T, respectively. Environmental pollution is one of the most serious issues that the twenty-first century is dealing with. Restoration and rehabilitation of contaminated sites have attracted a great deal of interest from the scientific community, with bioremediation as the main methods in such endeavors. Based on our genome analysis, all three strains, WHY3^T, WH131^T, and WH158^T, show they have the potential for bioremediation, as they contain certain important genes that have already been proven to be involved in bioremediation processes. The here described bacterial strains might have great potential for the bioremediation of certain pollutants either by absorbing or degrading certain pollutants. Therefore, we invite other researchers to join and to further explore the potential of the here described bacterial isolates in bioremediation processes and projects. The three novel species' protologue descriptions and etymologies are provided in descriptions.

Description of *Winogradskyella luteola* sp.nov.

Winogradskyella luteola (lu.te.o'la. L. fem. adj. luteola, light yellow). Cells are Gram-negative-staining, motile by gliding, rod-shaped, aerobic, no-spore-form, devoid of flagella, 0.3–0.4 µm width and 0.8–2.1 µm in length. Colonies are yellow. Temperature range for growth is 5–40 (°C) pH spectrum for growth 6–9 and NaCl (optimum) for growth 2.5 (%).

Positive for: catalase, oxidase, esterase (C4), esterase lipase (C8), phosphatase acid, naphthol-AS-BI-phosphohydrolase, acetoin, phosphatase alkaline, leucin arylamidase, valine arylamidase, assimilation of D-glucose, malic acid. There are also positive results (Biolog GEN III Micro Plate analysis) for glucuronamide, Gentiobiose, D-turanose, α-D-glucose, D-fucose, L-fucose, L-rhamnose, acetoacetic acid, acetic acid, L-malic acid, bromo-succinic acid, potassium tellurite, sodium butyrate, sodium bromate, D-glucuronic acid, D-fructose-6-PO4, L-histidine, 1% NaCl, 4% NaCl and 8% NaCl, 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, niaproof 4, vancomycin, nalidixic acid as well as susceptibility for chloramphenicol, thioestrepton, erythromycin, aztreonam. Major polar lipids are phosphatidylethanolamine (PE), unidentified glycolipid (GL), unidentified aminolipid (AL), and unidentified polar lipid (L). The predominant cellular fatty acids are C15:0, anteiso-C15:1 ω7c, iso-C15:0, C16:1ω7c. The menaquinone-6 (MK-6) is the major respiratory quinone.

The type strain WHY3^T (= DSM 111804^T = NCCB 100833^T) was isolated from Hemolymph of Pacific Oyster *Crassostrea gigas*, which was collected from Wilhelmshaven in Germany. The DNA G + C content of type strain is 34.50%. Genome size of strain WHY3^T indicates 3,53 Mbp. The GenBank/NCBI accession numbers for 16S rRNA Gene sequence and whole-genome sequence of strain WHY3^T are MW888983 and JAGSPD000000000, respectively.

Description of *Erythrobacter ani* sp.nov.

Erythrobacter ani (a'ni. L. gen. n. *ani*, of the anus, referring to anus area near the adductor muscle in *Crassostrea gigas*). Gram-negative, no-spore-form, non-flagellated and coccoid, ovoid or rod-shaped cell, 0.2–0.3 µm width and 0.6–3.1 µm length: Colonies are yellowish-orange. Temperature range for growth is 20–35 (°C) pH spectrum for growth 6–9 and NaCl (optimum) for growth 2.5 (%). Positive for: catalase, oxidase, esterase (C4), esterase lipase (C8), cystine arylamidase, trypsin, α-chymotrypsin, phosphatase acid, nitrate reduction, arginine dihydrolase, aesculin hydrolysis, tween 80 hydrolysis, phosphatase alkaline, leucin arylamidase, valine arylamidase, assimilation of D-glucose, D-mannose, D-mannitol, D-maltose, malic acid, phenylacetic acid. There are positive results (Biolog GEN III Micro Plate analysis) for glucuronamide, D-glucose-6-phosphate, D-fructose-6-phosphate, D-galacturonic acid, L-lactic acid, α-ketoglutaric acid, β-hydroxy-D, L-butyric acid, acetic acid, dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, 1% NaCl, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-aspartic acid, gelatin, glycyl-L-proline, L-glutamic acid, pectin, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, mucic acid, quinic acid, methyl pyruvate, D-lactic acid methyl ester, D-malic acid, L-malic acid, bromosuccinic acid, p-Hydroxy phenylacetic acid, tween 40, α-

ketobutyric acid, formic acid, potassium tellurite, aztreonam, sodium butyrate. The other substrates were absent or negative. Antibiotic susceptibility for gentamycin, kanamycin, chloramphenicol, spectinomycin, fusidic acid, thiostrepton, erythromycin and tetracycline. Major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), sphingoglycolipid (SGL), unidentified glycolipid (GL), unidentified aminolipid (AL), and unidentified polar lipid (L). The predominant cellular fatty acids are C_{14:0}-2-OH, t_{18:1}ω12. The ubiquinone-10 (Q-10) is their predominant isoprenoid quinone.

The type strain WH131^T (= DSM 112099^T = NCCB 100824^T) was isolated from Hemolymph of Pacific Oyster *Crassostrea gigas*, which was collected from Wilhelmshaven in Germany. The DNA G + C content of type strain is 59.70%. Genome size of strain WHY3^T indicates 3,15 Mbp. The GenBank/NCBI accession numbers for 16S rRNA Gene sequence and whole-genome sequence of strain WH131^T are MW888981 and JAGSPB000000000, respectively.

Description of *Erythrobacter crassostreae* sp.nov.

Erythrobacter crassostreae (crass.os'tre.ae. N.L. gen. n. *crassostreae*, referring to the giant oyster *Crassostrea gigas*). Gram-negative, no-spore-form, non-flagellated, and coccoid, ovoid or rod-shaped cell, 0.2–0.4 μm width and 0.5–2.4 μm length. Colonies are orange. Temperature range for growth is 20–35 (°C) pH spectrum for growth 6–9 and NaCl (optimum) for growth 2.5 (%). Positive for: catalase, oxidase, esterase (C4), esterase lipase (C8), cystine arylamidase, trypsin, α –chymotrypsin, phosphatase acid, Lipase (C14), Naphtol-AS-BI-phosphohydrolase, arginine dihydrolase, aesculin hydrolysis, phosphatase alkaline, leucin arylamidase, valine arylamidase, phosphatase alkaline, leucin arylamidase, valine arylamidase, assimilation of D-glucose, D-mannose, D-mannitol, D-maltose, phenylacetic acid. There are positive results (Biolog GEN III Micro Plate analysis) for dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, 1% NaCl, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, D-fucose, L-rhamnose, inosine, D-mannitol, D-glucose-6-phosphate, D-fructose-6-phosphate, glycyl-L-proline, L-glutamic acid, pectin, D-galacturonic acid, L-galactonic acid lactone, D-glucuronic acid, D-gluconic acid, glucuronamide, mucic acid, quinic acid, p-Hydroxy phenylacetic acid, methyl pyruvate, L-lactic acid, D-malic acid, L-malic acid, nalidixic acid, tween 40, α-ketobutyric acid, acetic acid, formic acid, aztreonam, sodium butyrate. The other substrates were absent or negative. Antibiotic susceptibility for gentamycin, kanamycin, chloramphenicol, spectinomycin, fusidic acid, thiostrepton, nalidixic acid, erythromycin, and tetracycline. Major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), sphingoglycolipid (SGL), unidentified glycolipid (GL), unidentified aminolipid (AL), and unidentified polar lipid (L). The predominant cellular fatty acids are C_{17:0} and C_{18:1}ω7c. The ubiquinone-10 (Q-10) is their predominant isoprenoid quinone.

The type strain WH158^T (= DSM 112102^T = NCCB 100877^T) was isolated from Hemolymph of Pacific Oyster *Crassostrea gigas*, which was collected from Wilhelmshaven in Germany. The DNA G + C content of type strain is 56.64%. The genome size of strain WHY3^T indicates 2,58 Mbp. The GenBank/NCBI accession numbers for 16S rRNA Gene sequence and whole-genome sequence of strain WH158^T are MW888982 and JAGSPC000000000, respectively.

Nucleotide sequences

The GenBank accession number for the 16S rRNA gene sequence of strains WHY3^T, WH131^T, and WH158^T are MW888983, MW888981, and MW888982, respectively. Draft genome of strains WHY3^T, WH131^T, and WH158^T was deposited at NCBI/GenBank under the accession numbers JAGSPD000000000, JAGSPB000000000, and JAGSPC000000000, respectively.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Conceptualization, performed experiments, data analyses, manuscript drafting, data analyses H.P, electron microscopy, M.M; pacific oyster samples preparation and technical support, P.J.S; the project supervisor and manuscript editor J.W.All authors read and edited the paper.

Funding

This scholarship awarded by Konrad Adenauer Stiftung (KAS) in Germany <https://www.kas.de/en/web/begabtenfoerderung-undkultur/promotionsfoerderung>

Institutional Review Board Statement

Not applicable

Acknowledgments

The authors appreciate the excellent effort of technical assistance, Mahshid Darab, prof. Kämpfer, Stephanie Schulz, Klaus Peter Conrad, Birte Trunkwalter, Wera Collisi, Ina Schleicher (for electron microscopy sample preparation), and Aileen Gollasch for recording the HRESIMS data. Special thanks to Jolanta Lulla for medium preparation.

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Tables

Table 8 is not available with this version

Figures

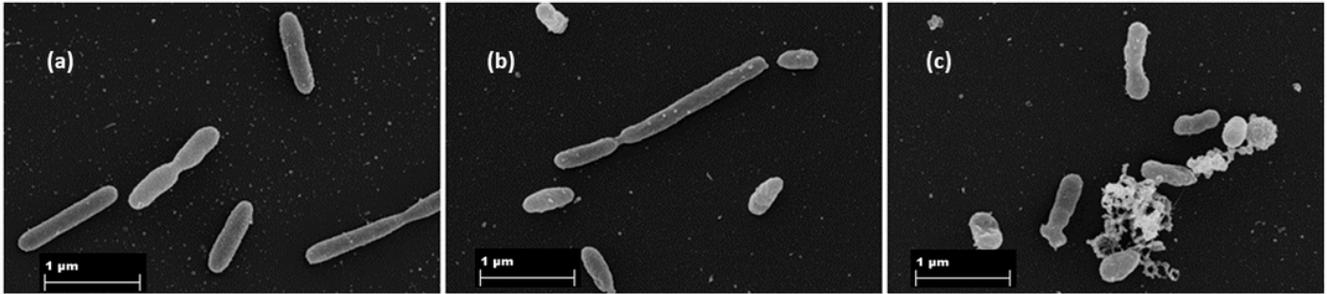


Figure 1

Scanning electron microscopy image of strains (a) WHY3^T, (b) WH131^T, and (c) WH158^T.

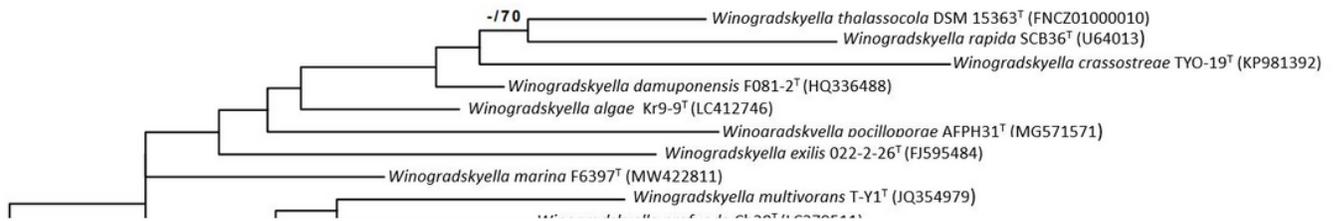


Figure 2

ML tree of strain WHY3^T and its closely related type strains inferred under the GTR+GAMMA model and rooted by midpoint-rooting. The numbers above the branches are support values (above 60%) from ML (left) and MP (right) bootstrapping. The ML and MP bootstrapping average support were 26.12% and 42.40%, respectively.

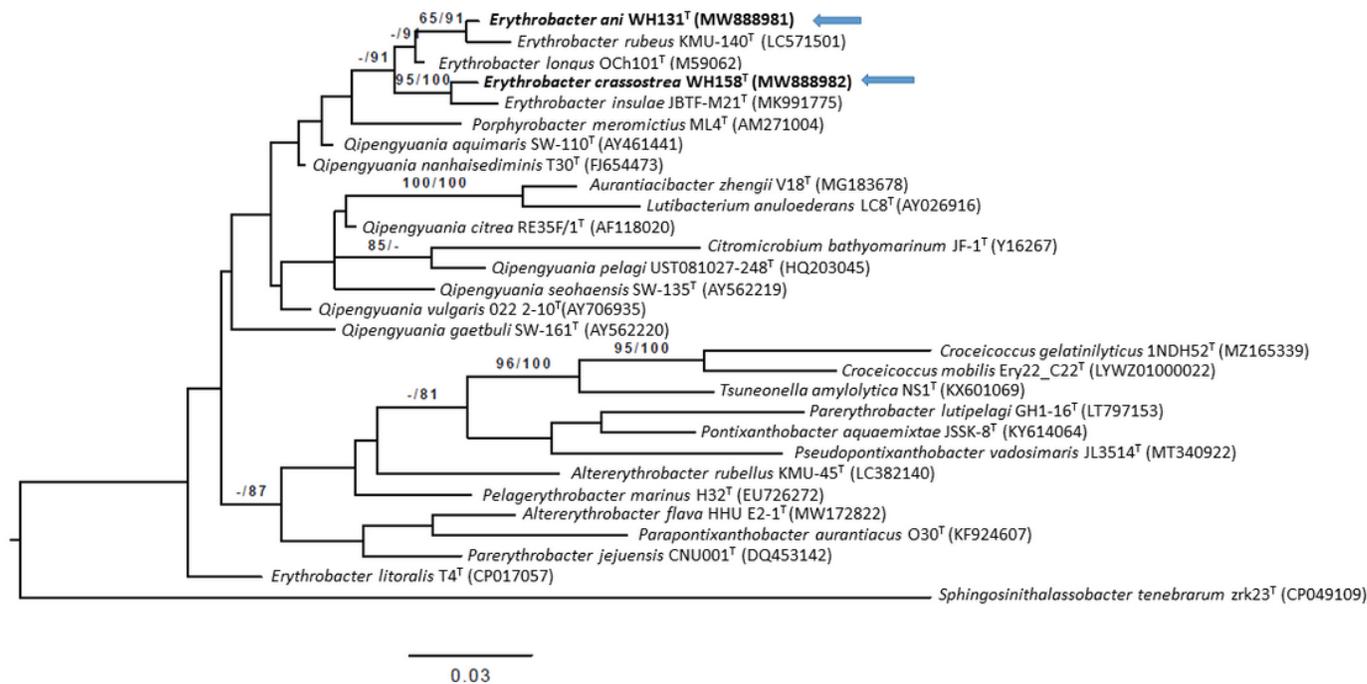


Figure 3

ML tree of strains WH131^T, WH158^T and its closely related type strains inferred under the GTR+GAMMA model and rooted by midpoint-rooting. The numbers above the branches are support values (above 60%) from ML (left) and MP (right) bootstrapping. The ML and MP bootstrapping average support were 50.92% and 61.73%, respectively.

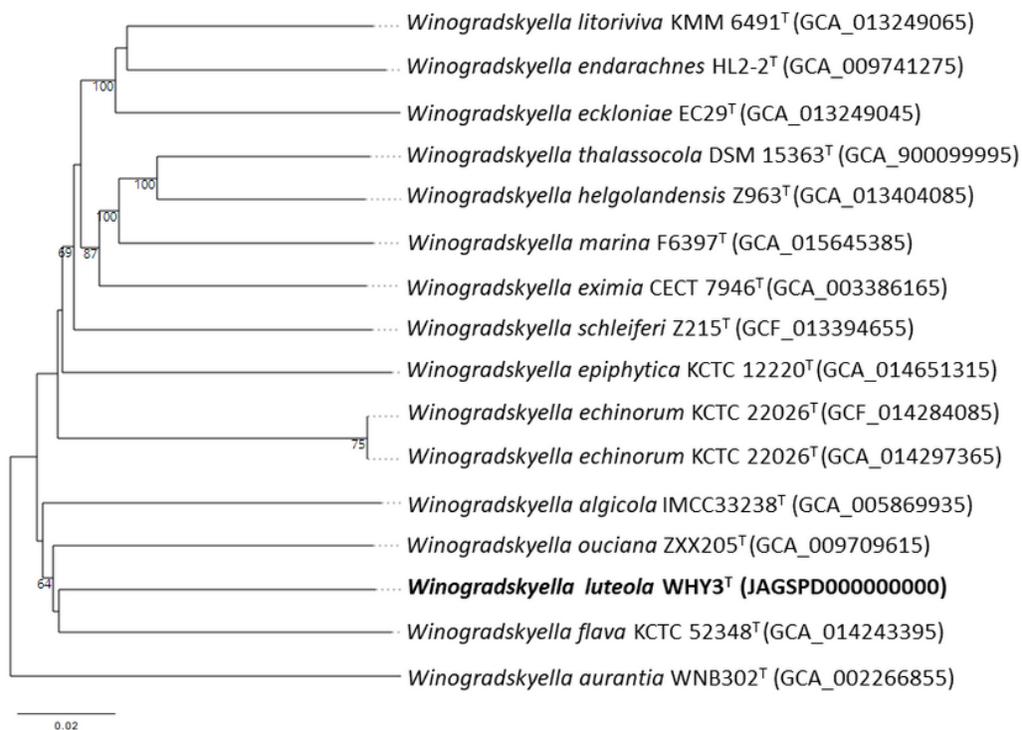


Figure 4

Phylogenomic tree showing relationships between strain WHY3^T and the most closely related type strains from the genus *Winogradskyella*. The number at the nodes are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with average branch support of 65.5%. Bar, 0.02 substitutions per site.

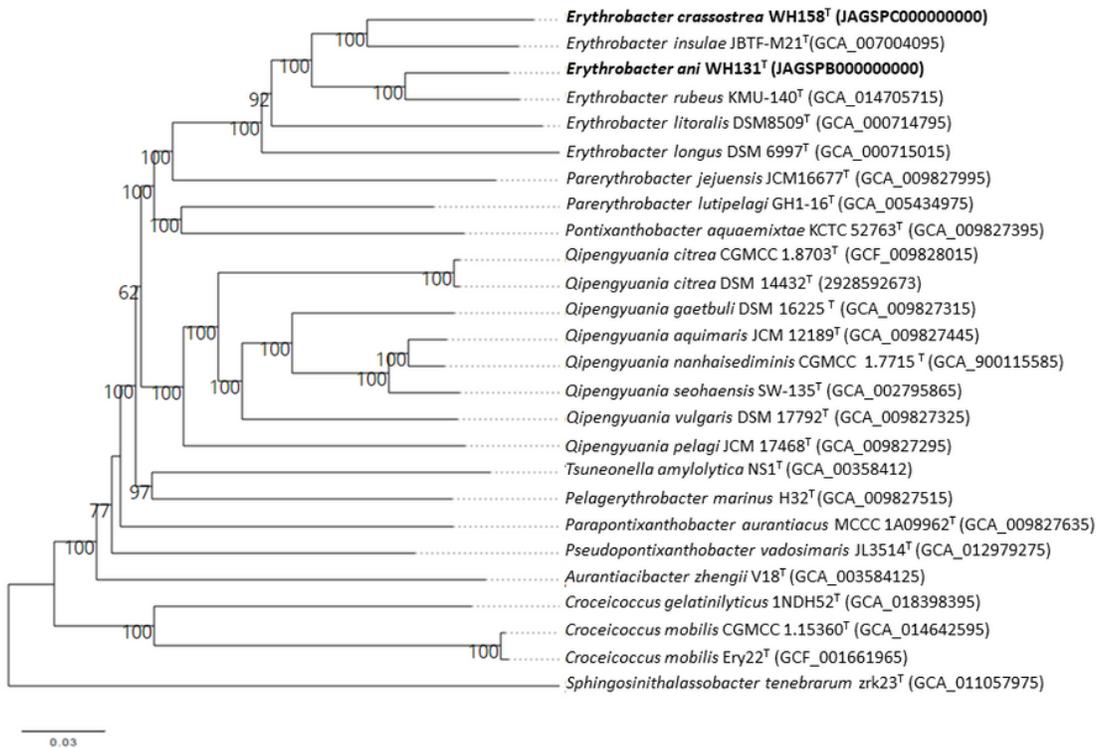


Figure 5

Phylogenomic tree showing relationships between strains WH131^T, WH158^T and the most closely related type strains from the genus *Erythrobacter* according to whole-proteome-base. The number at the nodes are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with average branch support of 94.5%.

Supplementary Files

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