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ORIGINAL PAPER

Parasalinivibrio latis gen. nov., sp. nov., isolated from the distal gut of healthy farmed Asian Seabass (*Lates calcarifer*)

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Abstract Asian Seabass (*Lates calcarifer*) is widely farmed as a sustainable source of protein for countries in the tropical Indo-West Pacific region. However, microbial species of the gut microbiome of healthy Asian Seabass remain largely uncharacterized and uncultured. Here, we analysed the microbial composition along the gastrointestinal tract of a farmed healthy Asian Seabass. We used different cultivation approaches to obtain isolates from the seabass intestinal tract and describe the isolation and characterization of a novel strain, TLL-SE01^T. Analysis of the strain's 16S rRNA gene indicates that the strain belongs to the family *Vibrionaceae* with *Photobacterium damselae* as its closest relative, albeit sharing only 94.8% (aligned region 1553 bp) nucleotide identity. Comparative genomic analysis with all validly published *Vibrionaceae* species with available genomes revealed average nucleotide identity (ANI) and DNA–DNA hybridisation (DDH) values of around 70% and 24% respectively to strain TLL-SE01^T, which are well below proposed thresholds for species delineation (ANI, 95–96%; DDH, 70%). The alignment fraction and ANI genus demarcation boundaries for all genera in the *Vibrionaceae* family were determined for which strain TLL-SE01^T is well below the calculated values, indicating that it belongs to a novel genus. Single- and core-gene phylogenetic analysis places strain TLL-SE01^T in a monophyletic clade, further supporting its designation to a novel genus. Phenotypic comparison between strain TLL-SE01^T and its close relatives indicated additional differences, such as growth response at different salt concentrations and different metabolic capabilities. Based on genotypic, phylogenetic and phenotypic differences to other *Vibrionaceae* species, we propose a novel species in a new genus, *Parasalinivibrio latis* gen. nov. sp. nov. and strain TLL-SE01^T (=BCRC 81435^T=JCM 36283^T) as the type strain.

Keywords Aquaculture microbiome · Pseudomonadota · Vibrionaceae · Asian Seabass · Biogeography of fish gut

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Introduction

The increasing demand for food fish to feed a growing world population has led to a rapid expansion of aquaculture facilities for farmed fish (Finegold 2009; Jennings et al. 2016; Lam 2016). Fishes in these facilities are often reared at high density, which may put the stock at risk of contracting and spreading diseases within facilities and beyond (Behringer et al. 2020; Krkošek 2010). Genomic selection for disease resistance is therefore considered to be a suitable approach to prevent or reduce the loss of fish stocks (Oikonomou et al. 2022; Song et al. 2023; Yáñez et al. 2022). However, selective breeding can often only be performed for one disease at a time and thus requires considerable time, i.e. multiple generations of fish (Gjedrem et al. 2012). Other means to develop disease resistance should therefore be considered. The fish's own microbiota may be altered to enrich for antimicrobial compounds producing microbes to fend off potential pathogens (Legrand et al. 2020; Zhou et al. 2022). However, the microbial composition of most fish species remains poorly studied and only a few cultured representatives have been obtained. This is particularly the case in tropical aquaculture systems, where the diversity of farmed fishes differs from that in temperate regions (Oyinlola et al. 2020; Soh et al. 2024).

In this study, we focused on the cultivation of the gut microbiota of the Asian Seabass (*Lates calcarifer*) which is farmed throughout the tropical regions of Southeast Asia and other locations of the Indo-West Pacific. In Singapore, aquaculture accounts for about 8% of total fish production meant for consumption and the Asian Seabass is one of the most farmed species of fish (SFA 2023). The Asian Seabass has been bred for rapid growth and, more recently, also for disease resistance (Liu et al. 2022; Wang et al. 2008; Ye et al. 2017). However, disease outbreaks from bacterial infections caused by members of the *Vibrionaceae* family remain commonly observed. This particularly concerns infections caused by *Photobacterium damselae* subsp. *damselae*, which causes lesions and haemorrhage in the fish, contributing to significant economic losses to aquaculture farms (Kanchanopas-Barnette et al. 2009; Labella et al. 2011; Sharma et al. 2017). The gut microbiota of healthy Asian Seabass remains grossly understudied despite its predominance in marine aquaculture. Likewise, in a recent study it was shown that the vast majority of fish-associated microbes from farmed and non-captive tropical fish remain uncultured (Soh et al. 2024). This presents a general knowledge gap that needs to be addressed before suitable probiotics may be developed for aquaculture applications. We therefore employed a comprehensive cultivation-based approach to isolate bacterial strains from the intestinal tract of a healthy Asian Seabass. Here, we report the isolation of a novel representative, strain TLL-SE01^T, of the family *Vibrionaceae*. The genome of the strain was sequenced and compared to that of related *Vibrionaceae* species and found to differ substantially in genomic and physiological features from representatives of related *Vibrionaceae* genera. While strain TLL-SE01^T was isolated from healthy Asian Seabass, some genomic features suggest that the strain may also have the potential to be an opportunistic pathogen. However, the exact role of the strain in fish health and disease remains to be elucidated. Based on the results of this polyphasic taxonomic study, it is concluded that the strain represents a novel genus.

Materials and methods

Sample collection and strain isolation

Five Asian Seabass with body weights ranging from 0.8 to 5.1 kg, were culled and collected from two commercial aquaculture facilities at different timepoints in Singapore. The fish did not show signs of disease at the time of collection and were deemed healthy. The fish were starved for 24 h prior to sacrifice to mitigate the direct influence of fish feed on gut microbiota composition. They were dissected aseptically on ice and gut contents were collected from the distal segment of the intestinal tract. The gut contents were homogenised by vortexing and flash-frozen in liquid nitrogen before storing at − 80 °C for downstream cultivation and amplicon sequencing. For each sample, approximately 10 µL of gut content was resuspended in 1 mL phosphate-buffered saline (1 M, pH 7.4) and ten-fold serially diluted up to four times. Then, 100 µL aliquots of each dilution were spread onto agar plates and incubated at 23 °C for six days under the following conditions: (1) Marine agar 2216 (MA, BD), incubated

aerobically in atmospheric air; (2) Gifu Anaerobic Medium (GAM, HiMedia) agar, incubated in a Coy anaerobic chamber (75% N_2, 20% CO_2, 5% H_2). Isolated colonies were re-streaked to ensure purity and resulting single colonies were inoculated in 5 mL Marine broth (MB) or GAM broth. A portion of the liquid cultures were cryopreserved in 25% (v/v) glycerol and remaining cultures were subjected to DNA extraction.

DNA extraction and strain verification

Genomic DNA (gDNA) of strain TLL-SE01^T was extracted for whole genome sequencing using QIAGEN Genomic-tip 20/G following the QIAGEN extraction protocol for bacteria (QIAGEN Genomic DNA Handbook 06/2015). The resulting gDNA was quantified and quality-checked using a Qubit 1.0 fluorometer and NanoDrop 2000 spectrophotometer.

For strain verification, gDNA was extracted using a phenol–chloroform bead-beating extraction method as previously described (Rius et al. 2012). DNA quantity and quality were measured using a NanoDrop 2000 spectrophotometer. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) primers (Lane 1991; Loy et al. 2005). The composition for each PCR reaction is as follows: 12.5 µL 2×GoTaq PCR master mix (Promega), 1.25 µL each of 10 µM 27F and 1492R primers, 3 µL of 10 ng/µL template DNA and topped up to 25 µL total volume with molecular grade water. PCR amplification was carried out according to the following conditions: initial denaturation at 95 °C for 3 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; final elongation at 72 °C for 10 min (T100 Thermal Cycler, Bio-Rad). The PCR amplicons were purified using QIAquick PCR purification kit (QIAGEN) and the purified products were subjected to Sanger sequencing using the same 27F and 1492R primers. The sequences were assembled and trimmed in SeqMan Pro 15 (DNASTAR) and aligned to sequences in the National Center for Biotechnology Information (NCBI) GenBank database using nucleotide Basic Local Alignment Search Tool (BLASTn) for isolate identification (Altschul et al. 1990).

Whole genome sequencing

Whole genome sequencing of strain TLL-SE01^T was conducted on an Illumina NovaSeq 6000 platform for 150 bp paired-end reads and on an Oxford Nanopore Technologies (ONT) MinION Mk1B device equipped with a R9.4.1 flow cell to obtain long-reads. Library preparation for Illumina paired-end sequencing was performed by an external vendor (NovogeneAIT) and that for Oxford Nanopore long-reads was performed according to ONT Native Barcoding protocol using the ligation sequencing kit (SQK-LSK109). Short-reads were quality filtered using fastp v0.23.2 to remove adapter sequences and trimmed as previously described (Chen et al. 2018; Low et al. 2023). Long-reads were quality filtered and barcodes removed using Guppy v3.2.10 installed in a MinIT MNT-001 device (ONT) (Wick et al. 2019). The average sequencing coverage achieved for paired-end and long-read sequencing were 221×and 112×, respectively. The whole genome was closed from the hybrid assembly of both the short- and long-reads using Unicycler v0.4.8 running on default parameters (Wick et al. 2017).

Phylogenetic analysis

The extracted full-length 16S rRNA gene sequence (1553 bp) and the whole genome sequence of strain TLL-SE01^T were used for subsequent phylogenetic and genomic analyses. Type strains of all validly published *Vibrionaceae* species were included in all analyses. Table S4 shows the accession numbers for the genomes used in this study. Genomes were retrieved from NCBI (accessed 01 Oct 2024) filtered according to the following parameters: (1) from *Vibrionaceae*, (2) from type material only, (3) CheckM completeness ≥ 90% and (4) CheckM contamination ≤ 6% (raised to 6% to include genomes from type species of *Aliivibrio* and *Enterovibrio*). The genomes were then cross-validated with List of Prokaryotic names with Standing in Nomenclature (LPSN; https://www.bacterio.net/) to ensure that only validly published strains were included. The phylogenomic tree was inferred by maximum likelihood using IQ-TREE v2.3.6 with 1000 bootstrap replications supported with ultrafast bootstrap (UFBoot) on a concatenated multiple sequence alignment of 81 single-copy core genes generated from the UBCG2 pipeline using default

parameters (Hoang et al. 2018; Kim et al. 2021a, b, c; Minh et al. 2020). Similarly, the maximum likelihood phylogenetic tree based on 16S rRNA gene sequences was constructed using IQ-TREE supported with 1,000 UFBoot replications. The multiple sequence alignment was generated using MAFFT v7.525 (L-INS-I option) and trimAl v1.5.0 (-automated1 flag) (Capella-Gutiérrez et al. 2009; Katoh and Standley 2013). The 16S rRNA genes were retrieved from LPSN database (accessed 04 Oct 2024). "*Coralli-bacterium pacifica*" OS-11M-2^T was included in the single gene phylogenetic analysis due to a high 16S rRNA gene sequence similarity (98.8%) with strain TLL-SE01^T; however, it does not have a publicly available whole genome sequence, thus, excluded from subsequent genomic comparisons.

Comparative genome analysis and genome annotation

Several overall genome relatedness indices (OGRIs) were used for comparative genomic analysis. Average nucleotide identities (ANI), digital DNA-DNA hybridisation (dDDH), amino acid identities (AAI) and percentage of conserved protein (POCP) values were determined by the OrthoANIu algorithm (Yoon et al. 2017b), Genome-to-Genome Distance Calculator (GGDC) by DSMZ following the recommended BLAST+ tool (Meier-Kolthoff et al. 2021), EzAAI pipeline (Kim et al. 2021a, b, c) and an open-source Ruby script (https://github.com/hoelzer/pocp), respectively. The alignment fraction (AF) and ANI values used in the combined OGRI approach were also derived from the OrthoANIu algorithm.

The genomes of strain TLL-SE01^T and the compared strains were uploaded to the Rapid Annotation using Subsystem Technology (RAST) webserver for structural and functional annotation (Aziz et al. 2008). Strains with the highest 16S rRNA gene sequence identity representing each genus from Table S2 were chosen for comparison. *Salinivibrio costicola* LMG 11651^T (type species) was included as well as *Salinivibrio* is phylogenetically close to strain TLL-SE01^T. Biosynthetic gene clusters were predicted using the online antiSMASH tool v6.1.1 with 'relaxed' strictness (Blin et al. 2021). Bacterial secretion systems were predicted using TXSScan v1.1.0 via the online Galaxy@Pasteur platform using default settings (Abby et al. 2016; Mareuil et al. 2017).

Phenotypic and biochemical characterisation

Gram staining was performed using Gram Staining Kit (Sigma) according to manufacturer's protocol. Cell morphology was determined by brightfield microscopy (Zeiss Axioplan 2 Upright) and cell size was derived from analysis of the 2D image results using open-source ImageJ software (Schneider et al. 2012).

Growth response was determined under the following conditions: (1) Lysogeny broth (LB) containing 0, 1, 2, 4, 6, 8, 11% (w/v) NaCl; (2) MB adjusted to pH 5, 6, 7, 8, 9, 10 using 1 M HCl (for pH 7 and below) and 1 M NaOH (for pH 8 and above); (3) Marine medium in a Coy anaerobic chamber. The experiments were performed in triplicates and growth response was evaluated by measuring the OD_{600nm} of the liquid cell cultures. Briefly, 120 µL of overnight liquid culture of strain TLL-SE01^T was inoculated into 6 mL of the respective media for each test condition and incubated at 23 °C, shaking at 200 rpm. OD_{600nm} was measured twice a day for three consecutive days. Temperature growth range and optimum temperature were determined by using the drop plate method at 4 °C, 15 °C, 23 °C, 30 °C, 37 °C, 40 °C, 45 °C. This was done by dispensing 10 µL of overnight liquid culture of strain TLL-SE01^T as a single drop onto MA agar plates in quadruplicates and growth was monitored every 24 h over a period of 3 days.

Growth under anaerobic conditions was determined by re-streaking an aerobic plate culture of strain TLL-SE01^T onto anoxic MA in an anaerobic chamber to eliminate residual oxygen, then inoculated into anoxic MB.

Catalase activity was determined by production of effervescence upon dousing an isolated colony of strain TLL-SE01^T with 25 µL of 2% (v/v) H_2O_2; an uninoculated plate of MA was used as negative control. Oxidase production was determined using oxidase test discs (Sigma-Aldrich) containing *N*,*N*-dimethyl-p-phenylenediamine oxalate and α-naphthol. API 20E, API 50 CH and API ZYM (bioMérieux) tests were used to evaluate the biochemical traits of strain TLL-SE01^T. All API and cellular fatty acids analyses were carried out by DSMZ Services (Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkuturen GmbH, Braunschweig, Germany). An outline of the methods employed for cellular fatty

acids analysis can be found on their website (www.dsmz.de).

Amplicon sequencing and data processing

DNA for amplicon sequencing was extracted from the gut contents (same five fishes used in the cultivation experiment) using a phenol–chloroform bead-beating extraction method as previously described (Rius et al. 2012). The extracted DNA was quantified using Quant-iT Picogreen (Thermo Fisher Scientific). PCR amplification targeting the V4 region of the 16S rRNA gene using 515F (Parada) and 806R (Apprill) primers was carried out according to the 16S Illumina Amplicon Protocol from the Earth Microbiome Project (Thompson et al. 2017). For each sample, PCR was performed in triplicates and a no-template control was included as well. Each PCR reaction contained 25 µL of: 1×Q5 reaction buffer (New England BioLabs), 0.4 U Q5 High-Fidelity DNA polymerase (New England BioLabs), 30 ng template DNA, final concentrations of 80 µM dNTP mix (Promega), 0.2 µM forward and reverse primers each, and molecular grade water. The PCR reactions were carried out in a T100 thermal cycler (Bio-Rad) under the following conditions: initial denaturation at 94 °C for 3 min; 34 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s; final elongation at 72 °C for 10 min. PCR products were pooled together and purified using AMPure XP beads (Beckman Coulter Life Sciences). Purified PCR products were quantified and quality-checked using a Qubit 1.0 fluorometer and NanoDrop 2000 spectrophotometer. The resulting amplicons were sequenced using 150 bp paired-end sequencing chemistry with 20% PhiX spike-in on the Illumina Miseq platform at the Genome Institute of Singapore.

Demultiplexed sequence files were provided by the sequencing vendor in FASTQ format. All sequence data were processed using QIIME 2 2021.4 unless otherwise stated (Bolyen et al. 2019). Forward reads were denoised and trimmed to 138 bases (position 13–150) using the 'q2-dada2' plugin (Callahan et al. 2016). Using the 'q2-feature-classifier' plugin (Bokulich et al. 2018) in QIIME 2 2022.8, taxonomy classification was performed on the resulting amplicon sequence variants (ASVs) with BLAST+(Camacho et al. 2009) against a pre-formatted SILVA SSU (version 138.1) V4 region non-redundant 99% identity database (Quast et al. 2012; Robeson et al. 2021) that included the reference sequence and taxonomy label of strain TLL-SE01^T. ASVs that were classified as archaea, eukaryotes, mitochondria, chloroplast or unassigned were then filtered out. Ordinary least squares regression was performed to determine the trend of the relative abundances of strain TLL-SE01^T across the gut segments. A P-value < 0.05 indicates significance of the fit of the model. All statistical analyses were performed in Python 3.9 using the 'statsmodels' module (version 0.13.5).

Results and discussion

Cultivation-dependent and -independent diversity of the gut microbiota

The cultivation approach undertaken in this study allowed us to identify 13 unique bacterial isolates from the distal intestinal tract of the Asian Seabass (see Table S1 for the composition of cultivated bacteria). Eight were isolated only on MA, three were isolated only on GAM agar and two were isolated on both media types. These bacterial isolates belonged to seven different families (*Bacillaceae*, *Dermacoccaceae*, *Fusobacteriaceae*, *Micrococcaceae*, *Ornithinimicrobiaceae*, *Shewanellaceae* and *Vibrionaceae*) corresponding to four distinct phyla *Actinomycetota* (formerly *Actinobacteria*), *Bacillota* (formerly *Firmicutes*), *Fusobacteriota* (formerly *Fusobacteria*) and *Pseudomonadota* (formerly *Proteobacteria*) (Oren and Garrity 2021). *Photobacterium* was the predominant genus, constituting 70% of the total cultivated microbial diversity. This is unsurprising as *Photobacterium* has been shown to be enriched in marine fish and dominated the gut of piscivores like the Asian Seabass (Huang et al. 2020; Kim et al. 2021a, b, c; Miyake et al. 2020; Soh et al. 2024; Zheng et al. 2019). Strain TLL-SE01^T, a putative novel taxon from the family *Vibrionaceae*, was isolated from one fish sample, representing 13.9% of the cultivated bacterial population in the distal intestine of this fish. Upon observing this, we were curious about the abundance of strain TLL-SE01^T and diversity within the fish's overall gut microbial community. As such, amplicon sequencing of the 16S rRNA gene was performed for all segments of the alimentary tract in the fish and the microbial community composition is presented in Fig. 1. Overall, 107,750 reads comprising 134 unique

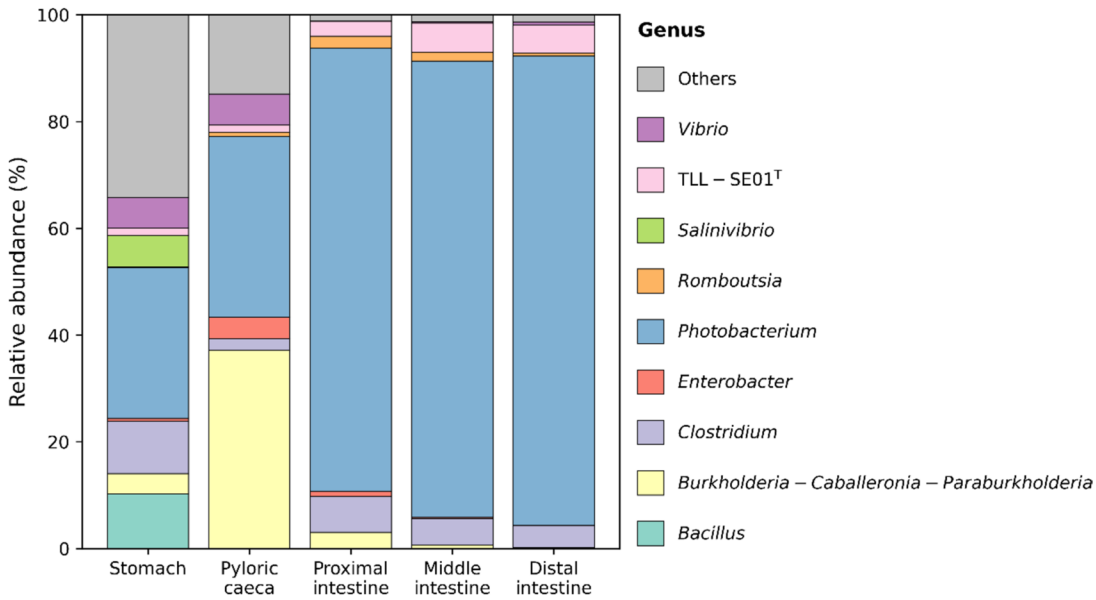

Fig. 1 Microbial community composition based on 16S rRNA gene amplicon sequencing data of each gut segment of the fish sample harbouring strain TLL-SE01^T. Relative abundances of dominant bacterial genera are displayed. The 'others' category include uncultured and low abundance genera (mean relative abundance across gut segments < 1%)

ASVs were obtained, of which, only one ASV was assigned to strain TLL-SE01^T. *Photobacterium* was the most abundant genus in the whole gut except the pyloric caeca which was dominated by *Burkholderia-Cabelleronia-Paraburkholderia*. The mean relative abundance of strain TLL-SE01^T across the gut segments was 4.25%. Notably, the relative abundance of strain TLL-SE01^T increased significantly along the gut ($P < 0.05$, F-test on OLS regression) from 1.4% in the stomach to 5.3% in the distal intestine, suggesting that it was able to colonise and proliferate in the host intestinal tract. Strain TLL-SE01^T was only detectable in one of the five fish that were analysed for this study. Since the fish were collected at different timepoints from different aquaculture facilities with different farming practices, it may indicate that the strain's presence in the collected fish could be reflective of a specific farming routine.

Single- and multi-locus based phylogenetic analyses of strain TLL-SE01^T

Using a near full-length 16S rRNA gene sequence obtained by Sanger sequencing, preliminary identification by BLAST showed that strain TLL-SE01^T belong to the family *Vibrionaceae*, having over 5% sequence divergence from the closest result. Subsequently, 12 identical copies of complete 16S rRNA gene sequences (1553 bp) were extracted from the whole genome sequence of strain TLL-SE01^T and aligned against the EzBioCloud database (Yoon et al. 2017a). The results revealed strain TLL-SE01^T to be closely related to *Photobacterium damselae* subsp. *damselae* ATCC 33539^T (identity 94.8%; coverage 100%). Other closely related genera were revealed to be *Enterovibrio*, *Grimontia* and *Veronia* with 16S rRNA gene sequence identities ranging from 93.9 to 94.2% (Table S2). The proposed 16S rRNA sequence similarity thresholds for genus and species delineation are 94.5% and 98.7% respectively (Chun et al. 2018; Yarza et al. 2008). While the 16S rRNA gene sequence similarity value of *P. damselae* ATCC 33539^T falls well below the species delineation threshold, it is only marginally above the genus delineation boundary. This suggests that strain TLL-SE01^T could be a novel species under the genus *Photobacterium*, however, the borderline deviation of the 16S rRNA gene sequence similarity between strain TLL-SE01^T and *P. damselae* ATCC 33539^T from the genus delineation boundary warrants further investigation of its taxonomic placement.

It was observed that strain TLL-SE01^T was also closely related to a non-validly published genus "*Corallibacterium*" comprising one representative, "*Corallibacterium pacifica*" OS-11M-2^T (98.8% 16S rRNA gene sequence identity). For this reason, "*C. pac*" OS-11M-2^T was included in the 16S rRNA gene sequence-based phylogenetic tree, but it was omitted from the phylogenomic and subsequent comparative genomic analyses as it did not have a publicly available genome sequence at the time of writing. The phylogenetic tree revealed that strain TLL-SE01^T formed a monophyletic clade with "*C. pac*" OS-11M-2^T with 71% bootstrap confidence, insinuating that both strains could be classified into the same genus (Fig. S1). It should be noted that the bootstrap values for most nodes in the phylogenetic tree based on the 16S rRNA gene are relatively low and many fall below 70%, indicating weak support for its topology (Hillis and Bull 1993). Subsequently, following a guideline by Chun et al. (2018) which recommended the implementation of at least 31 genes for phylogenomic analysis, a genome-based phylogenetic tree was constructed based on 81 conserved, single-copy genes. This resulted in a phylogenomic tree where almost all nodes had 100% bootstrap values (Fig. 2). Indeed, strain TLL-SE01^T was placed in an

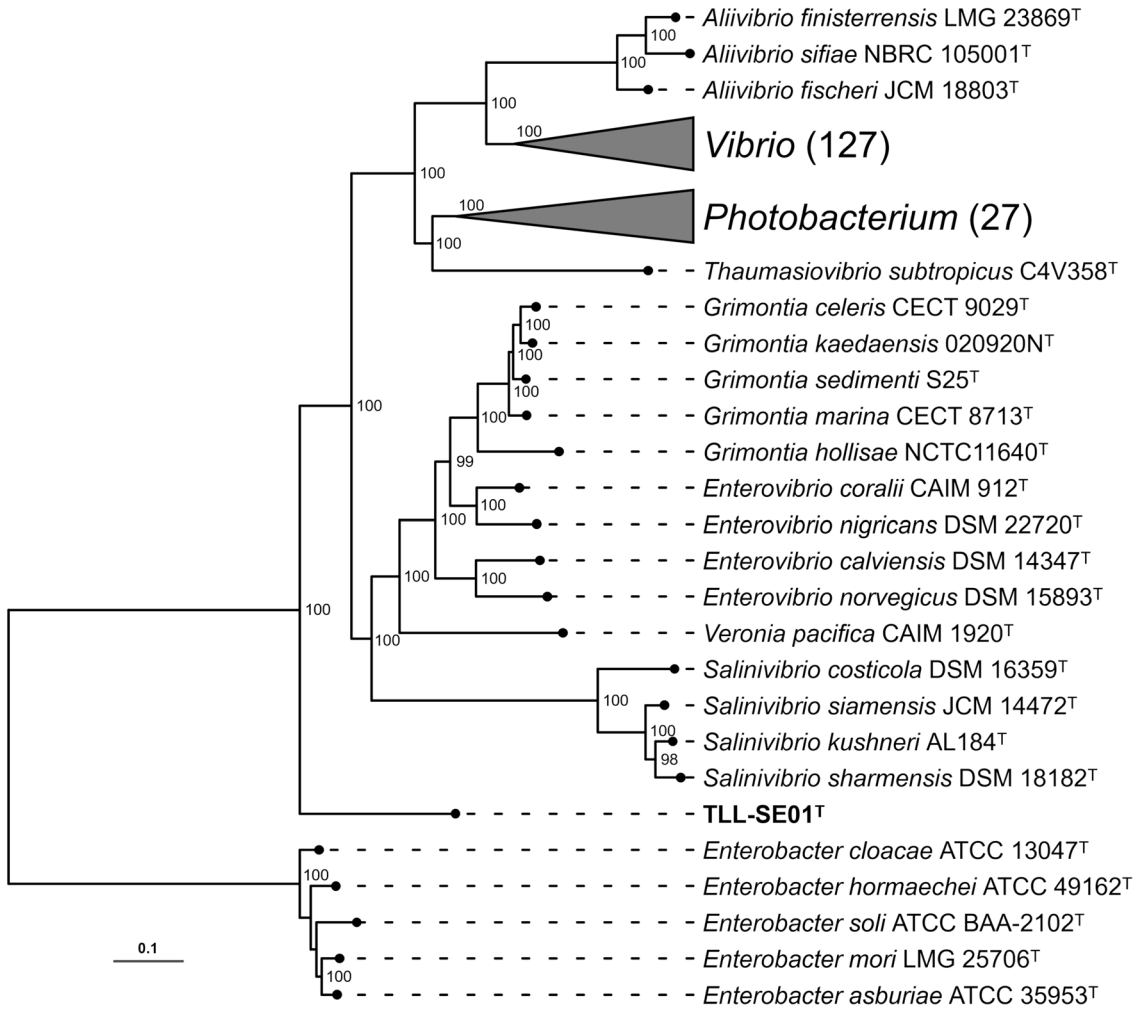

Fig. 2 Multi-locus phylogenomic tree based on 81 single-copy core genes showing the relationship of strain TLL-SE01^T with all type strains within the family *Vibrionaceae*. Five *Enterobacter* species from *Enterobacteriaceae* were used as the outgroup. GenBank accession numbers can be found in Table S4. Branch node labels represent bootstrap values (UFBoot expressed as percentages of 1000 replications) that are 70% and above. Scale bar: 0.1 nucleotide substitutions per site

independent clade diverging from the rest of the representatives within *Vibrionaceae*.

Comparative genome analyses of strain TLL-SE01^T

Hybrid assembly of the whole genome using Illumina short reads and Oxford nanopore long reads revealed the presence of two chromosomes (Chr) and one plasmid (Chr I, 3,781,055 bp; Chr II, 1,862,288 bp; Pl, 25,782 bp) within strain TLL-SE01^T (Table S3). The whole genome contained 5216 coding sequences (CDS), of which, 1867 (35.8%) were annotated as hypothetical proteins. A total of 159 RNAs were present in the genome comprising 122 tRNAs and 37 rRNAs. Of these 37 rRNAs, 34 were found on Chr I (12 5S, 11 16S and 23S each) and 3 on Chr II (1 5S, 16S, and 23S each). No RNAs were identified in the plasmid sequence.

The genome of strain TLL-SE01^T was compared to the draft genomes of four other closely related type strains of different genera (Table 1 and Table S3). Chromosome duality is a common feature of *Vibrionaceae* species, which is evident in strain TLL-SE01^T and at least two of the other strains—*P. damselae* and *Salinivibrio costicola* (Dikow and Smith 2013; Jha et al. 2012; Lilburn et al. 2010). Strain TLL-SE01^T

Table 1 Differential characteristics of strain TLL-SE01^T and representative strains of closely related genera

Characteristics	1	2	3	4	5
GenBank accession no	CP098812 – CP098814[b]	GCF_900055185	GCA_003026815	GCF_000565345	GCA_001707825
Size (Mbp)	5.67	5.61	4.91	3.38	5.29
G + C content (mol%)	49.55	48.38	40.50	49.29	45.49
Proteins (hypothetical proteins)	5216 (1867)	5204 (1876)	4561 (1475)	4300 (1283)	5310 (2529)
tRNAs	122	109	127	81	73
rRNAs (5S, 16S, 23S)	37 (13, 12, 12)	15 (11, 2, 2)	14 (9, 2, 3)	10 (7, 1, 2)	14 (10, 2, 2)
Colony morphology[a]	Circular, flat, smooth border	Round, convex, regular border	Circular, convex	Circular, convex, opaque	Round, smooth, entire margin
Cell length[a] (μm)	0.9–1.6	1.8–2.8	nd	nd	2.7–2.9
Cell morphology[a]	Short rod	Curved rod-shaped	Rod-shaped	Curved rod-shaped	Elliptical
Temperature[a] (°C)	10–37 (37)	15–40	25–37 (25)	5–45 (37)	10–37 (28)
NaCl[a] (%, w/v)	1–6	0 (-), 9 (max)	1–6	0.5–20 (10)	1–7 (3–4)
pH[a]	5–10	nd	5–10	5–10 (7.5)	7–9 (8)
API 20E					
Indole	+	−	−	−	−
API ZYM					
Cystine-arylamidase	wp	−	−	−	−
API 50 CH					
Ribose	−	+	+	+	+
Inositol	+	−	−	−	−
Sorbitol	+	−	−	−	−
Gentiobiose	+	−	−	−	−
L-Fucose	+	−	−	−	−

Values in parentheses represent the optimum condition

+, positive; −, negative; wp, weakly positive; nd, not determined

[a]Data from: 1, Strain TLL-SE01^T (this study); 2, *Grimontia celeris* CECT 9029^T (Pujalte et al. 2016); 3, *Photobacterium damselae* ATCC 33539^T (Love et al. 1981; Smith et al. 1991); 4, *Salinivibrio costicola* LMG 11651^T (Mellado et al. 1996); 5, *Veronia pacifica* CAIM 1920^T (Gomez-Gil et al. 2021)

[b]Accession numbers are as follows: Chr I (CP098812), Chr II (CP098813), Pl (CP098814)

was found to have the largest combined genome size (5.67 Mbp) and highest G + C content (49.55 mol%) among the five genomes. Despite having the largest genome, strain TLL-SE01^T had fewer coding sequences than *Veronia pacifica* CAIM 1920^T but more than those in the other three compared genomes (Table 1). The genome of strain TLL-SE01^T contained the most tRNAs among all the compared genomes, excluding *P. damselae* ATCC 33539^T. Notably, strain TLL-SE01^T has substantially higher 16S and 23S rRNA gene copy numbers (12 copies of each gene) than the other four genomes which contain only between one to three copies of each gene. The frequency of occurrence of 16S rRNA genes in a genome has been found to be potentially suggestive of its phylogenetic relationship whereby closely related organisms tend to share similar 16S rRNA gene copy numbers (Acinas et al. 2004; Rastogi et al. 2009; Schirrmeister et al. 2012). Hence, the large disparity in gene copy numbers observed here is concordant with the phylogenetic placement of strain TLL-SE01^T. In addition, the fact that strain TLL-SE01^T possesses a high copy number of identical 16S rRNA genes is intriguing. The preservation of the gene sequence homology could perhaps be reflective of a strong selection pressure exerted on the 16S rRNA gene, indicating the importance of conserving the functional role of the gene in response to adaptation to sudden shifts in environmental conditions (Tourova 2003). For example, the transition from free-living to gut-associated bacteria upon ingestion by a host results in vast changes in pH and osmolarity, on top of exposure to hostile conditions in the gut like bile salts, antimicrobial peptides and host immune defence mechanisms (Conner et al. 2016; Pazhani et al. 2021).

Biosynthetic gene cluster (BGC) prediction by antiSMASH identified a total of eight BGCs in the genome of strain TLL-SE01^T, of which, five (aryl polyene, thiopeptide, redox-cofactor, beta-lactone and RiPP-like) were found on Chr I and three (homoserine lactone, siderophore and ectoine) on Chr II. The aryl polyene cluster is one of the most commonly found BGCs in Gram-negative host-associated bacteria and plays an important role in oxidative stress response and biofilm formation (Johnston et al. 2021). Strain TLL-SE01^T was also predicted to possess the complete ectoine biosynthesis system responsible for protecting the cell against osmotic stress which is expected of halophilic bacterial strains (Czech et al. 2018). Interestingly, out of the five compared genomes, only *P. damselae* ATCC 33539^T did not contain the ectoine BGC. However, genes encoding for a family of osmo-regulated transporters, betaine-carnitine-choline transporter (BCCT), were identified in the genome of *P. damselae* ATCC 33539^T, hence, suggesting that it likely utilises a different mechanism to protect against osmotic stress which may be more energetically efficient for the cell (Gregory et al. 2020; Oren 1999).

Functional annotation by RAST assigned 29.8% (1,554) of all CDS in the genome of strain TLL-SE01^T to subsystem categories. When comparing the distribution of CDS involved in the various subsystem categories, just four categories were found to account for over 50% of the assigned CDS: Carbohydrates (19.24%), Amino Acids and Derivatives (14.67%), Protein Metabolism (12.60%), and Cofactors, Vitamins, Prosthetic Groups and Pigments (7.29%) (Fig. S3). Additionally, strain TLL-SE01^T had the greatest percentage of assigned CDS responsible for the Motility and Chemotaxis (6.30%), Iron Acquisition and Metabolism (1.08%), and Carbohydrates (19.24%) categories among the compared genomes. In contrast, *P. damselae* ATCC 33539^T placed the lowest among the five genomes in these three categories (1.28%, 0.20%, 10.86% respectively). The Motility and Chemotaxis category comprised between only 1.28% and 4.05% of assigned CDS in the other four strains as compared to strain TLL-SE01^T. Further investigation revealed that strain TLL-SE01^T was predicted to contain genes coding for proteins involved in both bacterial chemotaxis (27 genes) and flagellar motility subsystems (119 genes). Apart from strain TLL-SE01^T, *V. pacifica* CAIM 1920^T harbours the most genes encoding flagellar motility (74 genes), albeit still 1.6-fold lower than in strain TLL-SE01^T. The enrichment of flagellar motility genes offers strain TLL-SE01^T with a stronger fitness advantage over other bacterial competitors in the host gut through an enhanced ability to resist peristaltic flow of intestinal contents, traverse across the mucosal lining to facilitate adhesion and colonisation of the epithelium, and translocate along the lumen to access nutrient niches (Akahoshi and Bevins 2022). With regards to carbohydrates, only strain TLL-SE01^T and *Grimontia celeris* CECT 9029^T were predicted to possess genes coding for ribulose-1,5-bisphosphate

carboxylase/oxygenase (Rubisco), which is often conserved in other marine microorganisms, such as cyanobacteria (Hagemann et al. 2016; Orf et al. 2016).

A prediction of secretion systems identified T1SS, T2SS, T4aP, T5SS and Tad in the genome of strain TLL-SE01^T, which were also shared among the other reference genomes, except *S. costicola* LMG 11651^T (only T2SS is present). Strain TLL-SE01^T was found to possess two copies of the *tad* locus whereas the other reference genomes only contain one copy. The *tad* locus has been empirically shown to play an essential role in initial surface attachment, biofilm formation and translocation along the intestinal epithelium (Pu et al. 2018; Pu and Rowe-Magnus 2018). Another property of the Tad pilus is that it has low immunogenicity, allowing the bacterial cell to evade the host's immune system and remain viable in the gut (Duong-Nu et al. 2019). Possessing two copies of the *tad* locus enriches strain TLL-SE01^T for initial adherence to host cells which may be crucial for promoting colonisation of the fish's alimentary tract. As strain TLL-SE01^T was isolated from a presumably healthy fish, whether it is pathogenic to its host remains unknown but should be further investigated.

A comprehensive pairwise genomic analysis based on several OGRIs was performed between strain TLL-SE01^T and 172 *Vibrionaceae* type strains (Fig. 3). The ANI and dDDH values ranged from 69.23 to 72.22% and 18.8 to 24.0% respectively—well below the recommended thresholds (ANI, 95–96%; dDDH, 70%) for delineating new species (Chun et al. 2018). ANI and dDDH generally do not have sufficient resolution to discriminate genomes at the genus level, hence, genus delineation can be complemented by protein-based OGRIs instead, namely AAI and POCP. The AAI and POCP values between strain TLL-SE01^T and the compared strains ranged from 65.0 to 70.7% and 46.7 to 66.1% respectively. The proposed genus delineation boundary for AAI corresponds to 60–65% and that for POCP corresponds to an arbitrary value of 50% (Konstantinidis and Tiedje

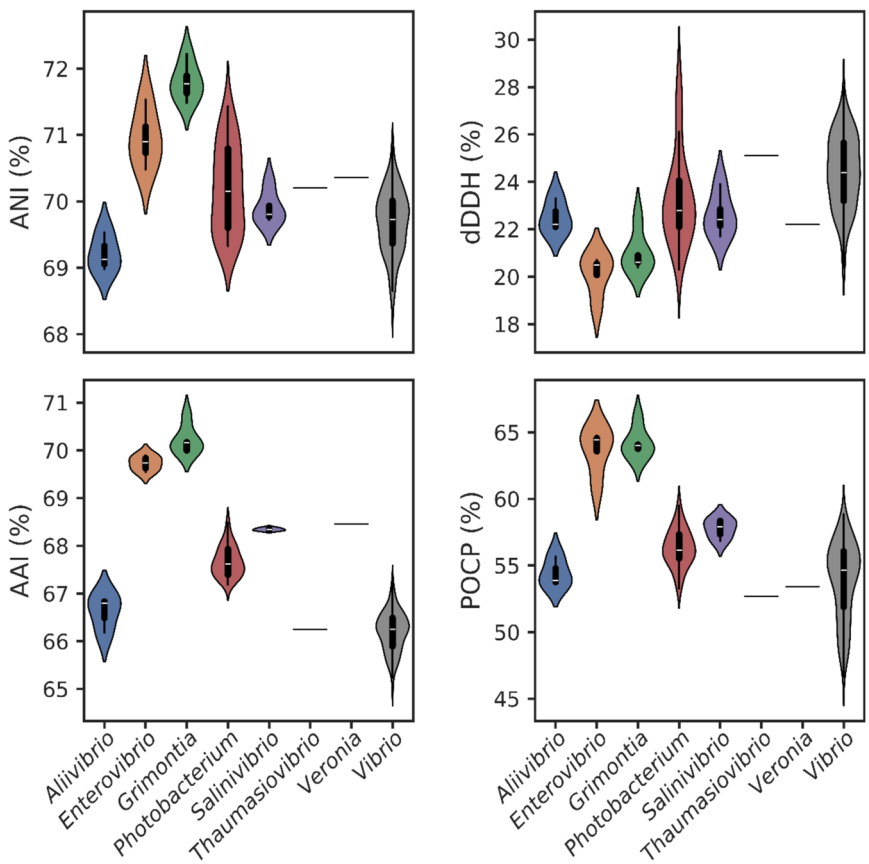

Fig. 3 Violinplot representing pairwise comparisons between strain TLL-SE01^T and *Vibrionaceae* genomes for several OGRIs (ANI, dDDH, AAI and POCP) stratified at the genus level—*Aliivibrio* (n = 3), *Enterovibrio* (n = 4), *Grimontia* (n = 5), *Photobacterium* (n = 27), *Salinivibrio* (n = 4), *Thaumasiovibrio* (n = 1), *Veronia* (n = 1) and *Vibrio* (n = 127). Vertical black bars depict box-and-whisker plots and medians are represented as horizontal white lines. (Color figure online)

2005; Luo et al. 2014; Qin et al. 2014; Riesco and Trujillo 2024). It is evident that most of the AAI and POCP values fall above the proposed genus delineation boundaries; all genomes had AAI values at least 65% and above, and only 19 genomes (all from *Vibrio*) shared less than half of their conserved proteins with strain TLL-SE01^T (POCP < 50%). As a result, a definitive conclusion cannot be drawn from the protein-based OGRI analyses as to whether strain TLL-SE01^T belongs to a novel or extant genus.

As such, we decided to probe further and evaluate the suitability of AAI and POCP for circumscribing genus boundaries by performing intra- and inter-genus pairwise comparisons for all validly published type strains with available genomes in the family *Vibrionaceae*. With respect to AAI, the mean inter-genus value was 67.5% and a narrow distribution for inter-genus genome pairs were observed—about 96% genome pairs were distributed within AAI values of 65–70% and the remaining 4% had AAI values of more than 70% (Fig. S2a). There was also minimal overlap between the intra- and inter-genus AAI values. The pairwise AAI analysis of *Vibrionaceae* genomes supports the genus delineation threshold of 70%. In fact, increasing the threshold to 70.9% would cover ~99% of the inter-genus pairs. Consequentially, the AAI values between strain TLL-SE01^T and the compared genomes all fall below this new boundary, hence, supporting the circumscription of strain TLL-SE01^T into a new genus. With regards to the POCP analysis, there was extensive overlap between the intra- and inter-genus values; the intra-genus POCP values ranged from 46.0% to 94.1% while the inter-genus POCP values ranged from 41.0 to 83.2% (Fig. S2b). This shows that POCP does not have sufficient resolution for genus delineation within *Vibrionaceae*. Moreover, the poor discriminatory power of POCP for genus delineation is also evident in other taxonomic families such as *Enterobacteriaceae*, *Morganellaceae* and *Cystobacteraceae* whereby the inter-genus POCP values for each of these families exceeded 58% (Gupta 2019). Nonetheless, it should be recognised that there are no absolute thresholds for demarcating genera as they are heterogeneous among taxonomic families, hence, family-specific thresholds should be derived individually as with the case of the AAI analysis in this study.

Recognising the ambiguity within the protein-based ORGIs, we consequently sought to characterise strain TLL-SE01^T at the genus level with higher resolution through a combined OGRI approach recently introduced by Barco et al. (2020). This index employs the use of both AF and ANI in parallel to complement the taxonogenomic characterisation of TLL-SE01^T. Pairwise comparisons were performed for (1) all type species of each genus (i.e., primary reference) within *Vibrionaceae* against strain TLL-SE01^T, (2) type species of other genera in the same family and (3) non-type species from the genus of the primary reference (Fig. 4). In all cases, strain TLL-SE01^T was placed below the AF and ANI genus demarcation boundaries. This supports the notion that strain TLL-SE01^T does not belong to any extant genera within *Vibrionaceae* and thus, may be representative of a new genus.

Physiology and chemotaxonomy

Key physical, physiological, and biochemical characteristics of strain TLL-SE01^T were compared to that of other closely related strains reported in literature or experimentally determined in this study (Table 1). The cells of strain TLL-SE01^T are Gram-negative short rods ranging from 0.9 to 1.6 µm in length (Fig. S4a) and exhibited weak growth under anaerobic conditions. The optimum growth temperature of strain TLL-SE01^T was found to be 37 °C. Strain TLL-SE01^T grew well between 1 and 4% (w/v) NaCl and pH 6–10 but weaker growth was observed at 6% (w/v) NaCl and at pH 5 (Fig. S4b-d). No growth was observed beyond 8% (w/v) NaCl and 45 °C. From the biochemical API assays, strain TLL-SE01^T was found to utilise a wide variety of carbon sources compared to the representative strains. Notably, only strain TLL-SE01^T was able to metabolise tryptophan to indole, as well as utilise inositol, sorbitol, gentiobiose and L-fucose as sole carbon sources. Conversely, strain TLL-SE01^T was the only strain not able to utilise ribose among the strains compared. API ZYM analysis also revealed that only strain TLL-SE01^T possessed cystine-arylamidase activity.

The unique capability to utilise a variety of alternative energy sources places it in a versatile position to adapt to different environment niches. In congruence with the biochemical assay, genome prediction confirmed the presence of a gene cluster for inositol catabolism in strain TLL-SE01^T only. Inositol is predominantly a eukaryotic metabolite and its biosynthesis has been shown

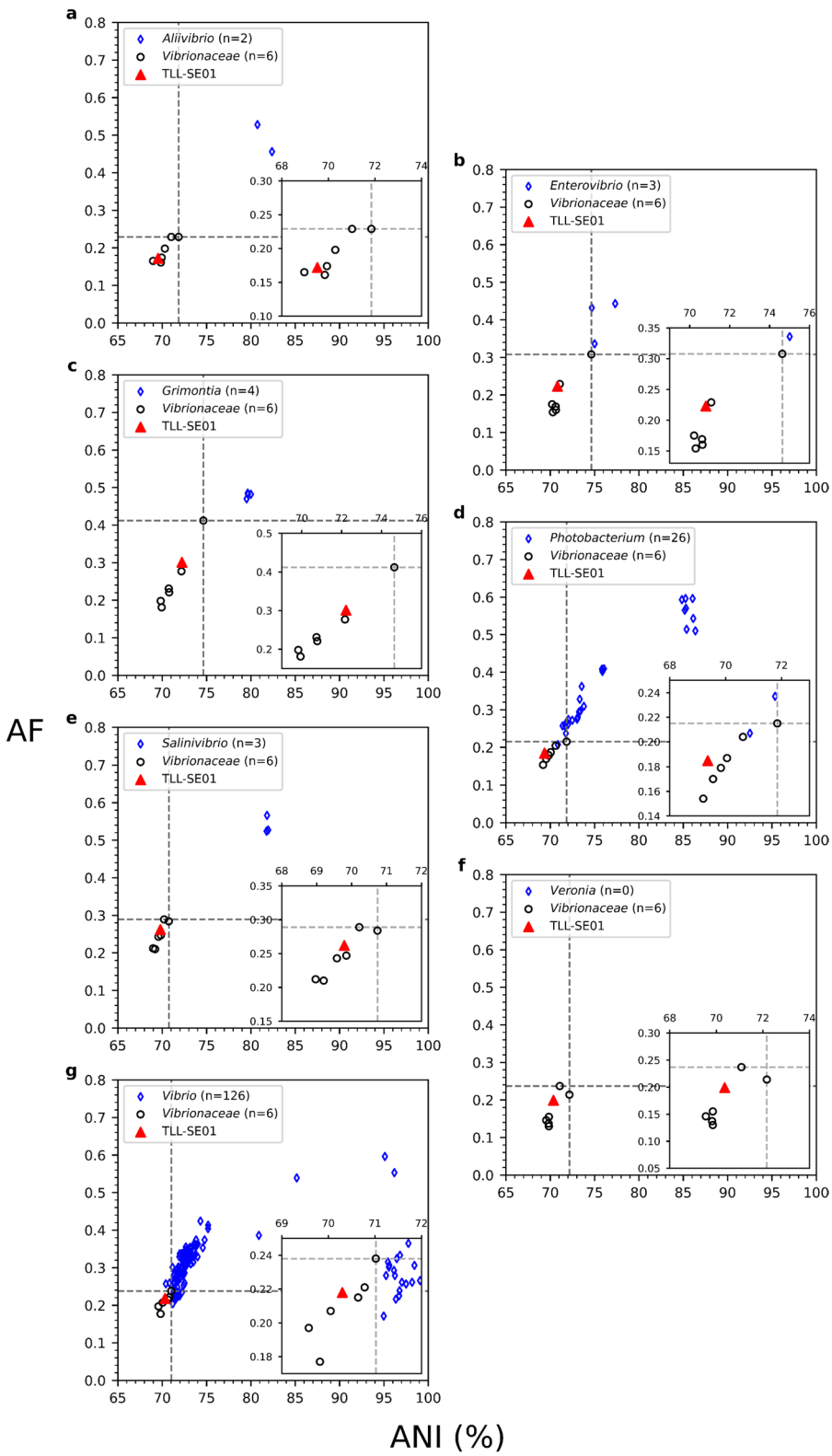

Fig. 4 Pairwise AF and ANI comparisons to primary references. **a** Primary reference, *Aliivibrio fischeri* ATCC 7744^{T}. Blue diamonds, non-type species (n = 2) of genus *Aliivibrio*. **b** Primary reference, *Enterovibrio norvegicus* DSM 15893^{T}. Blue diamonds, non-type species (n = 3) of genus *Enterovibrio*. **c** Primary reference, *Grimontia hollisae* NCTC11640^{T}. Blue diamonds, non-type species (n = 4) of genus *Grimontia*. **d** Primary reference, *Photobacterium phosphoreum* JCM 21184^{T}. Blue diamonds, non-type species (n = 26) of genus *Photobacterium*. **e** Primary reference, *Salinivibrio costicola* DSM 16359^{T}. Blue diamonds, non-type species (n = 3) of genus *Salinivibrio*. **f** Primary reference, *Veronia pacifica* CAIM 1920^{T}. Blue diamonds, non-type species (n = 0) of genus *Veronia*. **j** Primary reference, *Vibrio cholerae* FDAARGOS 103^{T}. Blue diamonds, non-type species (n = 126) of genus *Vibrio*. Red triangles, strain TLL-SE01^{T}. Black circles, type species (n = 6) of genera within *Vibrionaceae*. Dashed lines refer to the AF and ANI demarcation boundaries. The inset plots show the zoomed-in view of the intersection of genus demarcation boundaries. (Color figure online)

to be upregulated in teleost fish during periods of osmotic stress (Kalujnaia et al. 2010; Kültz 2015; Lichty et al. 2024). The ability to exploit this niche food source provides strain TLL-SE01^T with a competitive advantage over other gut colonisers and this advantage would be more pronounced during periods of thermal, osmotic or pH stress that could affect host-microbiota interactions. Furthermore, expression of the tryptophanase enzyme (catalyses degradation of tryptophan to produce indole) is unique in strain TLL-SE01^T as it is an inhabitant of fish intestinal tract, a stark contrast from the other reference strains which were isolated from marine environments or skin lesion of a fish host. Indeed, tryptophanase synthesisers are better equipped to thrive in the host gut (Boya et al. 2021; DeMoss and Moser 1969). The enteric habitat might be the main selection pressure driving evolution of strain TLL-SE01^T towards favouring genes that enhance proliferation within the gut, rather than genes conferring fitness towards environmental niches, hence, plausibly explaining its distinct phenotypic traits.

The predominant cellular fatty acids (> 5%) identified in strain TLL-SE01^T were summed feature 3 ($C_{16:1\ \omega6c}$/$C_{16:1\ \omega7c}$), summed feature 8 ($C_{18:1\ \omega6c}$/$C_{18:1\ \omega7c}$), *iso*-$C_{16:0}$, *iso*-$C_{17:0}$, $C_{16:0}$ and *iso*-$C_{15:0}$ whereas that for the other compared strains were summed features 3, 8 and $C_{16:0}$ except *V. pacifica* CAIM 1920^T which also comprised $C_{12:0\ 3OH}$ as a major fatty acid. *Iso*-$C_{17:0}$ and *iso*-$C_{15:0}$ were present only in strain TLL-SE01^T but not detected in the other strains (Table S5). Overall, the cellular fatty acid composition of strain TLL-SE01^T varied considerably from the representative strains of closely related genera.

Conclusion

In summary, this study describes the isolation of a new *Vibrionaceae* strain from the intestine of a healthy Asian Seabass. The strain is likely to be a representative of a novel genus. However, the role of the strain in fish health and physiology as well as its abundance in other fish or environmental sites remain to be investigated.

Description of *Parasalinivibrio* gen. nov.

Parasalinivibrio (Parasalinivib'rio Gr. prep. *para*, beside, near, like; N.L. masc. n. *Salinivibrio*, a bacterial genus name; N.L. masc. n. *Parasalinivibrio*, beside the genus *Salinivibrio*.). Cells are uniform, rod-shaped (0.9–1.6 µm long), Gram-negative, facultative anaerobes, catalase- and oxidase-positive. Members can metabolise tryptophan to indole, as well as utilise inositol, sorbitol, gentiobiose and L-fucose as carbon sources. The type species is *Parasalinivibrio latis*.

Description of *Parasalinivibrio latis* sp. nov.

Parasalinivibrio latis (la'tis. N.L. gen. n. latis, of the seabass genus *Lates*, the host from which the type strain was isolated). Colonies grown on Marine agar 2216 (Difco) are smooth, flat and circular after incubation for two days under atmospheric conditions. Growth occurs over the range of 10 °C to 37 °C. Moderately halophilic, growth occurs between 1 and 6% (w/v) NaCl, but not at 0% and 8% NaCl. Growth also occurs between pH 5 and pH 10. The DNA G + C content is about 49.5 mol%. The major cellular fatty acids are summed feature 3 ($C_{16:1\ \omega6c}$/$C_{16:1\ \omega7c}$), summed feature 8 ($C_{18:1\ \omega6c}$/$C_{18:1\ \omega7c}$), *iso*-$C_{16:0}$, $C_{17:0}$, $C_{16:0}$ and *iso*-$C_{15:0}$. API 20E analysis showed positive reactions for ß-galactosidase and arginine dihydrolase activities, and production of indole. API 50 CH assay indicated positive reactions for the utilisation of glycerol, galactose, glucose, fructose, mannose, inositol, mannitol, sorbitol, N-acetylglucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, starch, glycogen, gentiobiose, L-fucose and gluconate. API ZYM testing showed positive reactions for alkaline phosphatase, leucine-arylamidase and acid phosphatase; weakly positive reactions for esterase, esterase lipase, valine arylamidase, cystine arylamidase, naphtol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-ß-glucosaminidase.

The type strain is TLL-SE01^T (= BCRC 81435^T = JCM 36283^T), isolated from the distal intestine of a healthy farmed Asian Seabass (*Lates calcarifer*) in Singapore. The GenBank accession numbers for the 16S rRNA gene sequence and complete genome of strain TLL-SE01^T are OP963388 and CP098812 – CP098814, respectively.

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Author contributions S.E and H.S. designed experiments; S.E. and M.S. performed sample collection; S.E. isolated the novel strain; M.S. processed samples for amplicon sequencing; S.E. and A.L. processed samples for DNA sequencing; S.E. conducted cultivation-based analysis of strains; S.E. analysed data with input from H.S., A.L. and M.S; S.E. and H.S. wrote the manuscript with input from all other authors.

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Data availability The de-multiplexed, pair-matched amplicon sequences are deposited in NCBI SRA under the accession number PRJNA1110041. The complete TLL-SE01 genome and 16S rRNA gene sequence has been deposited in GenBank under accession numbers CP098812 – CP098814 and OP963388 respectively.

Declarations

Conflict of interest The authors declare no competing interests.

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