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Glowing pathogenic bacteria discovered in the Aral Sea – a harmless evolutionary feature or a hidden threat?

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Glowing pathogenic bacteria discovered in the Aral Sea – a harmless evolutionary feature or a hidden threat?

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ABSTRACT

Bioluminescent bacteria are widespread in marine ecosystems, yet whether they can establish stable populations in long-isolated inland saline lakes has remained unknown. Here we report species-level identification and comprehensive characterization of the first isolated bioluminescent bacteria from the Aral Sea, a formerly extensive inland water body that has remained isolated from the world ocean since its Holocene formation while experiencing extreme fluctuations in salinity, temperature and water level. We show that the luminescent microbiota comprises two marine species, *Vibrio cholerae* and *Vibrio vulnificus*. *V. cholerae* isolates displayed enhanced tolerance to low salinity and possessed a thermolabile luciferase. In contrast, isolated *V. vulnificus* strains grew and emitted light over an exceptionally broad elevated salinity range (5–70‰) and retained luminescence at temperatures exceeding 40 °C, indicating remarkable adaptation to the highly variable physicochemical conditions of the Aral Sea. Phylogenetic analyses place isolates close to contemporary type strains, and the retention of virulence-associated genes indicates limited evolutionary divergence, suggesting a relatively recent colonization rather than long-term independent evolution. Together, these results demonstrate that bioluminescent bacteria from pathogenic species can successfully colonize ecological niches in isolated inland saltwater ecosystems typically occupied by other Vibrio species in the open sea.

INTRODUCTION

Bioluminescent bacteria are characteristic members of marine microbial communities, occurring from tropical to polar oceans and occupying diverse ecological niches through free-living, commensal, mutualistic and pathogenic lifestyles (Dunlap & Urbanczyk, 2013; Urbanczyk et al., 2007, 2008). Although the ecology of marine luminous bacteria has been extensively investigated, remarkably little is known about their ability to colonize geographically isolated inland saline ecosystems. Consequently, the extent to which marine bioluminescent bacteria can disperse across major geographic barriers, establish persistent populations and adapt to novel environmental conditions remains largely unresolved.

The Aral Sea provides an exceptional natural experiment in microbial biogeography. Following its Holocene formation as an inland endorheic basin, it has remained isolated from the global ocean while undergoing profound environmental change (Svitohc, 2009). Over both geological and historical timescales, the lake has experienced large fluctuations in salinity, water level and temperature, changes that have accelerated dramatically during the past century. These extreme and rapidly varying physicochemical conditions impose strong ecological filters on microbial communities, making the Aral Sea an ideal system for investigating microbial dispersal, environmental adaptation and community assembly in isolated saline ecosystems.

In a recent survey, we reported the first occurrence of bioluminescent bacteria in the Aral Sea and identified the isolates as members of the genus *Vibrio* (Bazhenov et al., 2024). This finding was unexpected because luminous vibrios are considered typical representatives of marine microbial communities (Urbanczyk et al., 2007, 2008). Their presence in an inland saline lake that has remained isolated from the world ocean for thousands of years raises fundamental questions concerning microbial colonization and evolution. Do these bacteria represent relic populations that have persisted since ancient marine connections, or have they colonized the Aral Sea through recent long-distance dispersal? If the latter, how rapidly have they adapted to one of the world's most environmentally unstable saline ecosystems? Furthermore, can multiple luminescent species coexist under such conditions by partitioning ecological niches?

Several mechanisms could explain the occurrence of marine bacteria in isolated inland waters. Marine microorganisms may persist following historical connections between aquatic basins, whereas more recent colonization could occur through long-distance dispersal by migratory birds or other natural vectors, or

through anthropogenic transport. Distinguishing among these scenarios requires combining phylogenetic evidence with detailed physiological characterization of natural isolates.

Bioluminescence itself may facilitate successful colonization of environmentally stressful habitats. Besides its well-established roles in ecological interactions with multicellular hosts (Nyholm et al., 2009; Soto, 2022), bacterial light production has been proposed to protect cells against oxidative stress and ultraviolet radiation (Czyz et al., 2000; Melkina et al., 2015). Such functions may enhance survival under the highly variable physicochemical conditions characteristic of the Aral Sea and contribute to the persistence of luminescent microbial populations.

An additional aspect of particular interest is that many luminous marine bacteria belong to the genus *Vibrio*, which includes both environmentally important microorganisms and opportunistic pathogens of humans and aquatic animals (Baker-Austin et al., 2018; Park & Lee, 2018; Yun & Kim, 2018; Haftel & Sharman, 2023; Ofek et al., 2023). Environmental isolates of *Vibrio cholerae* and *Vibrio vulnificus* exhibit considerable diversity in virulence-associated traits, reflecting adaptation to different ecological niches rather than obligate pathogenicity. Assessment of newly discovered populations therefore requires not only taxonomic identification but also evaluation of physiological characteristics associated with environmental fitness and of virulence-related properties, including adhesion (Jones et al., 1976), extracellular hydrolytic enzyme production (Lydon et al., 2021) and biofilm formation (Yildiz & Visick, 2009).

Here we provide the first species-level characterization of bioluminescent bacteria isolated from the Aral Sea. We identify the isolates as *Vibrio cholerae* and *Vibrio vulnificus*, demonstrate that they occupy distinct physiological niches through contrasting responses to salinity and temperature, evaluate their virulence-associated characteristics, and use phylogenetic analyses to infer their evolutionary history. Our results indicate that these bacteria are closely related to contemporary marine populations, supporting recent colonization of the Aral Sea rather than long-term isolation. Together, these findings demonstrate that marine bioluminescent bacteria can successfully disperse to, establish and partition ecological niches within an isolated inland saline ecosystem, providing new insights into microbial biogeography, environmental adaptation and the assembly of microbial communities in extreme environments.

Materials and Methods

Sample Collection

Sample collection and luminescent strains isolation were described previously in (Bazhenov et al., 2024). Samples were collected from various sources including water and storm castings along the coastal zone of the Small Aral Sea near the village of Bogen (46.2081, 61.0727), water and shore soil in the strait between the Small and Large Aral Seas and in small saline lakes near the strait (46.2081, 61.0727), and water and sediment from a salt flat (46.0980, 60.7441). Additionally, fish caught by local fishermen in the southeastern part of the Small Aral Sea were also sampled.

Bacterial Cultivation

Bacteria were cultivated in liquid or on solid agar (15 g/L bactoagar) SWT medium (5 g/L tryptone, 2.5 g/L yeast extract, 3 g/L glycerol, and marine salts). For sampling from the Small Aral Sea, agar plates with SWT15 and SWT35 media containing 15 and 35 g/L sea salt were used, while for samples from desiccating saline coastal ponds, we used plates with 35 and 70 g/L marine salts. During an expedition, the cultivation of the initial bacterial cultures was carried out at room temperature for approximately 20 °C. Routine cultivation of isolated strains was performed at 30°C, using the same salt concentration in a medium as it was in plates from which these strains were isolated. Optical density (OD_{600}) of liquid cultures was measured using a Synergy H4 multimode plate reader (Biotek, USA).

Species Identification and phylogenetic analysis

Primary screening of isolated clones was conducted using mass spectrometry fingerprint analysis on a Microflex MALDI-TOF MS (Bruker Daltonics, Germany). The species identification methods for the numerous isolated strains and the sample preparation and analysis procedures followed the protocol outlined in (Bazhenov et al., 2019). Briefly, all isolated strains underwent fingerprint mass spectrometry analysis to group isolates with identical mass spectra, which were then compared with reference spectra in the Bruker Daltonics database. For several isolates, identification was verified through phylogenetic analysis.

Whole-genome sequencing was performed at Syntol (sequencing of strains RK2 and RK27) and at TN-Biotech (sequencing of strains RK1, RK3, and RK7). Sequencing at Syntol was performed on an Illumina MiSeq instrument using the MiSeq Reagent Kit v2. Libraries were prepared using the standard Neb Next Ultra2 DNA Library Prep Kit for Illumina whole-genome sequencing method. Sequencing at TN-Biotech was performed on a GeneMind GenoLab M instrument and the GenoLab M Sequencing set V1.0 FCH reagents. Libraries were prepared using the shotgun method using Raissol Bio SG GM Plus reagents. The whole genome sequences of isolated strains were deposited in GenBank: PRJNA1449535/SUB16102110 (*V. cholerae* RK2 and RK7), SUB16209717 (*V. cholerae* RK4, RK7, RK19, and RK24), and PRJNA1449567/SUB16105328 (*V. vulnificus* RK27).

Phylogenetic trees for assessment of divergence between isolated and other known strains of *V. cholerae* and *V. vulnificus* were constructed using the dataset of concatenated housekeeping genes *gapA, gyrB, mreB, recA, rpoA, topA,* and *ftsZ.* Multiple sequence alignment was performed with MEGA-X software (Kumar et al., 2018) using ClustalW and then MUSCLE algorithms. The data used for the analysis are presented in Table S1. Phylogenetic relationship between isolated and other known strains were examined separately for *V. cholerae* and *V. vulnificus* with Bayesian analysis (BA) using MrBayes 3.2.7 (Ronquist et al., 2012) with the best model found by iq-tree ModelFinder (GTR+F+G4). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 10,000

generations. The average value of the Potential Scale Reduction Factor (PSRF) was 1.0013 and the average standard deviation of split frequencies was 0.0056 at the end of the analysis, indicating convergence was achieved and a good sample from the posterior probability (PP) distribution was obtained. The resulting trees' effective sampling sizes (ESS) were more than 200 and were calculated using Tracer software ver. 1.7 (Rambaut et al., 2018).

Determination of Halotolerance and Thermotolerance

Halotolerance of the isolated strains was assessed by cultivating them on agarised and in liquid SWT media with varying sea salt concentrations. The studied strains were suspended in liquid SWT medium and then plated onto SWT agar medium with different salt contents (0, 5, 10, 20, 30, 40, 50, 60, 70, and 80 g/L). Once optimal salt concentration was determined for the strain, it was used for further routine culturing. For tests with measurements of OD and luminescence during cultivation in media with varied salt concentration, individual colony of tested strain was suspended in liquid SWT with optimal salt concentration. The suspension was incubated at 30°C for approximately 30 minutes with constant stirring. The culture was then diluted with fresh medium to obtain an OD_{600} of ~ 0.1, 1 mL of the diluted culture was divided into 0.1 mL portions in ten microcentrifuge tubes. Tubes were centrifuged at 10,000 rpm for 1 minute, and the supernatants were discarded. The cell pellets in each tube were resuspended in 1 mL SWT with various sea salt concentrations (0, 5, 10, 20, 30, 40, 50, 60, 70, and 80 g/L), and 0.2mL portions were placed in 96-well plate with white walls and clear bottom. Cell growth and luminescence at different salt concentrations were monitored using a Synergy H4 instrument (each measurement performed in triplicate). The temperature during measurements was maintained at 32°C. Negative controls consisted of SWT with the same salt concentrations but without cells.

Thermotolerance of the isolated strains was evaluated by their ability to grow and luminesce when plated on agar media and incubated at different temperatures. Individual colony tested strain was suspended in SWT medium to a final OD_{600} of ~ 0.2. Equal amounts of cells were taken from this suspension and plated on a series of Petri dishes with agarised SWT. Plates were then incubated at three different temperatures: 30, 37, and 42°C. Cell growth and luminescence were monitored and recorded after 18 and 24 hours of incubation. To document the results, the plates were removed from the incubators, and the number of colonies, their size, and luminescence were visually assessed at room temperature.

Determination of *in vivo* Luciferase Thermostability

Individual colonies of RK2 were suspended in liquid SWT with 10 g/L salt to a final OD of ~ 0.3. Similarly, RK27 were suspended in SWT with 40 g/L marine salt to a final OD of ~ 0.02. The culture densities were selected based on cell luminescence to ensure that the measured values fell within the linear range of the luminometer. Thermoinactivation of luciferase in cells was assessed by measuring luminescence at increasing temperatures. For this, 200 µL of the cell suspensions were added to the wells of a 96-well plate, and the plate was incubated in a Synergy H4 reader at 42 or 47 °C for 50 minutes with periodic luminescence measurements. Each strain was tested triple.

Determination of Pathogenicity Factors

The potential pathogenicity of the strains was evaluated based on the following factors: adhesion index/percentage of epithelial cells involved in adhesion, hemolytic activity, urease activity, mannitol fermentation, plasma coagulase activity, phospholipase activity, proteolytic activity, pro- and antioxidase activity, cytotoxicity of cell-free filtrates, and catalase activity. Detailed description of the pathogenicity factors determination methods is provided in the Supplementary Materials (Text box S1).

Study of Luminescence Dependence on the Growth Phase of Cell Cultures

Overnight cultures of RK2 and RK27 were diluted 10,000-fold in liquid SWT medium containing 10 and 40 g/L sea salt, respectively, and incubated at 32°C with constant shaking at 200 rpm. Optical density and luminescence were periodically measured. Specific luminescence was calculated by dividing luminescence by optical density. When luminescence values exceeded the linear range of the luminometer, the cell sample used for measurement was diluted 10 or 100 times, and the measured value was multiplied by 10 or 100, respectively.

Metagenomic studies

Sample collection, DNA sequencing and analysis were described in (Alexyuk et al., 2021). Briefly, 10 L of water samples were collected from the surface of the Small Aral Sea in sterile containers in May 2019. The sampling location was near the coast (46°37'22.0"N 61°28'25.0"E). Seawater samples were filtered through 3 μm filters to remove zooplankton and phytoplankton, and the resulting filtrate was concentrated using tangential flow filtration (TFF) with a 50 kDa cartridge (Vivaflow 200, Sartorius, PES) to a final volume of 500 mL. The concentrate was centrifuged to sediment bacterioplankton at 29,000 rpm for 2 hours at 4˚C in an Avanti J-30I centrifuge (Beckman Coulter). The sediment was resuspended in a minimum volume of sterile phosphate buffer. The PureLink Genomic DNA MiniKit (Invitrogen, USA) was used to isolate total DNA from the obtained sample. Quantitative DNA measurements were performed using the Qubit dsDNA HS kit (High Sensitivity, Invitrogen, USA) according to the instructions for the Qubit 3.0 fluorimeter. DNA libraries were prepared from 1 ng DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, USA) according to the instructions. Libraries were sequenced on the Miseq platform (Illumina, Illumina, Cambridge, UK) to obtain 2 × 300 paired end reads. Sequencing data were analyzed using Geneious Prime 2023 and Kaiju.

Results

Isolation and taxonomic identification of luminescent bacteria from the Small Aral Sea

To investigate whether luminescent bacteria occur in the isolated ecosystem of the Small Aral Sea, samples were collected from coastal seawater, storm-cast plant debris, shoreline sediments, hypersaline coastal ponds and the intestines of freshly caught fish (16 individuals representing five fish species) (Fig. 1). Luminescent bacteria were detected exclusively in samples originating from the Small Aral Sea, including seawater, coastal storm deposits and fish intestines (Table S2). Colonization of fish was remarkably common, more than 90% of the fish tested contained luminescent bacteria in their intestines. Approximately 10% of colonies obtained from seawater samples were luminescent, indicating that these bacteria represent a substantial component of the culturable microbial community.

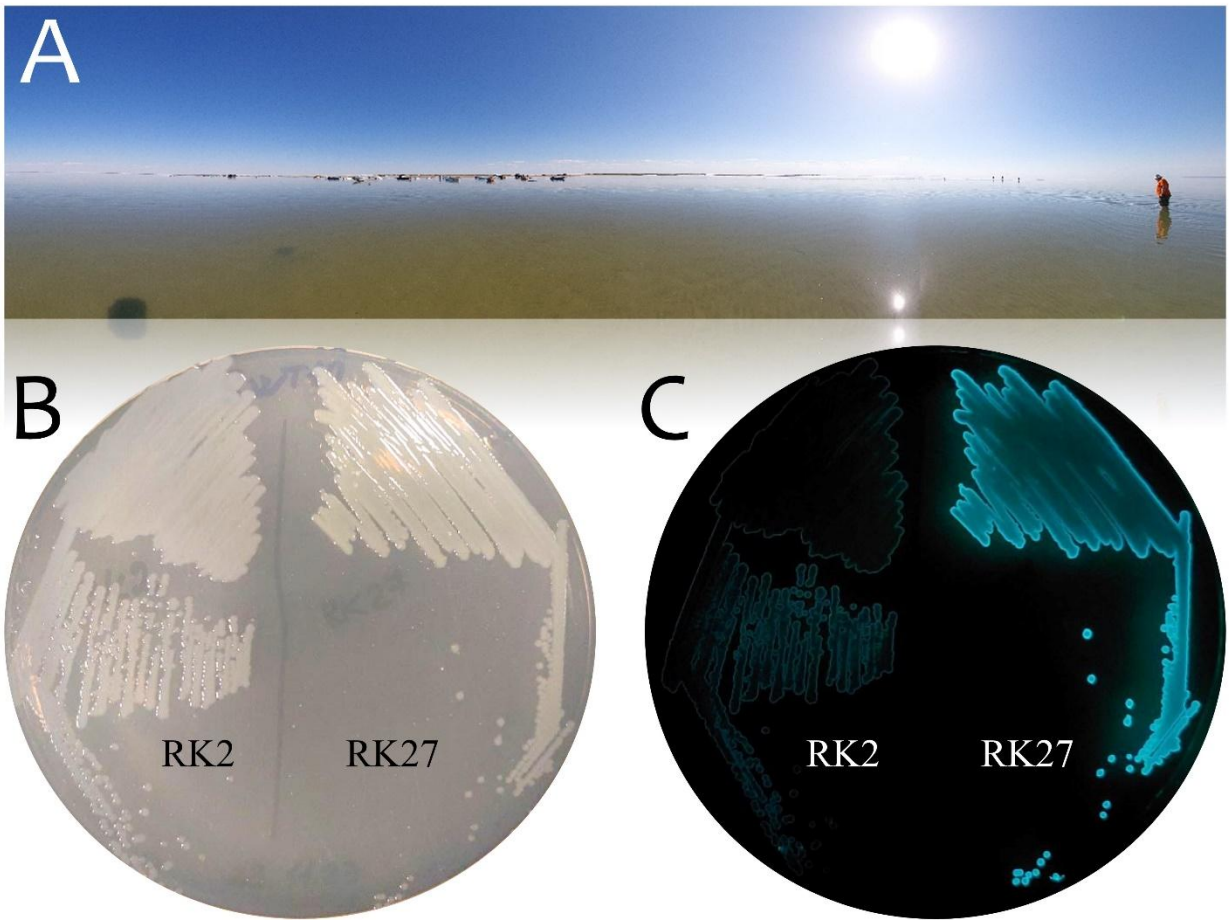


Figure 1. A panoramic photograph of the Aral Sea coastline in the sample collection area (A). In the center there are fishing boats, which provided freshly caught fish for intestinal microbiota sampling. On the right, the expedition leader I.V. Manukhov is inspecting the sampling site and conducting an organoleptic examination of the water for temperature and salinity. Photographs of cell cultures from isolated luminescent strains RK2 and RK27, grown at 30°C on the surface of SWT agar medium with 10 g/L salt, taken under external lighting (B) and in the dark (C).

The isolates separated into two clearly distinguishable phenotypic groups that differed in colony morphology and luminescence intensity (Fig. 1B,C). MALDI–TOF mass spectrometry showed that each group possessed a characteristic protein fingerprint (Fig. 2; Fig. S1). Database comparison identified the two groups as *V. cholerae* (formerly *Vibrio albensis*) and *V. vulnificus*, with high confidence at both the genus and species levels.

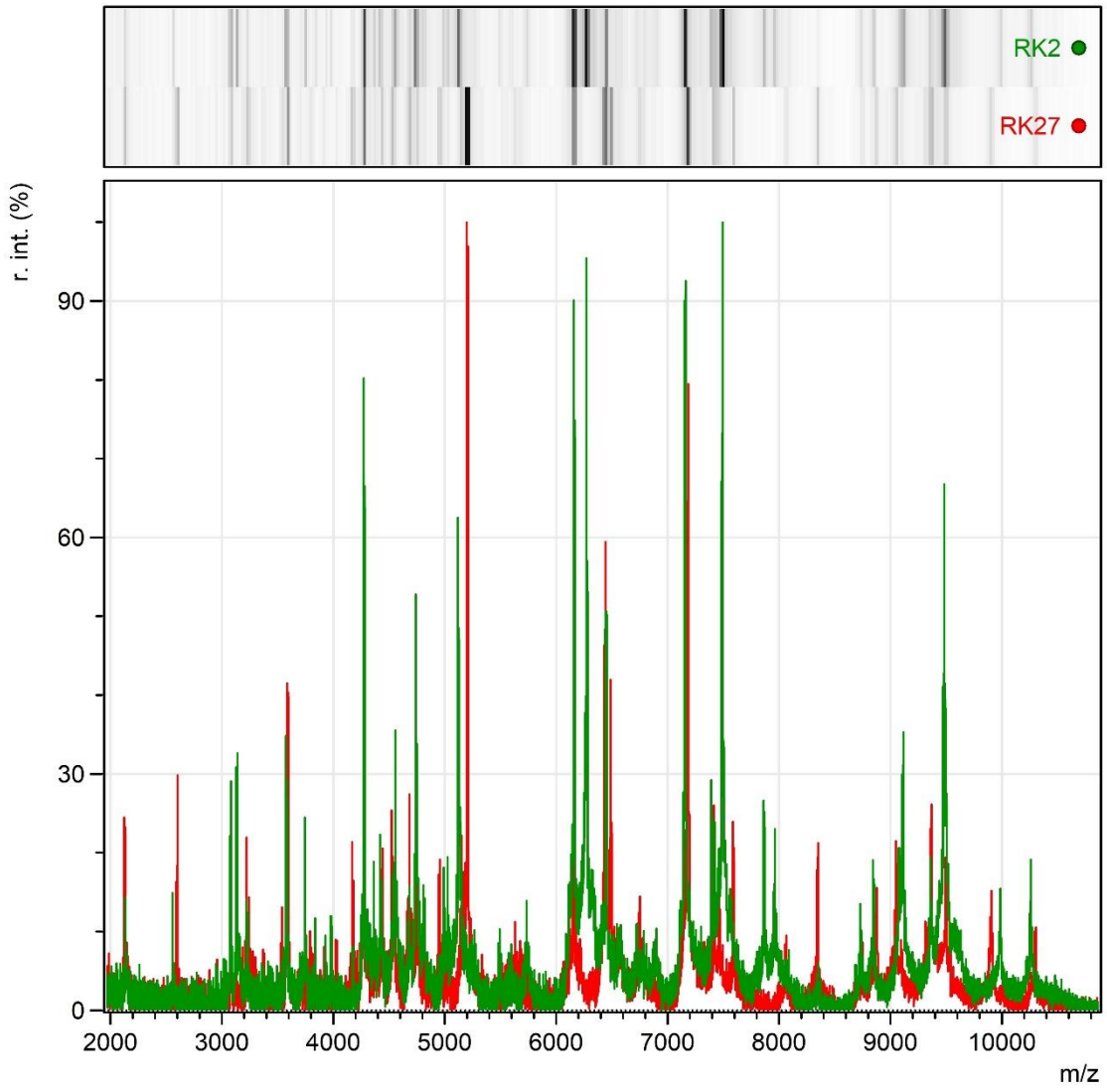


Figure 2. MALDI-TOF MS spectra obtained from the protein extracts of RK2 and RK27 strains. The upper part shows the Gel View of the mass spectra. Baseline correction followed by normalization was performed.

To confirm these assignments, representative isolates were subjected to whole-genome sequencing followed by multilocus phylogenetic analysis using seven conserved housekeeping genes (*gapA, gyrB, mreB, recA, rpoA, topA* and *ftsZ*). Five isolates (RK2, RK4, RK7, RK19 and RK24) clustered with *V. cholerae*, whereas RK27 grouped with *V. vulnificus* (Fig. 3A,B), fully confirming the MALDI–TOF identification.

Phylogenetic relationships indicate recent colonization and horizontal transfer of luminescence genes

Because the Aral Sea has remained isolated from the World Ocean since its reformation as an inland lake during the Holocene (about five thousand years ago), we asked whether its luminescent vibrios represent long-term endemic lineages or more recent colonizers.

Unexpectedly, the Aral Sea isolates did not form distinct phylogenetic clusters. Instead, *V. cholerae* isolates were interspersed among environmental strains from geographically distant aquatic ecosystems, whereas clinical isolates formed a separate lineage (Fig. 3A). Molecular dating was performed using the calibration proposed by Kumar *et al.* (2017) and the divergence constraint established by Feng *et al.* (2008). The resulting time-calibrated phylogeny indicated that contemporary environmental and clinical *V. cholerae* lineages diverged more than 600 years ago, whereas the Aral Sea isolates showed evolutionary distances comparable to those separating environmental strains from different parts of the world. These observations argue against prolonged independent evolution within the Aral Sea and instead suggest repeated historical introductions from external populations.

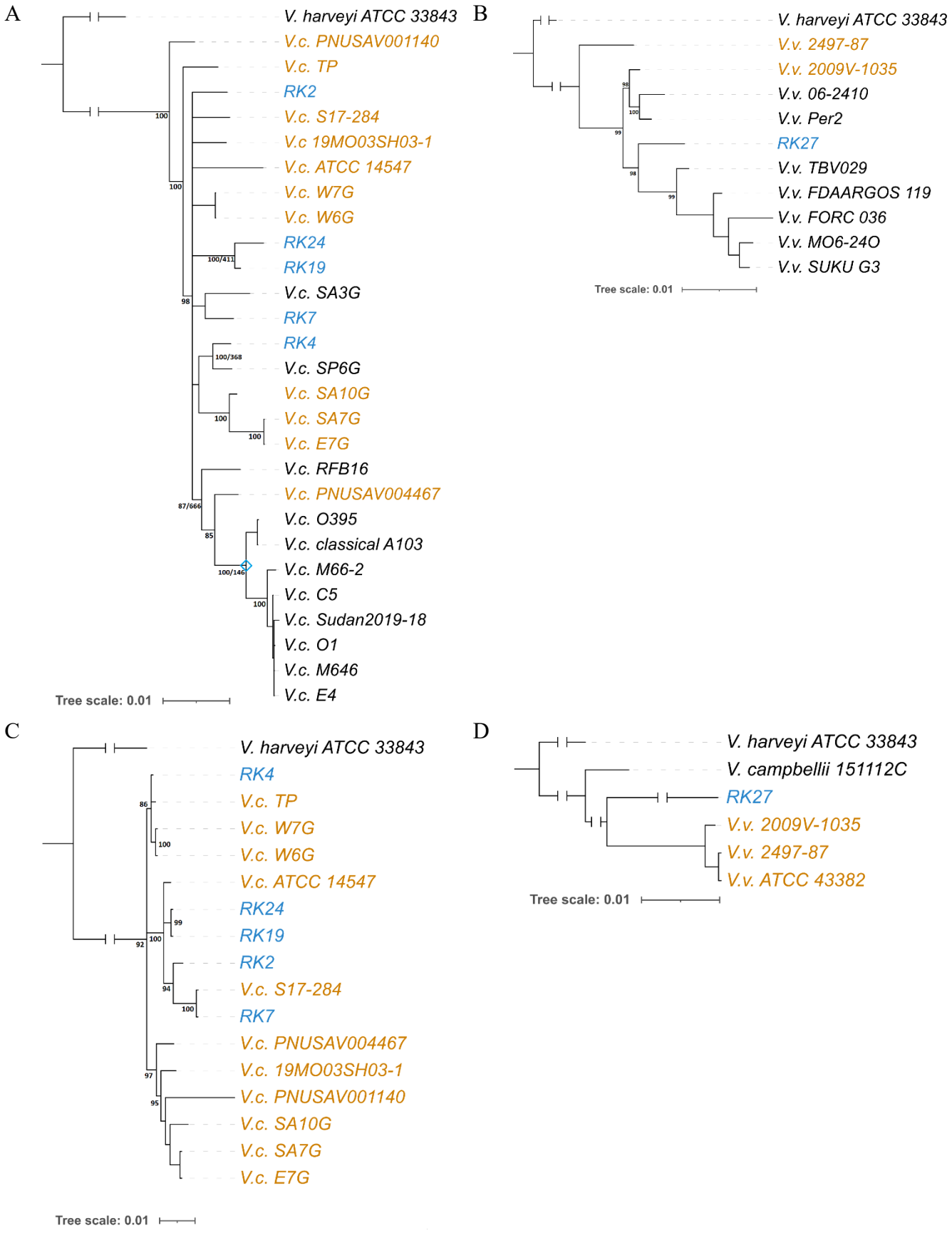


Figure 3. Phylogenetic trees of isolated strains. A - Phylogenetic analysis of *V. cholerae* strains based on a set of seven concatenated housekeeping genes. Bayesian posterior probabilities (expressed as percentages) are shown at nodes; where applicable, divergence years are provided after a slash (e.g., 100/146). The

constrained node is marked with a blue diamond. Branches are truncated (10x shorter). B – Phylogenetic analysis of *V. vulnificus* strains based on a set of seven concatenated housekeeping genes Branches are truncated (10x shorter). C and D - Phylogenetic analysis of *luxAB* genes of isolated *V. cholerae* and *V. vulnificus* strains, respectively, with addition of closest sequences from NCBI database. Branches are truncated (10x and 5x shorter, respectively) Bayesian posterior probabilities (expressed as percentages) are shown at nodes in all trees.

Analysis of the *luxAB* genes revealed a markedly different evolutionary history. The Aral *V. cholerae* isolates clustered with luminescent strains isolated from the Mississippi basin and the Elbe River despite being phylogenetically distant according to housekeeping genes (Fig. 3C). This incongruence provides strong evidence for horizontal transfer of luminescence genes among environmental vibrios.

Luminescent *V. vulnificus* strains are exceptionally rare worldwide. The *luxAB* genes of RK27 differed substantially from those of the few previously reported luminescent *V. vulnificus* isolates (Fig. 3D), again indicating independent acquisition of the luminescence operon.

Metagenomic analysis further revealed an unexpected ecological pattern (Fig. 4).

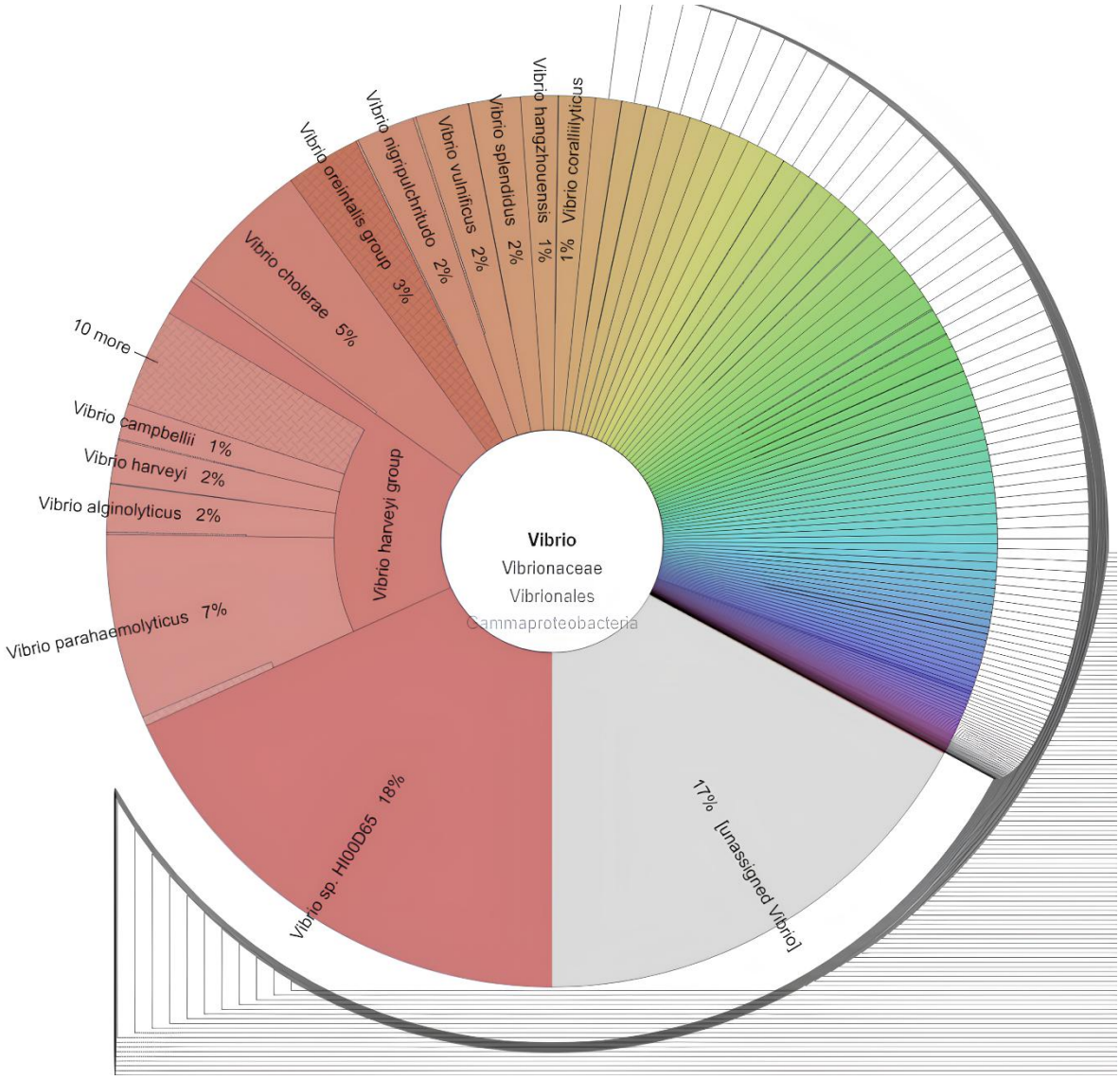


Figure 4. Frequency diagram of fragments of the genome sequences of the genus *Vibrio* in total DNA from water samples of the Aral Sea.

Members of the Harveyi clade comprise approximately 16% of all *Vibrio* sequences detected in the Small Aral Sea, yet none were recovered among cultured luminescent isolates, despite the presence of naturally luminescent species within this group. This discrepancy suggests that luminescent *V. cholerae* and *V. vulnificus* occupy ecological niches distinct from those of Harveyi-clade vibrios in the Aral Sea.

Environmental isolates retain virulence-associated traits

Because both *V. cholerae* and *V. vulnificus* include important human pathogens, we examined whether the Aral Sea isolates retain virulence-associated genes and phenotypes.

Metagenomic sequencing detected numerous virulence-associated genes characteristic of both species (Table 1), based on the pathogenicity markers described for *V. vulnificus* (Li *et al.*, 2019) and *V. cholerae* (Schmidt *et al.*, 2023). Whole-genome sequencing confirmed the presence of several of these genes in the isolated strains, including genes involved in quorum sensing, type VI secretion, hemolysis and extracellular protease production, whereas epidemic cholera-associated determinants such as *ctxA* and the toxin-coregulated pilus were absent.

Table 1. Presence of pathogenicity gene fragments in the metagenomic database of the Aral Sea water sample sequencing and in the results of whole-genome sequencing of isolated luminescent strains.

Gene's name	Description	Depth of coverage in metagenomic data	Presence in whole-genome sequences of isolated luminescent strains
V. vulnificus			
acfA	Accessory colonization factor	3	-
flaA	Flageline A	13	RK27
ompU	The outer membrane protein	444	RK27
tcp	Toxin co-regulated pilus	31	-
vvhA	*V. vulnificus* hemolysin	75	RK27
vvp	*V. vulnificus* protease	22	RK27
V. cholerae			
acfA *acfC*	Vibrio pathogenicity island 1 (VPI-1)/toxin-coregulated pilus. Accessory virulence factors	3 7	- -
	Type 6 secretion system (T6SS) core genes	0	RK2, RK4, RK7, RK19, RK24
cqsR	Quorum sensing/Autoinducer synthase	0	RK2, RK4, RK7, RK19, RK24
hcp-2	Hemolysin	54	RK2, RK4, RK7, RK19, RK24
hlyD	Cytotoxin/Hemolysin	7	RK2, RK4, RK7, RK19, RK24
icmF/vasK	Inner membrane protein vasK	15	RK2, RK4, RK7, RK19, RK24
luxS	S-ribosylhomocysteinase Quorum sensing/autoinducer	2	RK2, RK4, RK7, RK19, RK24
rtxABCD	Multifunctional autoprocessing repeat-in-toxins	3	RK2, RK4, RK7, RK19, RK24
tcpABCDEFHIJPQRST	Vibrio pathogenicity island 1 (VPI-1)/toxin-coregulated pilus		-
mglA	Type 6 secretion system (T6SS) core genes	0	RK2, RK4, RK7, RK19, RK24
zot	Zonula occludens toxin	0	-
ctxA	CTX prophage/Cholera toxin	94	-

ace	Accessory cholera enterotoxin (Membrane-acting toxin)	103	-

Phenotypic characterization further demonstrated that both representative isolates expressed multiple virulence-associated traits. *V. cholerae* RK2 exhibited β-haemolysis, plasma coagulase activity, mannitol fermentation and epithelial-cell adhesion, whereas *V. vulnificus* RK27 displayed α-haemolysis, plasma coagulase activity and an even higher adhesion index. These phenotypes represent recognized virulence-associated characteristics of pathogenic vibrios (Reyes *et al.*, 1985; Sasmal *et al.*, 1987; Mizuno *et al.*, 2019). Together, these observations indicate that environmental luminescent vibrios retain several traits associated with pathogenicity despite lacking the complete repertoire of epidemic virulence determinants (Table S3).

Complementary physiological adaptations support ecological niche partitioning

The Small Aral Sea experiences pronounced seasonal fluctuations in both salinity and temperature. We therefore investigated whether the two luminescent *Vibrio* species differ in their environmental tolerances. Both strains grew optimally at approximately 20 g L^{-1} marine salt (Fig. 5), but their tolerance ranges differed markedly. *V. cholerae* RK2 was capable of growth even in the absence of added salt but failed to grow at salt concentrations exceeding 60 g L^{-1}. In contrast, *V. vulnificus* RK27 tolerated salt concentrations up to 70 g L^{-1} but was unable to grow in salt-free medium. Thus, RK2 is adapted to lower salinity, whereas RK27 displays pronounced halotolerance.

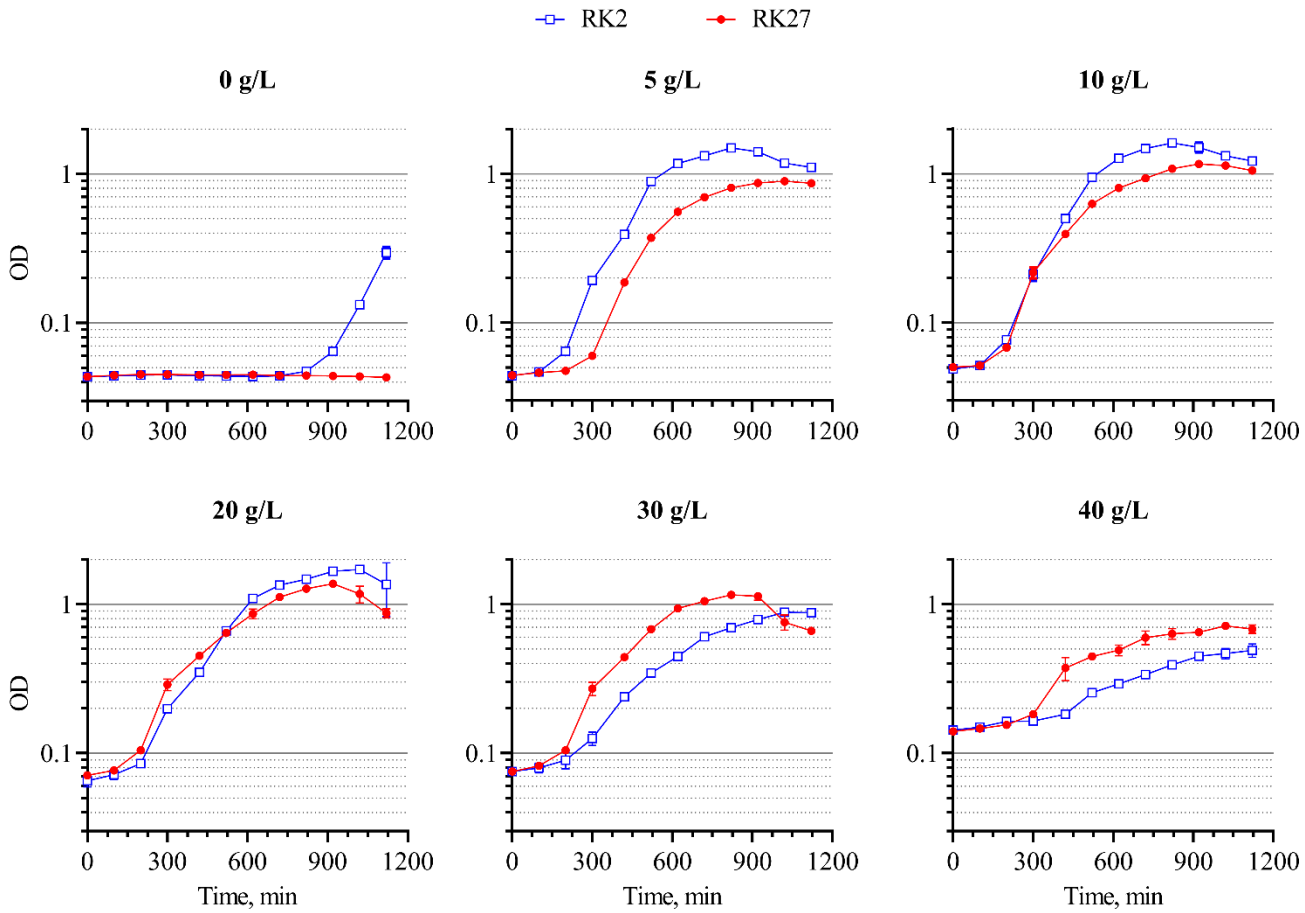


Figure 5. Growth of cell cultures of *V. vulnificus* RK27 and *V. cholerae* RK2 in liquid SWT medium at different salt concentrations. OD measurements over time were performed in a 96-well plate with three biological replicates, incubated at 32°C.

The two species also differed in thermal responses (Fig. 6). Although both remained viable at elevated temperatures, luminescence of RK2 declined rapidly above 30 °C despite continued growth, indicating thermal sensitivity of its light-emitting system. By contrast, RK27 maintained bioluminescence even at temperatures exceeding 40 °C, particularly under elevated salinity. Direct measurements of thermal inactivation confirmed substantially greater thermostability of luminescence in RK27 than in RK2.

A

T°C	Salt 5 g/L				Salt 40 g/L			
	Growth		Luminescence		Growth		Luminescence	
	RK2	RK27	RK2	RK27	RK2	RK27	RK2	RK27
30	Good	Good	Medium	Weak	Weak	Good	None	Good
37	Good	Weak	None	None	Good	Weak	None	Good
42	Good	None	None	None	Weak	Weak	None	Medium

B

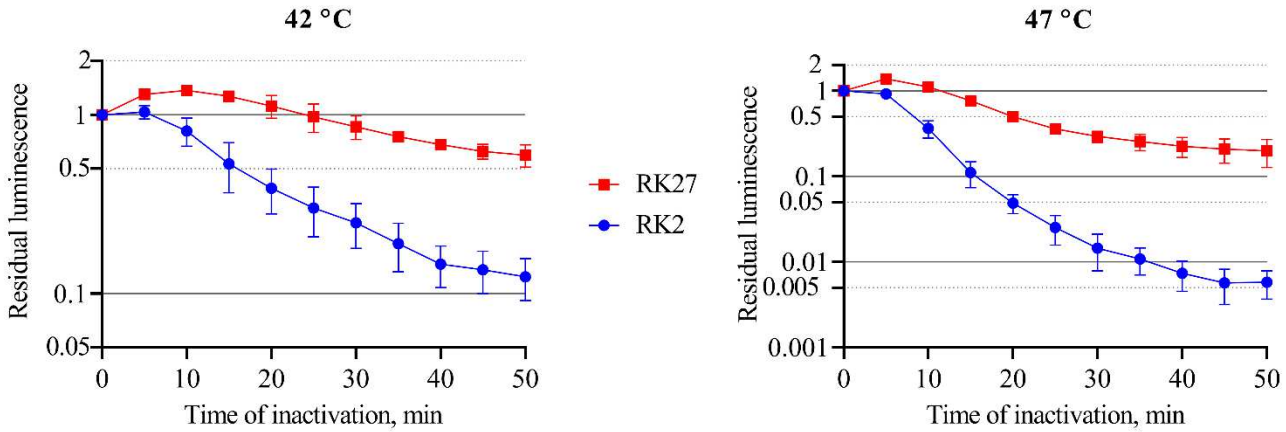


Figure 6. A - Growth and bioluminescence of isolated strains depending on temperature. "Good" – individual colonies of normal size are formed / bright glow; "medium" - individual colonies of small size are formed / visually well distinguished glow; "unstable" – formation of colonies and their glow vary significantly in biological replicates; "weak" – almost no individual colonies are formed / cells emit very little light, almost not visible. **B -** Graph of the decline in luminescence of RK2 and RK27 cells during prolonged exposure to elevated temperatures of 42°C and 47°C.

Together, these complementary physiological adaptations suggest that the two luminescent *Vibrio* species occupy partially distinct ecological niches within the highly dynamic environment of the Small Aral Sea, with *V. cholerae* favoured under lower salinity conditions and *V. vulnificus* under warmer and more saline conditions.

Luminescence is differentially regulated by quorum sensing

Both isolates exhibited cell-density-dependent luminescence characteristic of quorum-sensing regulation (Fig. 7). However, the regulatory responses differed substantially between the two species.

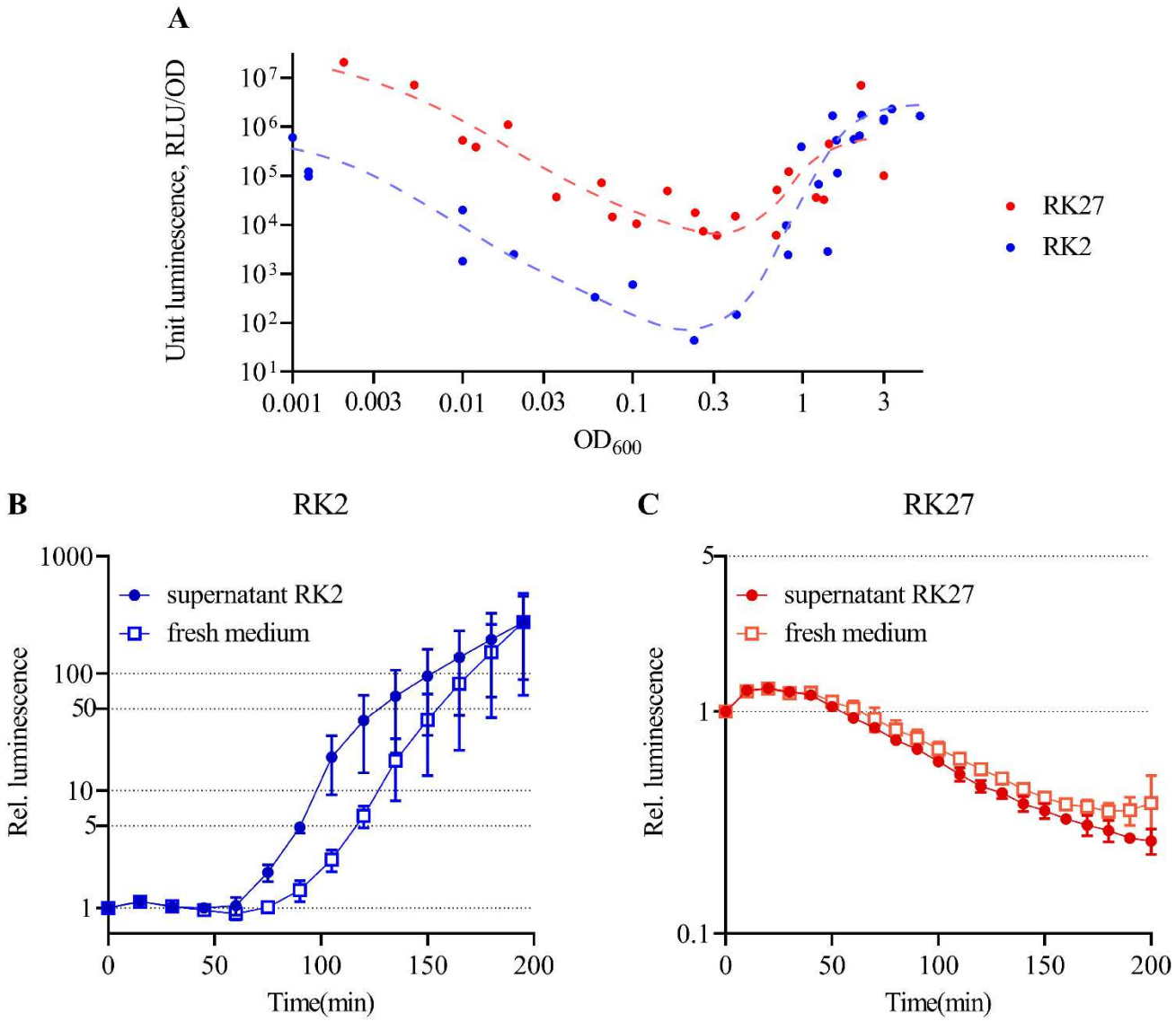


Figure 7. QS regulation of luminescence in *V. vulnificus* RK27 and *V. cholerae* RK2. Specific luminescence (lum/OD) (A) and relative change in luminescence of cultures (lum(t)/lum(0)) after the addition of supernatant from *V. cholerae* RK2 (B) and *V. vulnificus* RK27 (C) over incubation time in liquid medium. In Panel A, lines are drawn between points to visualize the processes of decline and increase in specific luminescence.

Specific luminescence of *V. cholerae* RK2 decreased sharply during early exponential growth before increasing at higher cell density, whereas *V. vulnificus* RK27 displayed a much less pronounced decline (Fig. 7A). Addition of cell-free stationary-phase supernatant strongly stimulated luminescence in RK2 but had little

effect on RK27 (Fig. 7B,C), indicating that luminescence in *V. cholerae* is regulated by diffusible quorum-sensing autoinducers, whereas *V. vulnificus* relies on a distinct or less responsive regulatory mechanism. The contrasting regulation of bioluminescence, together with the complementary salinity and temperature tolerances of the two species, provides further evidence that they occupy distinct ecological niches within the dynamic environment of the Small Aral Sea.

Discussion

The discovery of luminescent *V. cholerae* and *V. vulnificus* in the Small Aral Sea reveals that bioluminescent marine bacteria can successfully colonize and persist in an isolated inland saline ecosystem characterized by pronounced environmental instability. This finding considerably expands the known ecological range of marine luminescent bacteria and demonstrates that long-term geographical isolation does not preclude the establishment of functionally diverse luminescent microbial communities.

The evolutionary history of the Aral Sea makes this observation particularly intriguing. During the late Pliocene, approximately three million years ago, the present-day Aral basin formed part of a large marine system connected to the global ocean, whereas throughout Pleistocene and Holocene the lake experienced significant desiccation followed by repeated transgressions and regressions associated with dramatic changes in salinity, water level and temperature (Table S5) (Svitohc, 2009). Such prolonged isolation and recurrent environmental perturbations might have been expected to promote diversification and the emergence of an endemic luminescent microbiota. Instead, all luminescent isolates recovered in this study belonged to only two cosmopolitan species, *V. cholerae* and *V. vulnificus*, and clustered phylogenetically with contemporary strains from geographically distant marine environments rather than forming an evolutionarily distinct Aral lineage.

Equally unexpected was the taxonomic composition of the luminescent community. Marine luminescent microbiota are typically dominated by members of *Photobacterium* and by species of the *Vibrio harveyi* clade (Yetinson & Shilo, 1979; Urbanczyk et al., 2008; Calogero et al., 2022). By contrast, no luminescent representatives of the *V. harveyi* clade were isolated from the Aral Sea despite extensive sampling. This observation is surprising because brightly luminescent *V. cholerae* and *V. vulnificus* constitute only a small fraction of environmental populations, generally estimated at approximately 5–15% of isolates (West et al., 1986). Nevertheless, subsequent studies demonstrated that many environmental *V. cholerae* strains retain complete luciferase operons but express luminescence only under specific physiological conditions (Grim et al., 2008). Considering that *V. cholerae* and *V. vulnificus* themselves represent only a minor fraction of the total Vibrio community in the Aral Sea, their exclusive representation among cultured luminescent bacteria suggests either strong ecological selection against luminescent members of the *harveyi* clade or substantial seasonal and interannual restructuring of the luminescent microbiota. Future long-term monitoring combining

cultivation-independent metagenomics with targeted isolation will be required to distinguish between these possibilities.

Phylogenetic analyses further suggest that the Aral isolates are unlikely to represent ancient relicts retained since marine isolation. Instead, their close genetic relationship to globally distributed strains, together with the preservation of several virulence-associated genes and phenotypes (Tables 1 and S3), supports a relatively recent introduction into the Aral ecosystem. Because the Aral Sea has repeatedly experienced extensive human activity, fisheries and migratory bird traffic, several dispersal routes remain plausible. Human-mediated introduction appears particularly consistent with the recovery of strains belonging to pathogenic species, although dispersal by migratory birds or other vertebrates cannot presently be excluded.

The presence of luminescent representatives of two clinically important Vibrio species also raises ecological and public-health questions. Members of the family Vibrionaceae are increasingly recognized as emerging pathogens associated with aquatic environments, seafood and climate-driven expansion of marine pathogens (Vezzulli et al., 2017; Landrigan et al., 2020). Human infections are most frequently attributed to *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae* and *V. alginolyticus* (Baker-Austin & Oliver, 2018; Park & Lee, 2018; Yun & Kim, 2018; Haftel & Sharman, 2023). Although the Aral isolates retained several traits commonly associated with pathogenicity, no major epidemiological problems have been reported despite continuous fishing activities and frequent human contact with lake water. This observation suggests that the detected virulence determinants primarily contribute to environmental fitness rather than pathogenicity under present ecological conditions. Nevertheless, continued microbiological surveillance remains warranted given the dynamic environmental changes currently affecting the Aral Sea.

Our physiological analyses indicate that the two luminescent species could occupy partially distinct ecological niches. *V. vulnificus* RK27 displays exceptional halotolerance and maintains luminescence over a remarkably broad salinity range (5–70‰), while retaining luminescence at temperatures exceeding 40 °C. In contrast, *V. cholerae* RK2, despite its greater ability to grow at temperatures up to 42°C, exhibits luminescence only at lower temperatures and salinities. These complementary physiological characteristics likely reduce direct competition and permit coexistence under the highly variable hydrochemical conditions characteristic of the Aral Sea.

Seasonal succession driven by environmental variability may therefore represent an important mechanism structuring the luminescent microbiota. Comparable seasonal replacement of luminescent bacterial species has previously been documented for the Sea of Okhotsk and the Bering Sea, where members of *Aliivibrio* and *Photobacterium* alternate in dominance according to seasonal environmental conditions (Bazhenov et al., 2019). Our results suggest that *V. cholerae* and *V. vulnificus* may perform an analogous ecological partitioning in the Aral Sea, with fluctuations in salinity and temperature determining their relative abundance and contribution to the luminescent bacterial community.

The ecological significance of luminescence itself may also differ between the two species. In *V. cholerae*, quorum sensing coordinates luminescence with numerous physiological processes, including biofilm formation, colonization and regulation of virulence-associated genes. The quorum sensing-controlled luminescence observed in RK strains may therefore represent part of a broader ecological strategy facilitating

colonization of the gastrointestinal tract of fish and enhancing trophic transmission within the aquatic food web. The widespread occurrence of luminescent bacteria in more than 90% of fish intestines and water samples supports the hypothesis that luminescence remains an ecologically relevant trait in this inland ecosystem despite its isolation from marine environments.

Interestingly, previous metagenomic analysis of the Small Aral Sea detected a measurable *V. harveyi* component in environmental DNA collected in 2019 (Alexyuk et al., 2021), whereas our culture-based survey in 2023 recovered exclusively luminescent *V. cholerae* and *V. vulnificus*. Together with the remarkably high abundance of luminescent bacteria observed in both fish and water samples, these data suggest pronounced temporal dynamics in community composition, emphasizing the importance of integrating metagenomic and cultivation approaches for understanding microbial ecology in rapidly changing inland saline ecosystems.

Conclusion

This study provides the first comprehensive characterization of luminescent bacteria inhabiting the Small Aral Sea and demonstrates that two globally distributed species, *V. cholerae* and *V. vulnificus*, dominate the culturable luminescent microbiota of this isolated inland saline ecosystem. Their discovery considerably expands the known ecological distribution of marine bioluminescent bacteria and reveals their remarkable capacity to adapt to environments experiencing extreme and recurrent fluctuations in salinity and temperature.

The two species exhibit complementary physiological strategies that likely facilitate ecological partitioning within the Aral Sea. *V. vulnificus* RK27 combines exceptional halotolerance with thermotolerant luminescence, whereas *V. cholerae* RK2 is better adapted to lower salinities and displays quorum sensing-regulated luminescence mediated by a thermolabile luciferase. These contrasting traits provide a plausible mechanism for seasonal and environmental niche differentiation within the luminescent microbial community.

Phylogenetic analyses indicate that the Aral isolates are closely related to globally distributed strains and do not constitute an ancient endemic lineage. Together with the retention of selected virulence-associated genes, this finding supports a relatively recent colonization of the Aral Sea, most likely after its Holocene reformation as an inland endorheic lake, rather than continuous persistence since the late Pliocene marine connection.

Beyond documenting an unusual microbial community, our findings provide new insight into the ecological plasticity, dispersal capacity and environmental adaptation of potentially pathogenic luminescent bacteria to closed saline water niches. They also highlight the importance of inland saline lakes as previously overlooked reservoirs of bioluminescent and potentially pathogenic microorganisms. As the Aral Sea continues to undergo rapid environmental change, long-term monitoring of its microbial communities will provide a valuable model for understanding how climate-driven alterations in salinity and temperature shape the evolution, persistence and ecological functions of aquatic microorganisms.

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