Virulence and pangenome analysis of *Vibrio harveyi* strains from Greek and Red Sea marine aquaculture

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Abstract

Comparative genomic analysis of *Vibrio harveyi*, a leading pathogen in Mediterranean aquaculture, was conducted to assess the genomic plasticity of the species. *V. harveyi* is responsible for vibriosis outbreaks during the warmer months, resulting in significant economic losses that impact Greek aquaculture. Over a span of six years, we curated a diverse collection of bacterial strains associated with these outbreaks. Whole-genome sequencing was employed in 21 strains to uncover their evolutionary relationships and virulence factors. Pangenome analysis revealed significant gene gain/loss, with numerous unique genes within the strains. The core genome featured genes associated with pathogenicity, including secretion systems, flagella, pili, siderophores, and toxins. Furthermore, we examined the phenotypic traits and virulence of these strains using *in vivo* testing with gilthead seabream larvae. Our findings indicated variant metabolic profiles and virulence among the strains during these *in vivo* assays. By integrating genomic and phenotypic data, our study highlights the ongoing evolution of disease-associated *V. harveyi* strains, which pose a growing challenge to the aquaculture industry.

Keywords

1. Introduction

Vibrio spp. encompass a group of bacteria that are highly prevalent in aquatic environments, displaying adaptability to a broad spectrum of salinities and temperatures. This versatility has enabled them to inhabit a diverse array of ecological niches, including anthropogenic environments

44   like wastewater and aquaculture systems (Baker-Austin et al., 2017; Le Roux et al., 2015). They

45   thrive in anaerobic/aerobic, oligotrophic/nutrient-rich conditions, in biofilms and sediments, as

46   free-living cells, or associated with different hosts. This adaptability has been attributed to their

47   wide variety of genes and metabolic pathways. Vibrios can have various hosts, from microalgae

48   to vertebrates, and they act as symbionts or pathogens (Mougin et al., 2021; Sampaio et al., 2022).

49   Vibriosis is one of the oldest known diseases of fish, causing enormous economic losses to the

50   marine aquaculture industry (Mohd Yazid et al., 2021; Roberts, 2012). *V. harveyi* is one of the

51   species related to the disease and it can infect a wide range of fishes, including the catfish, grouper,

52   the rainbow trout, breams and basses (Austin and Zhang, 2006). The most relevant clinical signs

53   of vibriosis in fish include the presence of hemorrhages, skin lesions, fin erosion, ulceration and

54   gastro-enteritis. It can also cause systemic infection and mortality in fish, leading to septicemia

55   and organ failure (Mohamad et al., 2019; Zhang et al., 2020). The species is also notable for

56   causing mass mortalities in crustacean and mollusks aquaculture with systemic and severe

57   symptoms in shrimps, lobsters, oysters and abalones (Meibom et al., 2005; Saulnier et al., 2010;

58   Sawabe et al., 2007).

59   The *Vibrio* genus exhibits great genome plasticity within its members, even within populations of

60   the same species (Le Roux and Blokesch, 2018). In addition, among the different pathogens able

61   to colonize many niches, has disproportionately more virulence factors inside genomic islands

62   (GIs) (Ho Sui et al., 2009) underpinning how horizontal gene transfer (HGT) has created a

63   reservoir of genetic tools, readily available for use when an evolutionary cue arises. In the present

64   study, 21 new genomes mainly deriving from the European seabass are presented. Other fin fishes

65   that served as hosts were aquaculture species from the Mediterranean, such as the gilthead

66   seabream (*Sparus aurata*), the greater amberjack (*Seriola dumerili*), and the common dentex

67   (*Dentex dentex*). Moreover, two strains isolated from marine farms from the Red Sea were included

68   in the study, one from gilthead seabream and one from Nile tilapia (*Oreochromis niloticus*). We

69   conducted a comprehensive genome-wide comparison to elucidate both similarities and strain-

70   specific characteristics. The analysis focused on virulence and antibiotic resistance, and genomic

71   data were correlated with phenotypic traits. We present this set of draft genomes as a pangenome

72   of *V. harveyi* strains causing disease in marine fishes.

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2. Materials and Methods

74   **2. Materials and Methods**

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76   2.1 Sampling and Strain Isolation, Microbiology and Virulence

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78   The *V.harveyi* strains described in this study are all isolates from clinical cases and derived from

79   5 fish species, the *Dicentrarchus labrax, Sparus aurata, Seriola dumerili, Dentex dentex,* and

80   *Oreochromis niloticus.* They are members of a wider collection resulted from a big sampling effort

81   to study vibriosis caused by *V. harveyi* in Greece which has been published previously by our team

82   (Triga et al., 2023). The bacterial strains used here were routinely isolated on TCBS agar and then

83   re-cultures were purified on Tryptone Soy Agar (TSA) supplemented with 2% NaCl. All strains

84   were further cultured on Difco Marine Agar (MA) (Becton, Dickinson and Company, Le Pont de

85   Claix, France), MacConkey-AMP-Tween agar (Sigma-Aldrich, Co., St. Louis, MO, USA), MIO

86   media (Sigma-Aldrich, Co., St. Louis, MO, USA) and 5% seabass blood agar (blood taken from

87   healthy fish of the broodstock in the HCMR facilities). The phenotypic fingerprint of the strains

88   was obtained using the GEN III MicroPlate (BIOLOG, Hayward, USA) kit. Antibiotic

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89 susceptibility to antibiotics relevant to aquaculture practice was assessed with the disc diffusion

90 method according to CLSI M45 guidelines on Mueller-Hinton agar (MHA) (Difco, USA)

91 supplemented with 2% NaCl (CLSI, 2016). The antibiotic susceptibility discs (Oxoid Ltd.

92 Basingstoke, Hampshire, England) were ampicillin 10 μg (AMP10), oxolinic acid 2 μg (OA2),

93 flumequine 30 μg (UB30), florfenicol 30 μg (FFC30), oxytetracycline 30 μg (OT30) and

94 sulfamethoxazole/ trimethoprim 25 μg (SXT25). The gilthead seabream (*Sparus aurata*) larvae

95 model was used to compare the virulence of the 21 strains *in vivo* as described in Droubogiannis

96 *et al.* (Droubogiannis and Katharios, 2022) using eggs acquired from the HCMR broodstock

97 facility. Briefly, gilthead seabream eggs were placed individually in 96-well plates in sterile

98 seawater after being washed three times in sterile seawater. Infection was initiated after hatching

99 of all larvae, with the addition of 20 μL of bacterial suspensions of the strain tested adjusted to

100 achieve a mean final concentration of 1.66×10^6 cfu ml^{-1} in the wells. Mortality of the fish larvae

101 was monitored over a period of 5 days. The 96-well plates were incubated at a constant temperature

102 of 22°C in a cooling incubator. Kaplan-Meier survival curves and statistical analysis were

103 calculated using GraphPad Prism 10.0.0 (GraphPad Software, Boston, Massachusetts USA,

104 www.graphpad.com).

105

106 2.2 Genomic DNA Extraction, Whole Genome Sequencing, Genome Assembly and

107 Annotation

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109 Overnight cultures (25°C in TSB 2% NaCl) of the strains were processed with the DNeasy Blood

110 and Tissue kit (QIAGEN, Hilden, Germany) for DNA extraction, followed by quality control using

111   NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), Qubit (Thermo Fisher Scientific,

112   Waltham, MA, USA) instruments and 1% agarose gel electrophoresis. Whole genome sequencing

113   was conducted in the DNBseq platform (BGI Tech Solutions, Hong Kong) on the DNBSEQ-G400

114   sequencer using paired-end technology (PE100). The library preparation and filtering procedure

115   was done according to previously described method (Tsertou et al., 2023). The PATRIC platform

116   (Davis et al., 2019) was utilized for the genome assembly, and the following tools were involved

117   in the pipeline: Samtools 1.3 (Danecek et al., 2021), the Unicycler v0.4.8 (Wick et al., 2017), Pilon

118   1.23 (Walker et al., 2014) and Bandage 0.8.1 (Wick et al., 2015). The assembly was assessed with

119   BUSCO (Simão et al., 2015) and then the contigs were submitted to the GenBank and annotated

120   by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Li et al., 2021). Plasmid

121   sequences were explored with PLACNETw (Vielva et al., 2017). The corrected reads were also

122   submitted to the Sequence Read Archive (SRA) database, accession numbers are presented in

123   **Table 1**. The VH2 draft genome has been already published (Castillo et al., 2015), but for sake of

124   comparison and the same workflow being followed for all genomes, it got re-sequenced.

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126   2.3 Pangenome analysis and Phylogeny

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128   The pangenome analysis was performed in the web-based platform for comparative genomics

129   "Efficient Database framework for comparative genome analyses using BLAST score Ratios"

130   EDGAR 3.0 (https://edgar3.computational.bio.uni-giessen.de) (Dieckmann et al., 2021). In

131   EDGAR, the development of the pangenome was computed, along with the distribution and

132   categorization of gene sets into core, dispensable, and singletons. The core subset refers to the set

133   of genes present in all strains of a given species or population, representing the conserved genomic

content. The dispensable subset comprises genes present in some, but not all, strains, indicating variability within the population. Singleton genes are unique to individual strains, representing strain-specific elements. The construction of an approximately-maximum-likelihood phylogenetic tree was done after alignment of all core gene sets using MUSCLE (Edgar, 2004), and then with the FastTree software (http://www.microbesonline.org/fasttree/), as it is more discriminatory and effective than multi-locus or 16S rRNA based analysis. The genome similarity ANI-matrix was calculated with the Genome-based Distance Matrix calculator (Rodriguez-R and Konstantinidis, 2016), also including the *V. harveyi* reference strain ATCC 33843 (392 MAV), NCBI Biosample SAMN03075601. The EDGAR platform offered the construction of circular plot of the genes of the genomes using BioCircos (Cui et al., 2016) and the calculation of a POCP (percentage of conserved proteins) (Qin et al., 2014) matrix to estimate their evolutionary and phenotypic distance. In the EDGAR analyses that required a reference genome for the iterative calculation and the gene order of the output, VhP1-sp was set as one, as it was one of the oldest isolated strains. The multi-locus sequence typing (MLST) was used to allocate a sequence type for the 21 strains, and the isolates' alleles were submitted to the Public Databases for Molecular Typing and Microbial Genome Diversity (Jolley et al., 2018).

2.4 Functional annotations

The PATRIC workspace was employed for detecting virulence factors, and antibiotic resistance genes (ARGs), the CRISPRCasFinder v1.1.2 (Couvin et al., 2018) for CRISPR-Cas systems in the strains and DefenseFinder (Tesson et al., 2022) for anti-phage systems search. Gene sets of the pangenome were further analyzed with VFDB (Chen, 2004), EDGAR 3.0 and BlastKOALA

157 (Kanehisa et al., 2016) for functional category analysis. Genomic islands (Gls) were predicted with

158 the webtool IslandVewer v4 (Bertelli et al., 2017) using two independent methods that supported

159 the assembly level of the genomes, the SIGI-HMM and IslandPath-DIMOB, after the ordering of

160 the contigs against the reference genome *V. harveyi* ATCC 33843 (392 MAV). From the results

161 produced, the GIs not aligned to the reference were filtered out. Prophage regions were identified

162 and annotated using the PHASTER webserver (Arndt et al., 2016). The prophage regions located

163 at the end of the contigs were assigned manually as incomplete.

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165 **3. Results and Discussion**

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167 3.1 Strain Biochemical Characterization

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169 The 21 *V. harveyi* strains produced yellow colonies on the TCBS, and colorless to yellowish on

170 the MacConkey-AMP-Tween. They were all defined as motile in the MIO medium, had negative

171 phenotype for indole production, and 61% were positive for the ornithine decarboxylase (ODC)

172 (**Table S1**). A negative phenotype for ODC has been reported for other *V. harveyi* isolates (Brenner

173 et al., 2005; Pavlinec et al., 2022). Indole is an important intercellular signal molecule, produced

174 by many bacteria including vibrios and *V. harveyi* (Lee and Lee, 2010). When present in the

175 extracellular space, indole plays a role in decreasing the formation of biofilm and the virulence of

176 pathogenic *Vibrio* spp. (Li et al., 2014; Mueller et al., 2009; Zhang et al., 2022).

177 The Gen III MicroPlate assay results unveiled various carbon-utilization phenotypes. All strains

178 were cultivated within the tested NaCl gradient (2-8% NaCl) (Brenner et al., 2005). They exhibited

179 metabolic activity towards several substrates, including D-glucuronic acid, a constituent of

180 capsular polysaccharides (Riegert et al., 2021) and other bacterial and algae structures (Vinnitskiy

181 et al., 2015), as well as N-acetyl-D-glucosamine, a component of peptidoglycan and chitin.

182 Additionally, these strains metabolized the amino acids L-glutamic acid, L-serine, L-alanine, and

183 D-mannitol, commonly present in seaweed. These phenotypic profiles provide insights into the

184 degree of adaptation of *V. harveyi* isolates to the marine environment. Notably, there was

185 significant diversity in other metabolic traits, with 90% of isolates capable of fermenting sucrose,

186 14% metabolizing D-salicin, 86% utilizing D-cellobiose, and 67% D-galactose. Additionally, 33%

187 of the strains utilized L-arginine. However, none of the isolates exhibited the ability to utilize myo-

188 inositol, D-melibiose, or formic acid. It is noteworthy that formic acid has inhibitory properties

189 against various vibrios, including *V. harveyi*, and has been utilized as a bio-control agent against

190 vibriosis, particularly in shrimp aquaculture (Adams and Boopathy, 2013; Chuchird et al., 2015).

191 In the chemical resistance assays of Gen III MicroPlate, all of them showed growth at pH=6, and

192 four at pH=5, the Gal 90, ML 1, SA 6.1 and Sernef. The strains were sensitive to minocycline and

193 nalidixic acid, antibiotics that have been used in aquaculture practices but are not authorized for

194 use in Greece (Xu et al., 2022). Furthermore, the strains were resistant to 2% sodium lactate,

195 tetrazolium blue, and some antibiotics such as fusidic acid and rifamycin SV. Except for 23 carbon

196 sources utilization and 9 chemical resistance reactions that were constant for all the strains tested,

197 the other 62 reactions were variable. This aspect of the genus has been observed for many species,

198 and phenotypic identification must be complemented with genomic information (Thompson et al.,

199 2004). The Gen III MicroPlate assay results for carbon sources utilization and chemical resistance

200 are presented in **Table S1**.

201

3.2 Genomic characteristics, Pangenome and Phylogeny

The *V. harveyi* genome sizes and gene numbers among the strains were similar, with average length of 6,009,731 bps dispersed across 63 to 232 contigs. GC content ranges from 43.63% to 44.93% (**Table 1**). On average, the number of genes annotated per strain was 5,570 (ranging from 5,259 to 6,082), with 5,495 of these being coding DNA sequences (CDSs) and 75 being RNA sequences (ranging from 45 to 107). Among the annotated CDSs, an average of 664 hypothetical proteins (ranging from 514 to 1,050) were identified across the 21 strains. Additionally, an average of 66 pseudogenes (ranging from 28 to 106) were detected. The assembly did not provide chromosome level results, but the statistics reveal good quality contigs with completeness 99.4% and above, after BUSCO assessment, coverage around 203.69x and N50 between 228.328 and 507.658 (**Table S2**). The *V. harveyi* has been reported so far to possess two unequal in size chromosomes as well as plasmids. The family Vibrionaceae is one of the families known to possess chromids. This characteristic is associated with the plasticity of its bipartite genomes. The second chromosome, typically approximately 2 Mb, is considered a chromid. In *Vibrio* species, both the chromosome and chromid are similarly prone to gene acquitition (Sonnenberg and Haugen, 2023). Chromids appear more often to eukaryote symbionts or pathogens (diCenzo and Finan, 2017). In multipartite genomes, chromids have been attributed to facilitating bacteria in specific ecological niches (diCenzo and Finan, 2017; Riccardi et al., 2023). A limitation of our study is that both short-read sequencing and *de novo* assembly methods do not necessarily provide complete plasmid sequences. Furthermore, the PLACNETw tool failed to identify any nodes recognized as plasmids. Despite this, plasmids have been identified in complete genomes of the species, with sizes ranging from 9 to 185 kbps (Montánchez and Kaberdin, 2020). Examination of the public NCBI database

225 (accessed on 2024/02/09), which included 29 complete genomes of *V. harveyi* and their associated

226 plasmids, revealed that the genome lengths exhibited a similar range to our findings, regardless of

227 the presence of plasmids.

228 The pangenome consists of 10,418 CDSs, 4,336 core, 3,030 dispensable, 3,053 singletons (**Figure**

229 **S1**). The pangenome of the 21 strains comprised approximately 6.5% hypothetical proteins, while

230 a similar proportion, around 7.5% of the core genome's CDSs, were found to have unknown

231 functions. The average number of singletons per isolate was 94, and the largest strain-specific gene

232 sets were found in the strains SA 6.1 and SS 1, with 199 and 376 genes, respectively. Most the

233 CDSs are either core or singletons and very few dispensable are shared between more than 4 strains

234 (**Table S3)**. As additional genomes were incorporated into the dataset, the pangenome exhibited

235 continuous expansion without reaching a plateau (**Figure 1a**). Conversely, the core genome

236 decreased as the number of genomes increased along the x-axis. (**Figure 1b**). A similar

237 phenomenon has been observed in *Vibrio alginolyticus*, where strains from various geographic

238 regions and fish species also display an open pangenome (Chibani et al., 2020). The pangenome

239 evolution *V. harveyi* is characterized by gene gain/loss (De Mesa et al., 2023; Kayansamruaj et

240 al., 2018) and therefore the pangenome model is open, this is the case also for the 21 strains.

241 Species occupying diverse niches with many community interactions tend to have open

242 pangenomes where ecological factors are the key to bacterial gene sharing (Brockhurst et al.,

243 2019). Also, bipartite genomes of vibrios tend to be more open than monopartite genomes of

244 related families, therefore they are more prone to gene acquisition (Sonnenberg and Haugen,

245 2023).

246 All strains, including the ATCC 33843 reference strain, exhibited close relatedness, with pairwise

247 Average Nucleotide Identity (ANI) values exceeding 99%. The construction of the phylogenetic

248   tree for the 21 genomes was based on a core gene set comprising 4,336 genes (**Figure 2**). The

249   analysis formed three distinct major clusters. The Percentage of Conserved Proteins (POCP)

250   matrix closely matched the approximately maximum-likelihood tree in terms of the extent of small

251   groups with higher percentage identities (**Figure S2**). Specifically, Kef 80 exhibited a closer

252   relationship with MDO 7.2, Kef 22 with VhP1-li and ML 1, and Gal 88 with SM1. Importantly,

253   this clustering did not reflect geographic, temporal, or host source factors. In general, the high

254   protein similarities and conserved regions observed are characteristic of the species (Espinoza-

255   Valles et al., 2015). This explains why strains separated by both years and geographic distances

256   tend to cluster together.

257   The allele assignments within the PubMLST database for *gyrB, pyrH, recA*, and *atpA* displayed

258   significant diversity among the 21 strains, as detailed in **Table S4**, resulting in the classification

259   of distinct Sequence Types (STs). Among these strains, FL 9.1 was associated with ST=241, while

260   Serfr was linked to ST=216. Notably, both STs were attributed to *V. harveyi* isolates originating

261   from live marine animals located north of the Yangtze River, as well as other regions in Qingdao

262   and Hebei Provinces, China, spanning the years 2009 to 2016.

263

264   3.3 Metabolic enzymes, Virulence Factors, and HGT, from genotype to phenotype

265

266   Most of core genome proteins were found to be associated with COG superfamilies, with the most

267   prominent groups falling into three major categories: transcription, amino acid and carbohydrate

268   transport and metabolism (**Figure 3**). In contrast, fewer genes from the dispensable and singleton

269   fraction could be confidently assigned to the COG database. Interestingly, functions unique to

270 specific strains appeared to be primarily associated with processes such as replication, cell wall

271 and membrane formation, envelope biogenesis, intracellular trafficking, and the presence of

272 transposase elements.

273 The core genome of the strains encompassed crucial virulence factors, including transporters,

274 secretion systems, bacterial toxins, motility proteins, exosome proteins, antimicrobial resistance

275 mechanisms, prokaryotic defense systems, and siderophores. It is noteworthy that important

276 virulence elements are conserved within the core genome of *Vibrionaceae*, indicating that many

277 *Vibrio* species and strains in marine environments possess a fundamental repertoire of genetic tools

278 for potentially expressing pathogenic traits (Lilburn et al., 2010). However, it is important to

279 highlight that while numerous essential virulence factors were part of the core genome, singleton

280 gene sets also contained a subset of such factors (**Table S5**). This phenomenon is not uncommon

281 among pathogenic strains (Deng et al., 2019). For instance, SA 6.1 and SS 1, that exhibited the

282 largest singleton gene sets, contained genes that regulated processes such as metabolism,

283 transporters, two-component systems, quorum sensing and secretion systems.

284 Particularly important gene clusters of the T2SS, T3SS, T4SS, T6SS, T1SS, protein-export

285 proteins of the Sec-SRP system and flagellar-assembly proteins have been annotated. T3SS and

286 T6SS are closely associated with the pathogenicity of the species (Deng et al., 2019). Genes of

287 these systems are clustered together and share the same synteny in most of the genomes studied in

288 Fu *et al.,* who also incorporated the previous version of VH2 genome (Fu et al., 2021). This pattern

289 was observed in all 21 genomes as well. In addition to genes scattered throughout the genome, the

290 T3SS exhibited synteny with approximately 44 CDSs spanning 36.5 kbps in all 21 strains, while

291 the T6SS occupied 24.5 kbps.

292 The toxin genes in the core genome include an enterotoxin, the microbial collagenase *colA*

293 (K01387), the exfoliative toxin A/B *eta* (K11041), several lipases and phospholipases. Hemolytic

294 activity is very important for vibrios during infection (Ruwandeepika et al., 2012). Some

295 hemolysins of the core genome are the thermolabile hemolysin *tlh* (K11018) and the hemolysin III

296 *hlyIII* (K11068) the *tlyC* (K03699), hemolysin family protein. There are two loci of thermolabile

297 hemolysin in two strains, VhP1sp and SD1.1, with the second being a fragment, while most of the

298 strains contained one copy. However, more copies do not indicate higher hemolytic activity (Zhang

299 et al., 2001). No hemolysins were detected in the singletons. All strains were capable of β-

300 hemolysis (**Figure S3**). RTX toxin genes were found in the singletons of VhP1-li. It is a finding

301 that could be associated with higher mortalities induced by this strain to the gilthead seabream

302 larvae in the *in vivo* test (**Figure 4**). The *rtx* genes are detected in conserved areas of *V. harveyi*

303 (Espinoza-Valles et al., 2015) and between tandem repeats that complicate the assembly of short-

304 read sequencing (Tørresen et al., 2019). Therefore, the lack of these loci in the other genomes,

305 such as ML 1, might be attributed to sequencing/assembly error.

306 Iron uptake operon and vibrioferrin-related proteins, located in chromosome II, are conserved in

307 the species (Espinoza-Valles et al., 2015). In the 21 genomes, the MotA/TolQ/ExbB siderophore

308 complex is conserved and detected inside a cluster of related proteins. *V. harveyi* regulates the

309 accumulation of siderophores during growth phases through quorum sensing (QS) (McRose et al.,

310 2018). Other iron binding and transport proteins involved the FeO system, ferric ion ABC

311 transporters, and the enterobactin biosynthesis protein, *fecB*. All strains contain genes for the

312 metabolism of terpenoids, polyketides, xenobiotics, and hydrogen-peroxide-resistance proteins

313 such as *oxyR*. Urease activity in *Vibrio parahaemolyticus* serves as a mechanism for modulating

314 the pH within their habitat (Berutti et al., 2014). This capability is particularly significant during

315 the colonization of a host, whether as symbionts or pathogens, as it enables the bacterium to

316 influence the pH levels within its environment to establish a favorable ecological niche. *V. harveyi*

317 also demonstrates ureolytic activity (Defoirdt et al., 2017). Specifically, all 21 strains were found

318 to contain the genes *ureG/F/C/B*, and in some strains, additional *ureF/D/A* genes were identified.

319 Furthermore, the presence of the *chbG* chitinase gene, along with other genes related to chitin

320 metabolism, was observed. This observation is significant, as it suggests that *V. harveyi* possesses

321 a genetic toolkit for utilizing chitin, which allows it to adapt to a diverse range of environmental

322 conditions (Ran et al., 2023).

323 In all the strains examined, various virulence factors included genes associated with the

324 thioredoxin (TRX) system, which plays a role in maintaining redox homeostasis, as well as global

325 regulators like *luxR, luxS*, and *luxP*, known for their involvement in QS and luminescence. Quorum

326 sensing, a regulatory mechanism, plays a crucial role in controlling virulence in bacteria of the

327 Harveyi clade (Zhang et al., 2023) and anti-phage defense systems (Tesson and Bernheim, 2023).

328 Although vibrios are generally recognized as luminous marine bacteria with a conserved *lux*

329 operon (Vannier et al., 2020), which was primarily associated with *Vibrio campbellii*, it is

330 noteworthy that in this collection of strains, only VarA4 1.1 exhibited luminescence on MA.

331 Surprisingly, neither the entire *lux* operon (*luxCDABFEG*) nor individual genes from the operon

332 were detected in any of the genomes. The presence or absence of the *lux* operon is linked to

333 symbiotic and planktonic lifestyles in bacteria, with non-luminescent isolates typically lacking this

334 operon (O'Grady and Wimpee, 2008). The *V. harveyi* strains are serious pathogens, but

335 bioluminescence is not correlated with virulence (Defoirdt et al., 2008). The *luxT,* that encodes a

336 small RNA that regulates QS response (Eickhoff et al., 2021), was present in the strains Kef 62,

337 Kef 75, ML 1, Serfr, SD 1.1, SA 9.1 and VH2 and the luciferase only in the Gal 1. Aldehyde

338   metabolism genes were detected in all strains. Aldehyde serves as substrate for luciferase, playing

339   a biochemical role in light emission observed in many *Vibrio* species (Dunlap, 2014).

340   Antimicrobial resistance genes (ARGs) are prevalent in bacteria of the aquatic environment and

341   can be identified in many aquaculture pathogens (Bondad-Reantaso et al., 2023). A comprehensive

342   global study on ocean resistomes revealed that approximately 25% of ARGs are found within

343   putative plasmid sequences, with the majority residing on bacterial chromosomes (Cuadrat et al.,

344   2020). This underscores the critical role of both resistant bacteria dissemination and horizontal

345   gene transfer in the spread of antimicrobial resistance. In the case of the 21 strains under

346   investigation, the presence of ARGs was relatively limited, but as the strains are characterized by

347   an open pangenome and the ability to receive incoming ARGs from other species is still a major

348   concern. Deng et al. similarly observed a constrained distribution of ARGs among collected *V.*

349   *harveyi* isolates (Deng et al., 2020). Some ARGs were detected in the core genome of the 21

350   strains, including blaCARB (blaCARB-17, K19217), the outer-membrane protein tolC (K12340),

351   and genes homologous to parS (K18072), which are associated with beta-lactam and cationic

352   antimicrobial peptide (CAMP) resistance. The blaCARB-17 locus is believed to be an intrinsic

353   chromosomal gene of *V. parahaemolyticus*, conferring resistance to β-lactams (Chiou et al., 2015).

354   While isolates carrying *tet* and *qnr* genes are common in aquaculture, they do not exhibit high

355   levels of resistance to tetracyclines and quinolones (Deng et al., 2020). Interestingly, all the

356   examined strains were found to harbor *catB* (K00638), *tet35* (K18218), and *qnr* (K18555) genes.

357   ARGs display temporal patterns among the replicons, chromosomes and plasmids. Notably,

358   certain genes, such as β-lactamases and *tet* efflux pumps, tend to be prevalent on specific types of

359   chromids. Efflux pumps are typically part of the core genome (Wang et al., 2022), and demonstrate

360   multifunctional characteristics. The multidrug efflux pump modules annotated in the strains shared

361   homology to *mexA* (K03585), *mexB* (K18138), *mexL* (K18301), *mexJ* (K18302), *cueR* (K19591),

362   and *oprN* (K18300). When assessing the disk diffusion diameters, it was observed that the strains

363   displayed resistance to ampicillin and, for the most part, sensitivity to the tested antibiotics (**Table**

364   **2**). Due to the absence of established zone diameter breakpoints for the antibiotics tested against

365   *V. harveyi*, classification of strains based on inhibition zones was not feasible in our study.

366   Recently, epidemiological cut-off values for these antibiotics and the species have been published,

367   that range from ≤0.5 to 9.5 µg ml^{-1} (Smith et al., 2023). Additionally, the supplementation of NaCl

368   in agar media has been subject to controversy (Smith, 2019; Smith and Egan, 2018), and

369   standardized protocols recommend unmodified MHA for this species. These limitations

370   underscore the challenges encountered in antimicrobial susceptibility testing and emphasize the

371   need for further research in this area. Only Kef 75 exhibited resistance to oxytetracycline and

372   sulfamethoxazole/trimethoprim, without the presence of any other known antibiotic resistance

373   genes (ARGs) to account for this resistance. *Vibrio* strains can exhibit diverse antibiotic resistance

374   profiles, complicating disease management strategies (Deekshit et al., 2023; Rigos and Kogiannou,

375   2023). While ampicillin is commonly not effective, most antibiotics tested displayed low

376   resistance, a trend observed in Croatian isolates from European seabass (Pavlinec et al., 2022) and

377   Italian *Vibrio* isolates from shellfish (Mancini et al., 2023). It is worth noting that AMR of *V.*

378   *harveyi* appears to pose a more severe challenge in shrimp aquaculture in regions such as China

379   and Malaysia, where higher multiple antibiotic resistance (MAR) indices have been reported

380   (Deekshit et al., 2023; Yu et al., 2023).

381   The present study did not assume a direct and unequivocal correlation between genotype and

382   phenotype, a presumption that often proves challenging to uphold, even when employing

383   computational models and genome-wide analyses (GWAs) (Lees et al., 2020). For example, the

384 presence of genes associated with specific metabolic phenotypes, such as the positive ornithine

385 decarboxylase reaction, D-galactose, and sucrose fermentation (**Table S6**), was not predicted in

386 the 21 *V. harveyi* strains as observed in strains 1792 and 14126T (Amaral et al., 2014). Predicting

387 genotype-to-phenotype relationships is generally more reliable in cases involving antibiotic

388 resistance, while for virulence, it is imperative to also consider the influence of epistasis (Sailer

389 and Harms, 2017). Furthermore, many virulence factors were detected in the core genome of the

390 21 strains. However, their pathogenicity against seabream larvae was different (**Figure S4, Table

391 S7, S8**) and the strains were clustered according to their pathogenic potential (**Figure 4**). In the

392 virulence assay conducted, strain ML 1 emerged as the most virulent, resulting in approximately

393 80% larval mortality over the five-day period. In contrast, strain Kef 75 exhibited the lowest

394 virulence, causing less than 10% mortality. The strains can be broadly categorized into three

395 groups based on their virulence levels: the most virulent group (classified as "g" to "efg"),

396 comprising the top three strains; the intermediate group (classified as "def" to "abcd"), consisting

397 of twelve strains with moderate virulence; and the least virulent group (classified as "abc" to "a"),

398 comprising seven strains with the lowest virulence. This classification highlights the varying

399 degrees of pathogenicity among the tested strains. Harboring the same virulence genes did not

400 translate to equal morbidity. The 21 strains had most of the main virulence genes mentioned in Fu

401 *et al.*, except for *mshB* and *hutR* (Fu et al., 2021), but it is uncertain if the lack of them would affect

402 virulence. The three most virulent strains derive from eastern Aegean, ML1 and VhP1-li are

403 phylogenetically closer and showed high percentage of conserved proteins. It is important to

404 acknowledge that the diverse origins of the strains, particularly in relation to the host species, may

405 influence the observed variations in morbidity outcomes. Different hosts would exhibit distinct

406 mortality gradients when exposed to the same strain (Firmino et al., 2019). For example, certain

407 fish species like the European seabass are known to be more susceptible to *V. harveyi* infections

408 (Pujalte et al., 2003), which may account for the higher number of isolates obtained from this

409 species in this study. Our findings also suggest that strains of *V. harveyi* exhibit differential

410 behavior depending on the host, underscoring the complexity of host-pathogen interactions.

411 Further investigations are needed to elucidate the underlying mechanisms governing host

412 specificity and pathogenicity in this bacterium.

413 The genomic islands accounted for approximately 10% of the total genome in these strains, as

414 indicated in Table 3. The mean length of these GIs was estimated at approximately 16.4 kbps, and

415 the coding sequences (CDSs) within them encompassed a diverse array of genetic elements. These

416 elements included ABC transporters, transcription regulators, proteins associated with antibiotic

417 resistance and hydrogen peroxide resistance, toxin-antitoxin systems, metabolic enzymes, and

418 approximately 37% of hypothetical proteins. Notably, the presence of a natural competence-

419 inducing factor Tfox/Sxy family protein was annotated in the genomes, linking the ability to grow

420 on chitin with HGT (Meibom et al., 2005). Vibrios exhibit remarkable genetic adaptability,

421 characterized by genetic exchanges within and between species in the marine environment (Le

422 Roux and Blokesch, 2018; Lilburn et al., 2010). HGT plays a pivotal role in this adaptability,

423 allowing the exchange of genetic material and contributing to genetic diversity. It involves the

424 transfer of genes related to virulence and antibiotic resistance, often through prophage regions and

425 plasmids (Ruwandeepika et al., 2010). This genetic plasticity has enabled vibrios, such as *V.*

426 *harveyi*, to infect a broad spectrum of hosts, although rapid mutation rates have also influenced

427 their evolution (Thompson et al., 2004).

428 Moreover, each strain was found to possess a few prophage regions, with an average length of

429 approximately 19.7 kbps, as detailed in **Table 3**. Among these regions, the only intact prophage

430 was one of the strain Gal 1, comprising 63 CDSs (**Table S9**). Upon further analysis through a

431 BLAST search, it was revealed that this region exhibited significant similarity to chromosome two

432 of other *V. harveyi* strains isolated from fish (specific data not shown), and it shared common

433 syntenies with an induced prophage from a *V. alginolyticus* strain obtained from a crab farm,

434 designated as Vibrio phage vB_ValM-yong1 (**Figure 5**) (Qin et al., 2021). The annotations within

435 the intact prophage of Gal 1 included a hemolysin domain, *phrB*, a TonB-dependent receptor, and

436 several phage-related proteins. In *V. harveyi*, lysogenic conversion has been known to enhance

437 virulence, either by introducing new toxins or by regulating host virulence factors (Nawel et al.,

438 2022). The study of microbe-host interactions has expanded to encompass microbe-microbe-host

439 /microbe-phage-host interactions. These inter-species interactions reveal exchanges not only of

440 virulence factors through horizontal gene transfer but also of anti-phage systems (Blokesch, 2021;

441 McDonald et al., 2019). In *Vibrio*, 88% of defense systems lie in genomic islands (McDonald et

442 al., 2019). In the 21 genomes, 47% of the defense systems predicted lied inside GIs (**Table S10**).

443 Multiple defense systems can target the same phage or confer resistance against different phages,

444 the clustering of these antiviral systems into defense islands may not only facilitate horizontal

445 transfer but also enable simultaneous regulation, providing a synergistic effect (Tesson and

446 Bernheim, 2023).

447 Some genomes of this study exhibited elements of potential CRISPR/Cas type I, and Cas type I-E

448 systems identified (**Table S11**). Specifically, one CRISPR element of approximately 75 base pairs

449 was detected in FL 9.1 and Gal 90, while *cas3, cse2*, and *casB* genes were dispersed within the

450 contigs of eight additional strains. The CRISPR/Cas type I-E system, originally described in *Vibrio*

451 *cholerae*, has been identified in 10 *Vibrio* and 4 *Photobacterium* species (McDonald et al., 2019).

452 In *V. harveyi*, the system is often found within a GI alongside T3SS genes, often occupying a

453   single locus, although it may be absent in certain strains (McDonald et al., 2019; Parra et al., 2021).

454   It is important to note that having a complete set of *cas* genes is not the standard for the genus, as

455   CRISPR/Cas systems are not evenly distributed among vibrios. Orphan genes may represent

456   fragments of functional systems or serve alternative functions (Baliga et al., 2019).

457

4. Conclusions

459

460   The 21 *V. harveyi* strains demonstrate predominantly consistent sensitivity to aquaculture

461   antibiotics, alongside varied metabolic profiles across multiple traits and diverse outcomes in

462   virulence assays. Moreover, their pangenome exhibits an open structure characterized by

463   numerous mobile elements and a broad array of virulence genes. It is imperative to maintain

464   continuous whole-genome sequencing efforts to monitor and evaluate the evolutionary

465   trajectory of these bacteria concerning disease. The genetic and phenotypic pathogenic

466   properties observed in these strains underscore the likelihood of *V. harveyi* remaining a

467   significant concern for Greek aquaculture in the foreseeable future.

468

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476

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478

482

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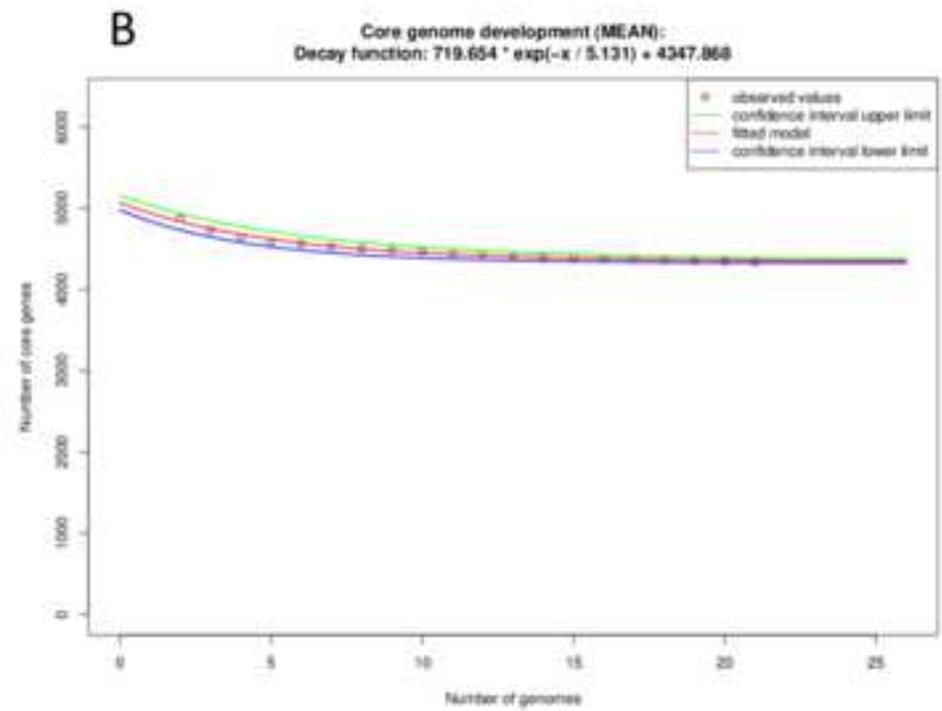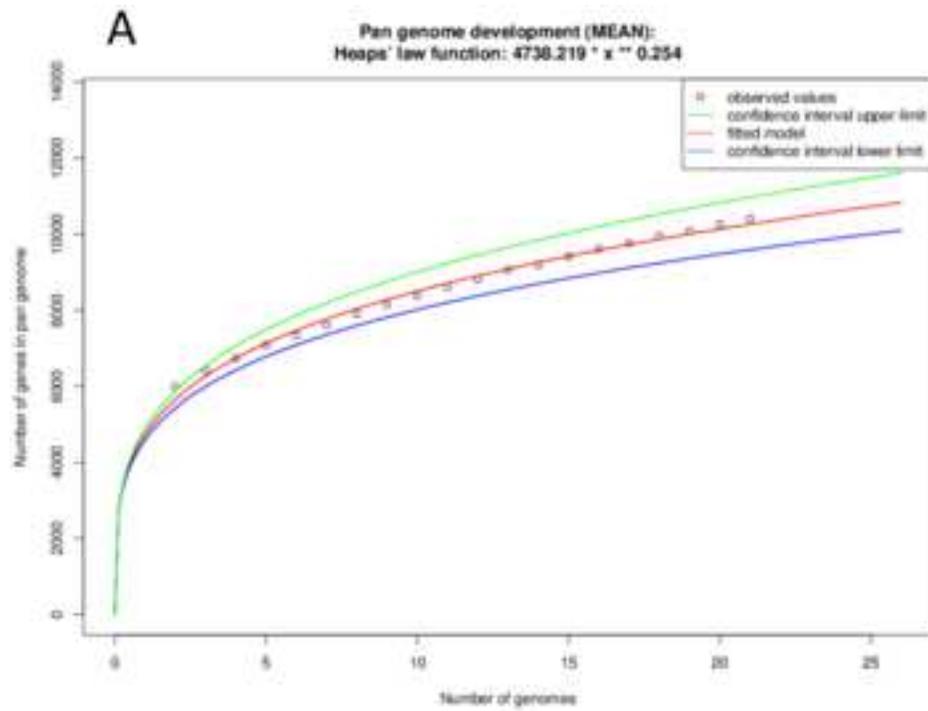804   the *Harveyi* clade. J Appl Microbiol 132, 167–176. https://doi.org/10.1111/jam.15227

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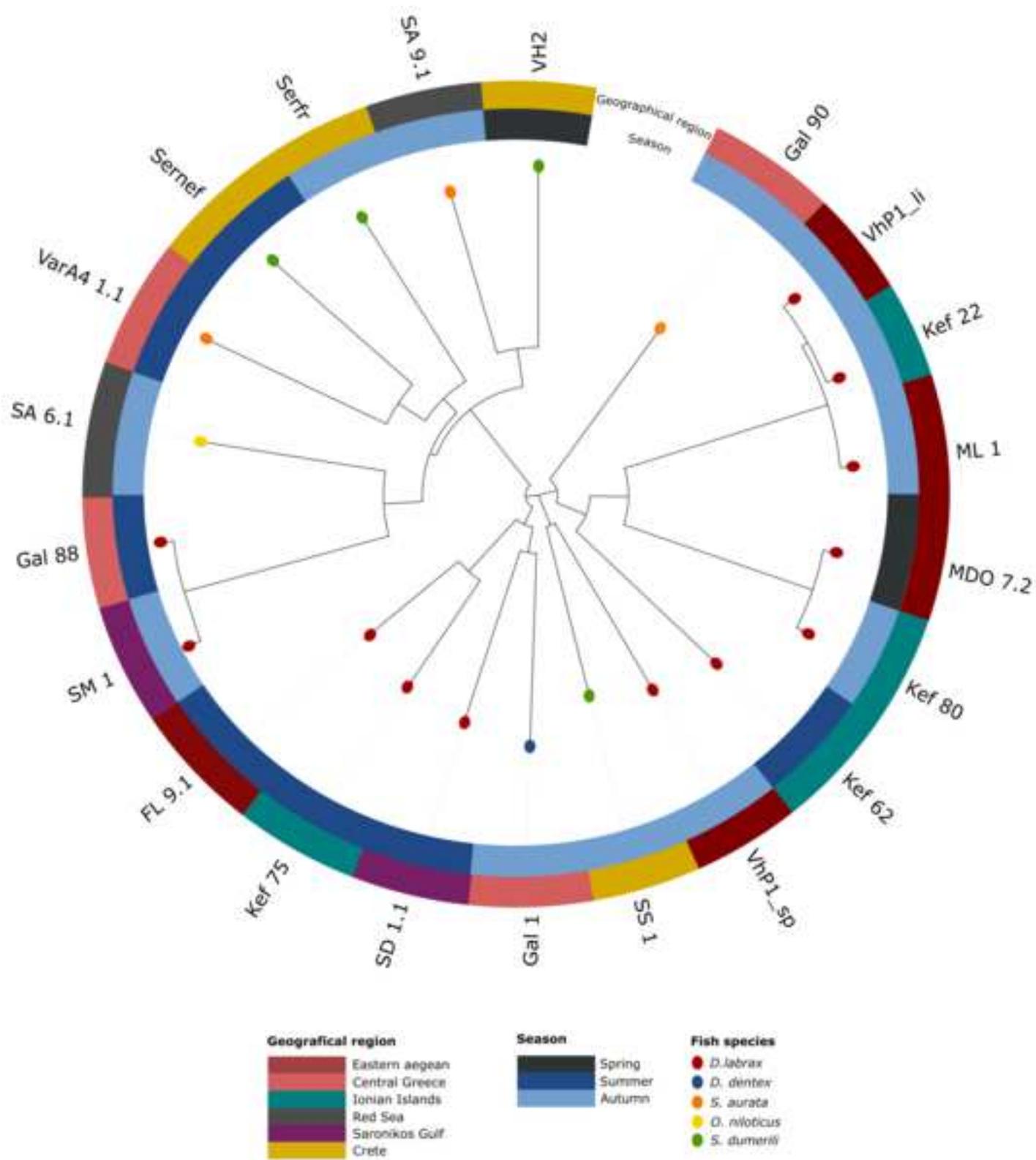
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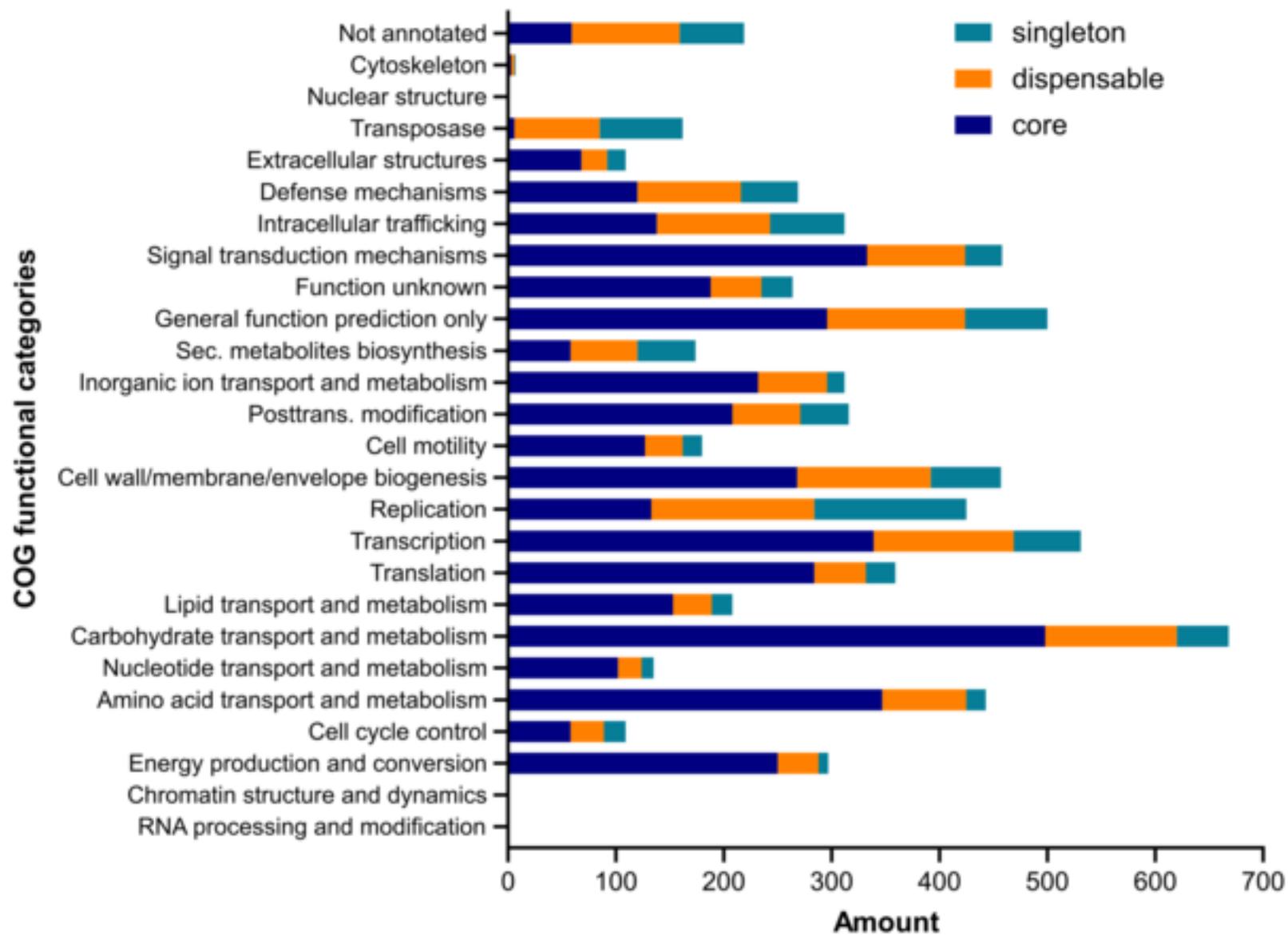
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812

813

figure 1

A Pan genome development (MEAN):
Heaps' law function: 4738.219 * x ** 0.254

B Core genome development (MEAN):
Decay function: 719.654 * exp(-x / 5.131) + 4347.868

figure 2

Click here to access/download;Figure;figure 2 plus.png ⬇

Geographical region

Season

Geografical region
- Eastern aegean
- Central Greece
- Ionian Islands
- Red Sea
- Saronikos Gulf
- Crete

Season
- Spring
- Summer
- Autumn

Fish species
- D.labrax
- D. dentex
- S. aurata
- O. niloticus
- S. dumerili

figure 3

Click here to access/download;Figure;figure 3.png ⬇

figure 4

Click here to access/download;Figure;figure 4.tif ⬇

Cumulative mortality (%)

figure 5

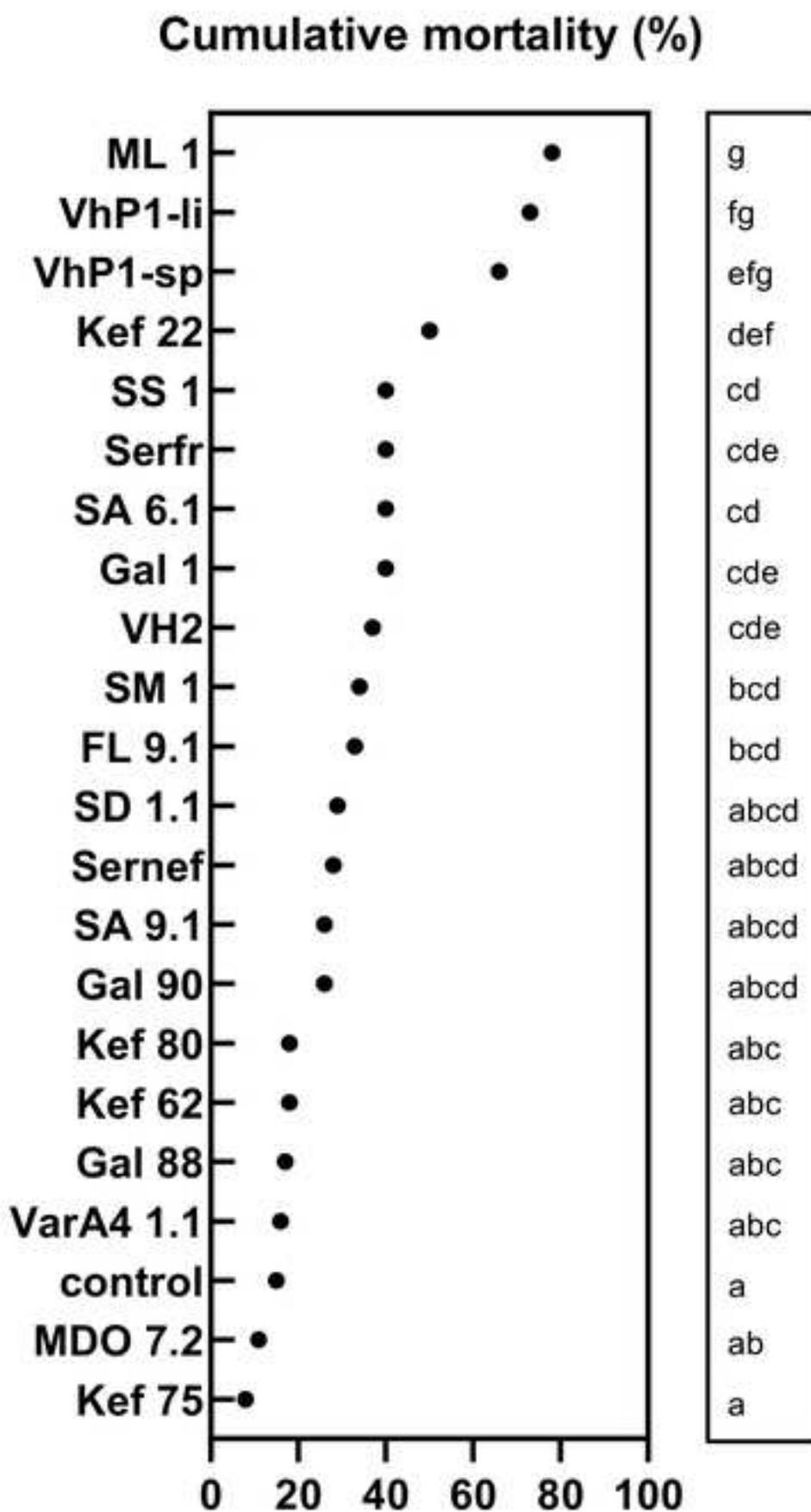Click here to access/download;figure;Figure 5.jpg

Figure 1. Curves showing the pangenome (A) and core genome (B) development for the 21 *V. harveyi* strains.

Figure 2. Phylogenetic tree of the 21 strains, constructed using the EDGAR platform based on core genes, with each genome consisting of 4,336 genes and a total of 1,411,553 amino acid residues. The outer ring represents the geographical origin of the strains, while the inner ring indicates the season of isolation. Host species are denoted by circles at the ends of the branches.

Figure 3. COG functional categories in the core, dispensable and singleton fractions of the pangenome.

Figure 4. Cumulative mortality percentages of seabream larvae at day 5 after virulence challenge with the 21 *V. harveyi* strains (n values available in Table S7). Following Log-rank (Mantel-Cox) analysis, letters indicate statistically significant difference of the pairwise comparisons (p<0.0001) between all strains.

Figure 5. The LCB 2 (44 kbps) of the Mauve genome alignment of Gal 1 prophage region (sequence 1) and Vibrio phage vB_ValM-yong1 NC_049477 (sequence 2).

Table 1

Table 1. Strain and genome information

	Host (tissue)	Location (year)	Size (Mbp)	GC (%)
FL 9.1	*D. labrax* (kidney)	North Aegean (2020)	6.03	44.77
Gal 1	*D. dentex* (kidney)	Central Greece (2019)	6.04	44.8
Gal 88	*D. labrax* (kidney)	Central Greece (2020)	5.98	44.87
Gal 90	*S. aurata* (kidney)	Central Greece (2020)	6.02	44.67
Kef 22	*D. labrax* (kidney)	Ionian Islands (2015)	6.09	44.71
Kef 62	*D. labrax* (spleen)	Ionian Islands (2020)	5.93	44.79
Kef 75	*D. labrax* (kidney)	Ionian Islands (2020)	5.82	44.86
Kef 80	*D. labrax* (brain)	Ionian Islands (2020)	6.02	44.82
MDO 7.2	*D. labrax* (kidney)	Eastern Aegean (2018)	6.04	44.83
ML 1	*D. labrax* (kidney)	Eastern Aegean (2020)	6.14	44.69
SA 6.1	*O. niloticus* (kidney)	Red Sea (2019)	5.96	44.83
SA 9.1	*S. aurata* (kidney)	Red Sea (2019)	5.93	44.89
SD 1.1	*D. labrax* (intestine)	Saronikos Gulf (2020)	5.71	44.93
Serfr	*S. dumerili* (fry)	Crete (2015)	5.85	44.86
Sernef	*S. dumerili* (kidney)	Crete (2017)	5.99	44.77

SM 1	*S. dumerili* (kidney)	Saronikos Gulf (2019)	6.39	44.6
SS 1	*S. dumerili* (kidney)	Crete (2021)	6.39	43.63
VarA4 1.1	*S. aurata* (brain)	Central Greece (2019)	5.86	44.78
VH2	*S. dumerili* (tail)	Crete (2015)	5.74	44.93
VhP1-li	*D. labrax* (liver)	Eastern Aegean (2015)	6.14	44.71
VhP1-sp	*D. labrax* (spleen)	Eastern Aegean (2015)	6.14	44.74

CDSs (total)	tRNA genes	rRNA genes	GenBank/RefSeq Accession (PGAP Accession)
5,523	67	3	JAIQZG000000000
5,499	74	3	JAIPUI000000000
5,439	66	4	JAIQXQ000000000
5,505	68	3	JAIULD000000000
5,587	69	3	JAIULE000000000
5,360	38	3	JAKGDN000000000
5,299	67	4	JAKGDM000000000
5,455	64	3	JAIULF000000000
5,461	64	3	JAIVBD000000000
5,625	65	3	JAIVBF000000000
5,461	61	3	JAJIRZ000000000
5,405	78	4	JAJISA000000000
5,191	69	3	JAKGDL000000000
5,354	54	4	JAKGDK000000000
5,571	78	3	JAIVBG000000000

5,998	76	4	JAIVBH000000000
5,898	98	4	JAKGDJ000000000
5,328	70	4	JAIWIV000000000
5,191	61	3	JAIVBE000000000
5,642	63	3	JAIWIX000000000
5,603	67	3	JAIWIW000000000

Table 2

Table 2. Antibiotic sensitivity of the 21 *V. harveyi* strains expressed in diameter of inhibition zones.

(mm)	OT30	OA2	SXT25	UB30	FFC30	AMP10
FL 9.1	28	16	20	26	27	0
Gal 1	23	18	22	26	28	0
Gal 88	22	21	23	28	30	0
Gal 90	20	15	21	25	29	0
Kef 22	23	17	18	24	26	0
Kef 62	25	18	25	28	28	0
Kef 75	0	15	0	25	30	0
Kef 80	23	16	20	22	28	0
MDO7.2	23	14	17	21	24	0
ML 1	24	17	21	25	28	0
SA 6.1	24	19	23	28	32	0
SA 9.1	24	16	21	24	30	0
SD 1.1	27	18	22	30	28	0
Serfr	25	16	22	27	32	0
Sernef	24	16	20	25	29	0
SM 1	29	25	29	34	35	0
SS 1	21	21	21	30	29	0
Var A41.1	23	16	22	25	35	0
VH2	22	16	21	23	28	0
VhP1-li	25	19	22	27	30	0
VhP1-sp	25	17	20	25	30	0

Table 3

Table 3. Genomic Islands and Prophage regions of the 21 V.harveyi strains.

	GIs/ WG (%)	Average Length (kbps)	Sum of GIs (bps)
FL 9.1	11	18.8	658,640
Gal 1	8	20.5	470,576
Gal 88	6	18.8	376,174
Gal 90	19	17.2	1,121,186
Kef 22	13	13.2	804,665
Kef 62	9	22	504,917
Kef 75	4	15.6	218,399
Kef 80	8	11.3	509,363
MDO 7.2	9	10.7	533,721
ML 1	13	12.9	812,241
SA 6.1	12	14.5	739,727
SA 9.1	9	12.2	510,603
SD 1.1	9	11.9	560,875
Serfr	6	20.3	345,422
Sernef	6	19.9	378,737
SM 1	13	16.2	827,530
SS 1	11	23.6	708,587
Var A4 1.1	11	19.1	668,102
VH2	8	10.5	440,817
VHP1-li	11	20.3	690,589
VHP1-sp	9	16.1	548,906

WG: whole genome, hp: hypothetical proteins, a: strain Gal 1 has also 1 intact 55.7 kbps prophage, b:

No. of hp in GIs/genes total (%)	No. of Incomplete Prophages	Average Length (kbps)
35	1	16.3
33	1[a]	12.9
37	3	8.2
40	1	30.8
36	2	18.4
35	1	30.9
33	2	31.2
30	1	30.8
33	1	12.9
34	1	27.5
38	4	18
40	1	14.4
45	3	9.5
37	4	8.2
38	2	19.1
53	2	26.2
49	2[b]	26.7
36	2	17.3
31	1	31.4
37	1	18.3
32	2	18.1

strain SS 1 has also 1 questionable 26.3 kbps prophage

Declaration of Interest Statement

Author Statement

The authors declare that this manuscript is original, has not been published previously, and is not currently being considered for publication elsewhere. Any sources of funding received for this research have been disclosed, and all conflicts of interest have been declared. The authors are accountable for the accuracy and integrity of the work presented herein.

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