Genomic variation among closely related \textit{Vibrio alginolyticus} strains is located on mobile genetic elements

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**Abstract**

**Background:** Species of the genus \textit{Vibrio}, one of the most diverse bacteria genera, have undergone niche adaptation followed by clonal expansion. Niche adaptation and ultimately the formation of ecotypes and speciation in this genus has been suggested to be mainly driven by horizontal gene transfer (HGT) through mobile genetic elements (MGEs). Our knowledge about the diversity and distribution of \textit{Vibrio} MGEs is heavily biased towards human pathogens and our understanding of the distribution of core genomic signatures and accessory genes encoded on MGEs within specific \textit{Vibrio} clades is still incomplete. We used nine different strains of the marine bacterium \textit{Vibrio alginolyticus} isolated from pipefish in the Kiel-Fjord to perform a multiscale-comparative genomic approach that allowed us to investigate [1] those genomic signatures that characterize a habitat-specific ecotype and [2] the source of genomic variation within this ecotype.

**Results:** We found that the nine isolates from the Kiel-Fjord have a closed-pangenome and did not differ based on core-genomic signatures. Unique genomic regions and a unique repertoire of MGEs within the Kiel-Fjord isolates suggest that the acquisition of gene-blocks by HGT played an important role in the evolution of this ecotype. Additionally, we found that ~ 90% of the genomic variation among the nine isolates is encoded on MGEs, which supports ongoing theory that accessory genes are predominately located on MGEs and shared by HGT. Lastly, we could show that these nine isolates share a unique virulence and resistance profile which clearly separates them from all other investigated \textit{V. alginolyticus} strains and suggests that these are habitat-specific genes, required for a successful colonization of the pipefish, the niche of this ecotype.

**Conclusion:** We conclude that all nine \textit{V. alginolyticus} strains from the Kiel-Fjord belong to a unique ecotype, which we named the Kiel-\textit{alginolyticus} ecotype. The low sequence variation of the core-genome in combination with the presence of MGE encoded relevant traits, as well as the presence of a suitable niche (here the pipefish), suggest, that this ecotype might have evolved from a clonal expansion following HGT driven niche-adaptation.

**Keywords:** Pangenome, Mega-plasmids, Mobile genetic elements, Horizontal gene transfer, Genomic islands, Niche-adaptation

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Background

The genomic variation within a bacterial species is reflected by accessory genes. Together with strain-specific singletons (genes found in only one strain) and the core-genomes that are shared by all strains of a species (such as housekeeping genes and genes required for essential cellular functions) the accessory elements form the pangenome [1]. Accessory genes are usually only found in a subset of genomes of a species and are attributed to habitat-specific functions, such as strain-specific virulence factors [2], antibiotic resistance [3], or niche-adaptation [4]. As such, accessory genes are often located on mobile genetic elements (MGEs), including plasmids, bacteriophages, transposons, integrative or conjugative elements (ICEs) and genomic islands (GIs) and are shared between species via horizontal gene transfer (HGT) [5]. Even though HGT between strains of the same species is more frequent, some MGEs have a broad host range enabling them to move genes across species boundaries thereby blurring the definition of a species [6].

Not only the size of pangenomes differs among species but also the relationship between the proportion of core-genes relative to the pangenome [7]. A large pangenome with a proportionally small core-genome and with high rates of HGT is considered open, whereas a small pangenome with a high proportion of core-genes and low HGT rates is considered to be closed [8]. For a wide range of bacteria, the pangenome size is positively correlated with the effective population size ($N_e$) [7]. Species with a high $N_e$ are likely to occupy different environmental niches where they experience a larger diversity of selection pressures, which will drive selection for increased genome diversity and ultimately lead to larger pangenomes [8]. However, niche adaptation in species with high $N_e$ can also select for distinct ecotypes (i.e. ecologically and evolutionary distinct subpopulations) and ultimately initiate a speciation process with a niche-specific pangenome [8]. It remains debated when ecotypes are sufficiently diverged to be considered different species [9].

An excellent model organism to study pan-core genomic signatures and niche adaptation resulting from HGT is the genus Vibrio. These bacteria constitute one of the most diverse bacterial genera with a large pangenome comprising more than 100 species from different clades, that occupy different ecological niches [10, 11], where they can exist as pathogenic, opportunistic, mutualistic or free-living forms [12]. The considerable large pangenome (> 26,500 genes) of the genus Vibrio, which is more than 50 times larger than the Vibrio specific core-genome [11], indicates a large reservoir of genomic diversity for this genus. In contrast, within a specific Vibrio clade, the pangenome to core-genome ratio decreases considerably. For instance, within the cholerae clade, the pangenome is only 4.5 times larger than the core-genome [11]. The relatively small cholerae-pangenome (6923 genes) compared to the entire Vibrio-pangenome, might reflect a specific set of genes, required to occupy the cholera-specific niche.

Niche adaptation and ultimately speciation in this genus has been suggested to be mainly driven by HGT [13]. Furthermore, MGEs, in particular filamentous phages, contribute significantly to the emergence of pathogenic vibrios from environmental populations [6] and the prophage repertoire of a given Vibrio species can account for a large fraction of the variation among strains [14, 15]. Our knowledge about the diversity and distribution of Vibrio MGEs is heavily biased towards human pathogens, e.g. V. cholerae and V. parahaemolyticus. While recent studies started to expand our knowledge on Vibrio-specific MGEs to other clades, such as V. anguillarum [16, 17], V. fischerii [18], or V. alginolyticus [19], our understanding about the distribution of core-genomic signatures and accessory genes encoded on MGEs within specific Vibrio clades is still incomplete. This might be because most of the available genomes are draft genomes comprising multiple contigs, whereas only a few complete genomes of environmental vibrios are available to date [19]. It is challenging to generate high-quality genomes of vibrios due to repetitive genomic regions such as rRNA operons present in the two chromosomes [8–12] and arrays of multiple integrated prophages. Especially multiple prophage arrays cannot be resolved using draft genome assemblies from short-read sequencing techniques. Due to the incomplete information concerning the genome organization stored in draft genomes, it is not possible to study genome dynamics of mobile genetic elements especially of prophages without long-read sequencing data [20].

The present study aimed to investigate how genome organization, as well as core-genomic signatures and accessory genes, differ within a group of closely related environmental Vibrio isolates. To do so, we performed a multi-scale comparative genomics approach using Vibrio alginolyticus as a model organism. V. alginolyticus, a ubiquitous marine opportunistic pathogen can cause mass mortalities in shellfish, shrimp, and fish, resulting in severe economic losses worldwide [3, 21, 22]. Vibrio pathogenicity is a complex interaction of abiotic and biotic factors, including high temperatures [23, 24] low salinities [25], host and bacterial genotypes [26, 27]. To date only six closed alginolyticus genomes are available, while 28 remain fragmented with 2 to 75 contigs (last accessed: December 2019). A detailed comparative genomic characterization of the alginolyticus species, including plasmid content, distribution of MGEs and virulence factors of this species is as yet missing. Here we analysed
core-and accessory-genomic signatures of \textit{V. alginolyticus} by comparing nine closely related \textit{V. alginolyticus} genomes, isolated from the pipefish \textit{Syngnathus typhle} in the Kiel-Fjord \cite{28}, (later named Kiel-alginolyticus ecotype) with less closely related \textit{V. alginolyticus} isolates (Table 1).

\textbf{Results and discussion}

\textbf{Genome features of Vibrio alginolyticus isolated from the Kiel-Fjord}

We sequenced the genomes of nine \textit{Vibrio alginolyticus} strains, previously isolated from the gut or gills of six different pipefish caught in the Kiel-Fjord (Germany, 54°7′57″N; 9°87′66″E) in May 2010 \cite{28}, using a combination of PacBio long- and Illumina short-read technology. The assembly resulted in eight closed genome sequences and one draft genome (strain K09K1), where both chromosomes have been assembled into a single contig due to multiple copies of an integrated filamentous phage. The replicon boundaries could not be resolved experimentally based on PCR thus strain K09K1 has been assigned a “permanent draft” status. All \textit{V. alginolyticus} genomes contain a \(\sim 3.47\) Mbp chromosome 1 and a \(\sim 1.88\) Mbp chromosome 2, with a %GC content of around 44% (Table 1), which is typical for the genus \textit{Vibrio} as well as for the species \textit{V. alginolyticus} \cite{29, 32}. We found extra-chromosomal replicons including plasmids and filamentous phages in seven isolates (Table 1).

\textbf{Species definition and phylogenetic relationship of the Kiel-Fjord isolates}

All nine strains isolated from the Kiel-Fjord share more than 98\% average nucleotide identity (ANI) with other \textit{alginolyticus} strains, suggesting that these strains belong, as previously suggested based on a multi-locus-sequencing-approach (MLSA) \cite{23}, to the species \textit{V. alginolyticus}. All \textit{alginolyticus} strains share \(\sim 92\%\) ANI with two other closely related \textit{Vibrio} species, i.e. \textit{V. diabolicus} and \textit{V. antiquarius} (Fig. 1), which is below the species threshold of ANI = 95\% \cite{33, 34}, indicating that \textit{V. alginolyticus} is a distinct species.

\textbf{The Vibrio alginolyticus pan/ core genome}

\textbf{Pangenome}

To compare the gene repertoire of the nine \textit{V. alginolyticus} strains from the Kiel-Fjord with the global \textit{V. alginolyticus} gene repertoire, we calculated their pangenome including the amount of core-and accessory genes as well as strain-specific singletons, i.e. genes unique to single strains \cite{35}. The analysis included in total 73,277 protein-sequences encoded on chromosomes and MGEs, including plasmids and extra-chromosomal phage replicons. Overall, we found that the size of the pangenome of \textit{V. alginolyticus} increased stronger (from 4997 to 8843 gene clusters) with the sequential addition of each new genome when we included all 15 strains from diverse habitats (Fig. 2a). In contrast, we only observed a slight increase in the pangenome (from 5044 to 5679 gene clusters) when we only included the nine \textit{V. alginolyticus} isolates from the Kiel-Fjord (Fig. 2b). An increase in the pangenome results from new strain-specific genes, found in each newly analyzed isolate. The much larger increase in the pangenome across all available \textit{V. alginolyticus} isolates relative to the increase in the pangenome of Kiel-Fjord isolates suggests that the genomic diversity of \textit{V. alginolyticus} within the Kiel-Fjord is very limited. However, the species \textit{V. alginolyticus} per se has a vast reservoir of genomic diversity.

We extrapolated both, the within-Kiel and the global \textit{V. alginolyticus} pangenome by fitting a least-squares curve based on Heaps’ Law \cite{1} and found that the within-Kiel pangenome is closed (\(\alpha = 1.12\)), whereas the global \textit{V. alginolyticus} pangenome is open (\(\alpha = 0.58\)). This means, that within the global \textit{V. alginolyticus} pangenome each newly sequenced \textit{V. alginolyticus} isolate will reveal a unique set of genes, irrespective of the number of strains included in the present analysis. This open \textit{V. alginolyticus} pangenome reflects the diversity in habitats in which this species exists. These distinct environments probably require different adaptations and/or promote high levels of HGT. In contrast, a closed pangenome, as has been detected for the within-Kiel \textit{V. alginolyticus} pangenome, suggests that the number of genes that will be obtained from any newly sequenced isolate will converge to zero. Here, we assume, that at least the sequenced pipefish associated \textit{V. alginolyticus} strains within the Kiel-Fjord contain the major part of the gene equipment that is requested to adapt to their habitat. Thus the genomes can be expected to be highly similar, potentially resulting from niche adaptation and strong selection thereof. Strong positive selection of such adaptive genes might have led to a clonal expansion of the Kiel-alginolyticus ecotype. Indeed, we found no sequence divergence based on core-genomic signature among all nine isolates from Kiel (Fig. 2c), indicating a clonal expansion of this ecotype. It remains to be investigated, whether free-living \textit{V. alginolyticus} strains or isolates from other eukaryotic hosts from the Kiel-Fjord share the same gene-pool or are more divergent from the pipefish-associated strains.

\textbf{Core-genome and singletons}

We observed a stronger decrease in the core-genome when we included all \textit{V. alginolyticus} isolates as opposed to when we performed the analysis only within the Kiel-alginolyticus ecotype. Comparative analyses between all 15 \textit{V. alginolyticus} strains and the Kiel-alginolyticus ecotype only, revealed that the core genome (4708 gene
| Strain          | GC%  | Replicon      | Size [bp] | CDS  | Ref       | Genbank    |
|-----------------|------|---------------|----------|------|-----------|------------|
| Kiel-Fjord Vibrio alginolyticus genomes |      |               |          |      |           |            |
| K01M1           | 44.6 | Chromosome 1  | 3,468,303| 3206 | This study| CP017889.1 |
|                 |      | Chromosome 2  | 1,883,748| 1668 | This study| CP017890.1 |
|                 |      | pL9064        | 9064     | 8    | This study| CP028135.1 |
| K04M1           | 44.31| chromosome 1 | 3,473,127| 3213 | This study| CP017891.1 |
|                 |      | chromosome 2 | 1,870,775| 1660 | This study| CP017892.1 |
|                 |      | pL19          | 19,690   | 28   | This study| CP017893.1 |
|                 |      | pL280         | 280,614  | 305  | This study| CP017894.1 |
|                 |      | vK04M1*       | 70,79    | 11   | This study| CP017895.1 |
| K04M3           | 44.31| chromosome 1 | 3,476,174| 3219 | This study| CP017896.1 |
|                 |      | chromosome 2 | 1,903,830| 1708 | This study| CP017897.1 |
|                 |      | pL294         | 294,086  | 325  | This study| CP017898.1 |
| K04M5           | 44.31| chromosome 1 | 3,470,916| 3211 | This study| CP017899.1 |
|                 |      | chromosome 2 | 1,900,618| 1688 | This study| CP017900.1 |
|                 |      | pL294         | 294,721  | 320  | This study| CP017901.1 |
| K05K4           | 44.34| chromosome 1 | 3,473,579| 3218 | This study| CP017902.1 |
|                 |      | chromosome 2 | 1,875,554| 1670 | This study| CP017903.1 |
|                 |      | pL289         | 289,065  | 315  | This study| CP017904.1 |
|                 |      | vK05K4_1*     | 21,012   | 34   | This study| CP017905.1 |
|                 |      | vK05K4_2*     | 13,327   | 23   | This study| CP017906.1 |
| K06K5           | 44.31| Chromosome 1  | 3,471,297| 3213 | This study| CP017907.1 |
|                 |      | Chromosome 2  | 1,879,729| 1662 | This study| CP017908.1 |
|                 |      | pL29          | 294,688  | 20   | This study| CP017909.1 |
|                 |      | pL291         | 291,285  | 322  | This study| CP017910.1 |
| K08M3           | 44.32| Chromosome 1  | 3,468,139| 3214 | This study| CP017913.1 |
|                 |      | Chromosome 2  | 1,886,577| 1675 | This study| CP017914.1 |
|                 |      | pL300         | 300,425  | 331  | This study| CP017915.1 |
| K09K1           | 44.61| Chromosome 1  | 1,897,210| 3209 | This study| CP017918.1 |
|                 |      | Chromosome 2  | 3,465,619| 1704 | This study| CP017919.1 |
| K10K4           | 44.6 | Chromosome 1  | 3,494,647| 3231 | This study| CP017911.1 |
|                 |      | Chromosome 2  | 1,894,531| 1682 | This study| CP017912.1 |
| all other closed V. alginolyticus genomes |      |               |          |      |           |            |
| ATCC 33787      | 44.48| Chromosome 1  | 3,362,673| 3190 | Wang et al. 2016 [29] | CP013484.1 |
|                 |      | Chromosome 2  | 1,851,538| 1674 | Wang et al. 2016 [29] | CP013485.1 |
|                 |      | pMBL128       | 128,112  | 144  | Wang et al. 2016 [29] | CP013486.1 |
|                 |      | pMBL287       | 286,750  | 301  | Wang et al. 2016 [29] | CP013487.1 |
|                 |      | pMBL96        | 95,866   | 109  | Wang et al. 2016 [29] | CP013488.1 |
| ZJ-T            | 44.67| Chromosome 1  | 3,535,128| 3301 | Deng et al. 2016 [30] | CP016224.1 |
|                 |      | Chromosome 2  | 1,870,966| 1657 | Deng et al. 2016 [30] | CP016225.1 |
| ATCC 17749      | 44.7 | Chromosome 1  | 3,334,467| 3128 | Liu et al. 2015 [31] | CP006718.1 |
|                 |      | Chromosome 2  | 1,812,170| 1640 | Liu et al. 2015 [31] | CP006719.1 |
| FDAARGOS_108    | 44.15| Chromosome 1  | 3,418,310| 3047 | Hoffmann, M (unpublished) | CP014053 |
|                 |      | Chromosome 2  | 1,810,594| 1587 | Hoffmann, M (unpublished) | CP014054.1 |
|                 |      | unnamed plasmid | 217,123 | 231  | Hoffmann, M (unpublished) | CP014052 |
| FDAARGOS_110    | 44.57| Chromosome 1  | 3,434,696| 3156 | Hoffmann, M (unpublished) | CP014040.1 |
|                 |      | Chromosome 2  | 1,833,981| 1622 | Hoffmann, M (unpublished) | CP014041.1 |
| FDAARGOS_114    | 44.68| Chromosome 1  | 3,372,141| 3054 | Hoffmann, M (unpublished) | CP014045.1 |
|                 |      | Chromosome 2  | 1,806,127| 1593 | Hoffmann, M (unpublished) | CP014044.1 |
clusters) is four times larger than the accessory genome (971 gene clusters) when we only included the nine Kiel strains. However, when we extended the analysis and included all 15 \textit{V. alginolyticus}, we found that the core genome (3876 gene clusters) becomes smaller than the accessory gene pool (4967 gene clusters). In other words, within the Kiel-Fjord, different \textit{V. alginolyticus} isolates have a large core-genome (83\% of the pangenome) with a limited accessory gene pool (17\% of the pangenome).

In contrast, despite all being members of the same species, the global accessory gene-pool is highly variable (56\% of the pangenome) and the \textit{V. alginolyticus} core-genome constitutes only 44\% of the pangenome.

\textbf{Habitat specific chromosomal regions}

The acquisition of entire gene-blocks (genomic islands, plasmids, prophages) by HGT can rapidly alter the lifestyle of a bacterium in quantum leaps \cite{36}. This

\textbf{Fig. 1} Average nucleotide identity (ANI) percentage analysis of closed Vibrio genomes. ANI analysis based on MUMmer alignment of the genome sequences was performed and visualized using PYANI. ANI-values ranging from 0 to 70\% no hits, 70–95\% ANI colored in blue and higher than 95\% ANI in red) All true \textit{V. alginolyticus} (including the nine strains from the present study) can be separated from \textit{V. diabolicus} and \textit{V. antiquaries} (small red square, bottom left).
mechanism seems to be particularly important for bacterial adaptation to new ecological niches but also for how bacteria diverge from each other, forming ecotypes and ultimately new species [37]. Genomic islands, which encode specific functions allowing for niche adaptation and maybe even speciation events are common within the genus *Vibrio* [38]. For the *Kiel-alginolyticus* ecotype, we could identify 19 chromosomal genomic regions (GRs) of which most are unique to the strains isolated from the Kiel-Fjord (Fig. 3, Table S2). Overall, these 19 GRs encode a total of 487 genes out of which 305 have only been found within the *Kiel-alginolyticus* ecotype. Out of these 19 GRs five could be assigned to integrated prophages. GR 13 and GR 14 correspond to *Vibrio* phage VALGΦ2/2b and *Vibrio* phage VALGΦ6, which are unique for the isolates from the Kiel-Fjord and have not been found elsewhere [19]. GR 3 corresponds to *Vibrio* phage VALGΦ1, which has so far only been detected in *V. alginolyticus* ATCC33787, with a relatively low query cover of 57%, thus making it a unique region for Kiel. In contrast GR 5, which corresponds to the filamentous phage *Vibrio* phage VALGΦ8 on chromosome 1, is absent in most Kiel strains, except K10K4 and K05K4, but present in some non-Kiel strains, such as ATCC17749 and FDAARGOS_114, suggesting that GR 5 is not unique for the Kiel isolates. Similarly, GR 15, which corresponds to a multiphage-cassette consisting of multiple repeats of a combination of the two filamentous phages *Vibrio* phage VALGΦ6 and *Vibrio* phage VALGΦ8 [19], is absent in most Kiel strains, except K04M3 and K04M5 but in parts present in ATCC17749 and thus not unique for the Kiel system. Of the remaining 14 GRs, which do not correspond to integrated phages, we could identify four genomic islands: GR 2, GR 6, GR 7 and GR 8 (> 10 kb, presence of integrase/ transposase, different GC content). According to functional COG categories, these islands contain a variety of proteins, most of them involved in replication, recombination, and repair [L] and transcription [K] (see Table S3 for functional annotation of all GRs). The high prevalence of those maintenance genes is however not surprising and might result from an identification bias, as they are usually better annotated for MGEs than accessory genes, which would provide a selective advantage to the host. As such, many proteins were classified as phage integrases, transposases or other proteins,
involved with viral integration into host DNA or DNA repair, suggesting that HGT might have played an important role during the acquisition of these GIs. GR 6 encodes the multi-drug transporter subunit MdtN and two other loci which were assigned to COG category [V] and predicted to be involved in defense mechanisms. This suggests that GR 6 might play a role in resistance. None of the other genomic islands could be identified as pathogenicity−/ metabolic- or resistance island. All other GRs were either smaller than 10 kb or did not contain integrases or transposases and are thus referred to as genomic regions instead of genomic island. Out of these GRs, GR 16 encoded three proteins associated with the type VI secretion system and GR 18 encoded a beta-lactamase protein suggesting an adaptive role in virulence and resistance, respectively. The acquisition of these unique GRs might have allowed the Kiel-alginolyticus ecotype to invade a new niche, which was then followed by clonal expansion of this ecotype. Clonal expansion has been observed for several pathogens, in particular within the genus *Vibrio*. The best characterized example of clonal expansion upon acquisition of virulence genes is *V. cholerae*. But also other *Vibrio* pathogens, for instance *V. parahaemolyticus* have been shown to experience similar evolutionary dynamics (for a review see [39]).

**Genomic differences within Kiel-alginolyticus ecotype**

To investigate genomic differences between the nine strains from the Kiel-Fjord, we focused on all those gene-clusters from the Roary analysis, which could only be found within the Kiel-alginolyticus ecotype but were absent in all non-Kiel isolates. We found that all Kiel-specific core-genes (*n* = 412) were located exclusively on one of the two chromosomes (Fig. 4). In contrast, the majority of the Kiel-specific accessory gene clusters (89%) were encoded on mobile genetic elements, in particular plasmids. These results support ongoing theory that accessory genes are predominately located on MGEs and shared by horizontal gene transfer (HGT) [5]. We found 490 gene clusters with no orthologous in any other Kiel strain, i.e. singletons out of which 40% were located on MGEs, in particular extrachromosomal replicating elements (170 on plasmids and 27 on extrachromosomal phages) and 60% (*n* = 293) were chromosomal (Fig. 4). All Kiel-specific *alginolyticus* gene clusters were further assigned to putative functional categories using the Clusters of Orthologous Groups of Proteins (COG) database [40] (Table S4). Even though a large fraction of the gene clusters could either not be assigned to a COG or was poorly characterized, we found differences in the relative distribution of super-functional COGs between core- and accessory genomes and singletons: The
majority of the singletons (37%) was predicted to be dedicated to cellular processes/signaling, while relatively small proportions of gene clusters (10 and 16%) belong to information storage-processing and metabolism. In contrast, the largest proportion of gene-clusters encoded on core-genes was predicted to belong to information storage-processing (24%), and only 16 and 13% of gene-clusters encoded on core genes belong to cellular processes/signaling and metabolism. Among the gene-clusters encoded on the accessory genome 22% could be assigned to information storage-processing as well as to cellular processes/signaling and only 6% to the metabolism (Fig. 4). Within the accessory genome most of the genes are involved in replication, recombination, and repair (COG [L], Table S4). These include mainly proteins involved in HGT, such as transposases, integrases, transferases, recombinases as well as proteins involved in immunity, such as CRISPR associated helicase Cas3 and restriction modification methylases. This extensive representation of proteins involved in HGT on the accessory genome suggests that HGT was potentially one of the driving evolutionary mechanisms underlying the diversification of the nine V. alginolyticus strains from the Kiel-Fjord.

The majority of the accessory gene-pool within the Kiel-alginolyticus ecotype is located on plasmids (Fig. 4). We could identify four different plasmids from all nine Kiel V. alginolyticus isolates. Three plasmid types isolated from the Kiel strains were unique for specific strains with a size of 0.9 to 2.9 kbp (Table 1). The fourth plasmid type was characterized as a mega-plasmid (Fig. 5a), which ranged between 280 and 300 kbp in size, and was present in six out of the nine strains (Fig. 5b). The six mega-plasmids share 296 core-genes, encode 129 accessory genes and between 5 and 26 singletons per plasmid (Fig. 5b). Together with the three small plasmids, plasmid-encoded singletons make up 34.5% of all 486 Kiel-specific singletons. The majority of the plasmid-specific singletons comprise hypothetical proteins. The remaining singletons include AAA proteins (K04M1_pL280), PFAM phosphoadenosine phosphosulfate reductase and ATPases (K04M5_pL294), spore germination and type IV secretion system (K5K4_pL289), endonuclease and site-specific methyl-transferase, potentially forming a restriction-modification system (K06K5_pL291), and DNA polymerase (K08M3_pL300). V. alginolyticus ATCC 33787 contains as well three plasmids including plasmid pMBL287, which is similar in size as the Kiel mega plasmids [29]. However, a comparison of ATCC 33787 plasmids revealed no sequence similarity to any of the plasmids from the Kiel strains.

Only 6.5% of the 870 Kiel-specific accessory genes and singletons are located on extrachromosomal phages. The within-Kiel variation caused by these phages can be explained by the absence of extrachromosomal replicons of Vibrio phage VALGΦ8 in all but two strains (K04M1 and K05K4). However, four strains (K04M3, K04M5, K05K4, and K10K4) had an intra-chromosomal version

Fig. 4 Genomic variation among all nine Kiel V. alginolyticus strains. Left: Kiel-specific core genes (shared by all nine isolates), middle: Kiel-specific accessory genes (shared by two or more isolates, but not by all), and right: Kiel-specific singletons (unique for one isolate) distributed across replicons (CR1: chromosome 1, CR2: chromosome 2, Phages: extrachromosomal phages, Plasmids) and assigned to Clusters of Orthologous Groups of Proteins (COGs): black: information storage and processing; orange: cellular processes and signaling; blue: metabolism; green: poorly characterized; white: not assignable
of this phage, while strains K01M1, K06K5 and K08M3 did neither contain an intra- nor an extrachromosomal version of *Vibrio* phage VALGΦ8 (for a detailed analysis of Kiel vibriophages see [19]). Parts of *Vibrio* phage VALGΦ8 could be identified on both chromosomes of ATCC 17749T, and on chromosome 1 of FDAARGOS_114 but not on any other non-Kiel *Vibrio*. Genome analyses of *Vibrio* phage VALGΦ8 revealed no significant virulence-associated genes nor any other genes that could be associated with a habitat-specific adaptation [19].

**Virulence and resistance of the Kiel-Fjord V. alginolyticus isolates**

We found an identical virulence and resistance profile among the nine *V. alginolyticus* isolates from the Kiel-Fjord. The Kiel-Fjord isolates encode a total number of 17 homologous resistance genes out of which the majority (*n* = 13) is located on chromosome 1 and four resistance genes are encoded on chromosome 2 (Fig. 6, Table S5). In comparison with other *V. alginolyticus* strains (~25–130 resistance genes on Chromosome 1 and 11–12 resistance genes on Chromosome 2), isolates from the Kiel-Fjord have significantly less resistance genes (*t*-test: *t* = 3.51, *p* = 0.007; Fig. 6) and are missing in particular genes conferring resistance to tetracycline, aminoglycosides, and quinolones.

In contrast to non-pathogenic strains, such as *V. fischerii*, which is a mutualist containing 112 virulence genes, the Kiel-Fjord isolates encode more virulence genes (150 virulence genes, exception strain K05K4, 149 virulence genes). This number is lower than what has been found for human pathogenic *vibrios*, such as *V. cholerae* (~165 virulence genes) and *V. parahaemolyticus* (162 virulence genes), but similar to what has been found in other *V. alginolyticus* strains, except (strain FDAARGOS_114: 165 virulence genes). Unique for the Kiel-Fjord isolates is the absence of genes involved in Vibriobactin biosynthesis, which are present in almost all other *V. alginolyticus* strains. Similarly, In contrast to other *V. alginolyticus* isolates, the strains from the Kiel-Fjord miss the gene flaC, which is involved in the regulation of stringent-response-induced toxin production [41] and vscP, a gene involved in the type III secretion system. In contrast to other *V. alginolyticus* strains, the Kiel-*alginolyticus* eco-type encodes the type IV secretion system effectors, the phytotoxin coronatine and the thermolabile hemolysin (tlh), both toxins, which could not be found in any other *V. alginolyticus* strain. This unique virulence profile of the Kiel-Fjord isolates separates them from other *Vibrio* species including other *V. alginolyticus* isolates abut also another strain from the Kiel-Fjord: *V. typhli*, K08M4 (Chibani C, Hertel R, Goehlich H, Mertens V, Bunk B, Overmann J, et al. Complete Genome Sequence of Vibrio *typhli* K08M4, a fish pathogen isolated from the Kiel Fjord. in preparation) (Fig. 6). This clear separation was further supported by a hierarchical cluster analysis (Fig. 6), which indicates, that only based on the presence/absence of virulence genes the Kiel-*alginolyticus* eco-type can be distinguished from all other *V. alginolyticus* strains, sequenced to date. The unique resistance and virulence profile within the Kiel-Fjord *V. alginolyticus* isolates might be a further indication for niche-adaptation followed by clonal expansion of this eco-type.
Conclusions

By using a multi-scale comparative genomic approach, we conclude that the nine *V. alginolyticus* strains isolated from pipefish in the Kiel-Fjord belong to a unique ecotype, which evolved from a clonal expansion following niche adaptation. We came to this conclusion based on the following observations. First, the nine strains from the Kiel-Fjord have a closed-pangenome reflecting low genomic diversity within its niche. Second, even though these strains have been isolated from different eukaryotic host species, they did not differ based on core-genomic signatures which has been previously suggested based on MLSA [23]. Third, niche adaptation and ultimately speciation in the genus *Vibrio* have been suggested to be mainly driven by HGT [13]. Likewise, unique GIs and a unique repertoire of MGEs within the Kiel-Fjord isolates (including unique plasmids and prophages) support ongoing theory that the acquisition of gene-blocks by HGT, one of the most important mechanisms driving the evolution of niche adaptation and clonal expansion also played an important role in the evolution of the Kiel-*alginolyticus* ecotype. Lastly, the unique virulence and resistance profile which clearly separates the Kiel-Fjord isolates from all other investigated *V. alginolyticus* strains, suggests, that these are essential
genes, required for a successful colonization of the pipefish, the niche of the Kiel-*V. alginolyticus* ecotype. To ultimately verify this theory, colonization experiments with knock-out mutants lacking the Kiel-specific virulence genes, or alternatively non virulent *Vibrio* strains equipped with the Kiel-specific virulence genes, would be required.

We are aware that our conclusion is limited by the low number of non-Kiel *V. alginolyticus* strains available included in the comparative genomic analyses. We could have included draft genomes, to increase the number of sequences available for comparison. However, due to the incomplete information especially for MGEs stored in draft genomes, the number of core-genomes drops considerably by approximately 50% compared to when only complete genomes are included in the analysis [20]. In favor of a more accurate analysis, we thus decided to only include complete genomes.

The present study extends our current knowledge of pan-and core-genomic signatures in the genus *Vibrio* which is currently heavily biased towards human pathogens, such as *V. cholerae* and *V. parahaemolyticus*. Based on the present results we suggest that also non-human pathogens might experience similar evolutionary dynamics, once relevant pathogenic traits (in the present case: *Vibrio* phage VALGΦ6 [19], plus three unique virulence genes (Fig. 6)) have been acquired. The availability of a suitable niche, in the present case, presumably the pipefish, might have led to niche adaptation and clonal expansion of the Kiel-*alginolyticus* ecotype, clearly separating it from other *V. alginolyticus* species.

**Methods**

**Bacterial genome data**

Using a combination of PacBio and Illumina sequencing, we generated eight closed genomes and one permanent draft genome of nine *Vibrio* strains isolated from different pipefish in the Kiel-Fjord [28] and previously characterized as *V. alginolyticus* based on multi-locus-sequence typing [23]. For sequencing and annotation information please see [23]. We additionally sequenced prophages induced with mitomycin C from each strain, for details see [19]. We compared all replicons from the nine *V. alginolyticus* strains to 10 closed *Vibrio* genome sequences downloaded from the NCBI nucleotide database; date of accession 1.12.2019 (Table S1).

**Comparative genome analysis**

**Average nucleotide identity**

The average similarity between the nine *V. alginolyticus* genomes from the present study, six other complete *V. alginolyticus* genomes as well as four other closely related vibrios (i.e. three *V. diabolicus* and one *V. antiquarius*) was measured as the average nucleotide identity (ANI) of all 19 *Vibrio* replicons (concatenated Chromosome 1 and Chromosome 2) using the ANIm mode from the PyANI python module [42] based on MUMmer (https://github.com/widdowquinn/pyani). Briefly, nucleotide sequences were extracted from each GenBank file using Biopython (https://biopython.org/) and subsequently used as input for PyANI for genome sequence alignment.

**Pan-Core genome prediction**

To determine the total gene-pool of *V. alginolyticus* within the Kiel-Fjord as well as the global gene repertoire of *V. alginolyticus*, we performed two pan-core genome analyses. In the first analysis, we only included the nine strains from the Kiel-Fjord. In the second analysis, we included all fully closed *V. alginolyticus* genomes available from NCBI (accession date 10.10. 2019) which were isolated from various regions (Table 1), in addition to the nine Kiel strains. Genbank files of all closed *V. alginolyticus* isolates were converted to GFF3 files and a nucleotide alignment was generated using Roary 3.8.0 [43] with standard settings (minimum BLASTP identity of 90% with paralogue splitting). We used concatenated files for each isolate including both chromosomes and if existing, mobile genetic elements (plasmids and episomal phages). To estimate whether we have an open or a closed pangenome we used Heaps’ Law: \[ \eta = \kappa \cdot N^{-\alpha} \] [1], implemented in the R package ‘micropan’ [44], where \( \eta \) is the expected number of genes for a given number of Genomes (N), and \( \kappa \) and \( \alpha \) are constants to fit the specific curve. The exponent \( \alpha \) is an indicator whether the pangenome is open (\( \alpha < 1 \)) or closed (\( \alpha > 1 \)). Lastly, a functional characterization of all protein-coding sequences was performed using the eggnog-mapper v2 database v5.0 [45, 46]. We subsequently determined the COG category (Cluster of Orthologous Groups of Proteins [40]) for Kiel-specific core- and accessory genes as well as singletons identified by the pan-core analysis based on the Roary clustering input table.

**Phylogenetic analysis**

To further infer the phylogenetic relationship among all *V. alginolyticus* isolates we generated a phylogenetic tree based on the core-genome alignment for all 15 strains obtained from the Roary analysis (length for all strains ~ 3.84 Mbp, which corresponds to approximately 72% of the average size of the input genomes). To do so, we used the Bayesian Markov chain Monte Carlo (MCMC) method as implemented in MrBayes version 3.2.5 [47, 48]. The TN93 [49] model plus invariant sites (TN93 + I), as suggested by the Akaike information criterion (AIC) given by jModelTest [50], was used as a statistical model for nucleotide substitution. The MCMC process was repeated for \( 10^6 \) generations and sampled every 5000 generations. The first 2000 trees were deleted as
burn-in processes and the consensus tree was constructed from the remaining trees. Convergence was assured via the standard deviation of split frequencies (< 0.01) and the potential scale reduction factor (PSRF~ 1). The resulting phylogenetic tree and associated posterior probabilities were illustrated using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

**Supplementary information**

**Additional file 1**: Table S1. Vibrio genomes accessed from NCBI 3.12.2019 used for PyANI analysis.

**Additional file 2**: Table S2. Genomic regions (GRs) unique for the nine *V. alginolyticus* strains from the Kiel-Fjord. For chromosome 1 (left) we used K10K4 as a reference, as this strain has more integrated prophages than the other Kiel isolates. For the same reason, we used strain K04M3 as a reference for chromosome 2. For a detailed comparison of GRs among all nine strains, see Fig. 3 main manuscript.

**Additional file 3**: Table S3. Functional characterization of all protein-coding sequences found on the Genomic Regions (using the eggNOG-mapper v2 software) with the resulting COG assignments. Similar to Table S2 we used strain K10K4 as reference for chromosome 1 and strain K04M3 as reference for chromosome 2. The column Kiel Specific indicates whether the GR is unique for the Kiel *alginolyticus* ecotype.

**Additional file 4**: Table S4. Functional annotation of Kiel specific gene clusters (core genome [C], accessory genes [A], singletons [S]), i.e. gene cluster which was only found within the Kiel *alginolyticus* ecotype with the resulting COG assignments.

**Additional file 5**: Table S5. Resistance genes found using IslandViewer for all nine *V. alginolyticus* strains from the Kiel-Fjord. Even though this table shows the locus tags for strain K04M3 only, all other strains have been found to contain the exact same resistance genes.

**Abbreviations**

GIs: Genomic islands; GRs: Genomic regions; ANI: Average nucleotide identity; MLSA: Multi-locus-sequencing-approach; COG: Clusters of Orthologous Groups of Proteins; MGEs: Mobile genetic elements; HGT: Horizontal gene transfer.

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**Authors’ contributions**

CCW, HL and OR designed the study. CMC and CCW analyzed the data. CCW wrote the manuscript. All authors commented on the manuscript and approved the final version.

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**Availability of data and materials**

The genomic sequences of the nine Kiel *V. alginolyticus* strains analyzed during the current study are available at GenBank (CP017889-CP017919).

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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