Microb Ecol (2010) 59:1–13
DOI 10.1007/s00248-009-9596-7

MINIREVIEWS

On the Origins of a *Vibrio* Species

Tammi Vesth · Trudy M. Wassenaar · Peter F. Hallin · Lars Snipen · Karin Lagesen · David W. Ussery

Received: 3 July 2009 / Accepted: 17 September 2009 / Published online: 15 October 2009

Abstract Thirty-two genome sequences of various *Vibrionaceae* members are compared, with emphasis on what makes *V. cholerae* unique. As few as 1,000 gene families are conserved across all the *Vibrionaceae* genomes analysed; this fraction roughly doubles for gene families conserved within the species *V. cholerae*. Of these, approximately 200 gene families that cluster on various locations of the genome are not found in other sequenced *Vibrionaceae*; these are possibly unique to the *V. cholerae* species. By comparing gene family content of the analysed genomes, the relatedness to a particular species is identified for two unspeciated genomes. Conversely, two genomes presumably belonging to the same species have suspiciously dissimilar gene family content. We are able to identify a number of genes that are conserved in, and unique to, *V. cholerae*. Some of these genes may be crucial to the niche adaptation of this species.

T. Vesth · T. M. Wassenaar · P. F. Hallin · L. Snipen · K. Lagesen · D. W. Ussery (✉)
Center for Biological Sequence Analysis,
Department of Systems Biology,
The Technical University of Denmark,
Building 208,
2800 Kgs. Lyngby, Denmark
e-mail: dave@cbs.dtu.dk

T. M. Wassenaar
Molecular Microbiology and Genomics Consultants,
Zotzenheim, Germany

P. F. Hallin
Novozymes A/S,
Krogshøjvej 36,
2880 Bagsværd, Denmark

L. Snipen
Biostatistics, Department of Chemistry, Biotechnology,
and Food Sciences, Norwegian University of Life Sciences,
Ås, Norway

K. Lagesen
Centre for Molecular Biology and Neuroscience and Institute
of Medical Microbiology, University of Oslo,
Oslo, Norway

Introduction

The species concept for bacteria has long been under siege from several angles, and now with thousands of bacterial genomes being sequenced, the disputes have intensified [8]. One frequently used definition of a bacterial species is "a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" [12]. Such independent features are usually phenotypes that can easily be tested. For a new species to be defined, amongst other criteria, inter-species DNA–DNA hybridisation has to be below 70%, although this rule is not without its limitations [18]. In the late 1970s and 1980s, the 16S rRNA gene sequence was introduced as a molecular clock that could be used to infer phylogenetic relationships [50]. Ideally, isolates belonging to the same species have identical or nearly identical 16S rRNA genes, and these differ from isolates belonging to different species [32, 44]. In practice, this is not always the case. Examples exist of different species sharing identical rRNA genes (for instance, *E. coli* and *Shigella* [37] that are even placed in different genera); in addition, isolates of one species can have different rRNA genes beyond the 97% that is considered to demarcate species [4]. Lateral transfer of genetic material (to which ribosomal genes are believed to be resistant) destroys the phylogenetic relationship, so that

phylogenies based on alternative housekeeping genes can differ from a 16S rRNA tree and frequently are not even in accordance to each other. Such observations question the validity of a phylogenetic tree as the most suitable model for bacterial ancestry, when multiple genetic transfers would produce a network-like evolutionary structure [6]. On the other hand, it is observed that lateral gene transfer is most frequent between genetically related members sharing a similar base content and occupying the same ecological niche [29]. Nevertheless, a core of genes can be recognised that produce coherent phylogenetic trees, though these may not represent the species' complete evolutionary history as they comprise only a minor fraction of the genetic content of the organism [35].

Whether a tree or a network is more accurate to describe phylogeny, in either case bacterial species may be considered as a cloud of isolates having a higher level of genetic similarity to each other than to organisms belonging to a different species. When such clouds have fuzzy and overlapping borders, the species concept falls apart but that will only apply to certain cases [7]. Since 16S rRNA genes are not informative on the level of diversity within a species, the 'density' of a cloud of isolates making up a species cannot be determined by this gene. Those genes shared by all isolates belonging to one species comprise the core genome of that species [39], and the degree of diversity in the remaining non-core genes determines the density of the species cloud.

We hypothesised that certain genes can be recognised as specific to a particular species, to be conserved in that species but not present in related species. We tested our hypothesis with complete genome sequences of the bacterial family *Vibrionaceae*, which belong to the γ-Proteobacteria and comprises eight genera. Most available genome sequences belong to the genus *Vibrio.* This genus contains 51 recognised species [10, 46] which are mainly found in marine environments, frequently living in association with marine organisms such as corals, fish, squid or zooplankton. Most of them are symbionts and only a few are human pathogens, notably particular serotypes of *V. cholerae* producing cholera, *Vibrio parahaemolyticus* (causing gastroenteritis) and *Vi vulnificus* (causing wound infections) [46]. Other *Vibrionaceae*, including *V. vulnificus*, *Aliivibrio salmonicida* and *V. harveyi*, are fish or shellfish pathogens and have major economic impact. *Photobacterium profundum*, representing another genus within the *Vibrionaceae*, was also included.

The gene content of 32 available sequenced *Vibrionaceae* genomes was compared and the results were analysed in various ways. The data allowed us to identify possible *V. cholerae*-specific genes, since this species was represented by 18 genomes that was a sufficient number to test conservation both within the species and across species. We found that a two-component signal transduction pathway is uniquely conserved in *V. cholerae* but is not found outside this species. Our findings further indicated that possibly a relatively small set of genes could confer niche specialisation allowing *V. cholerae* to be adopted to a unique environment, so that over time *V. cholerae* have become a distinct species.

Materials and Methods

Genomes and Gene Annotations Used

Publicly available genome sequences of *Vibrionaceae* were selected that were provided in less than 300 contigs and in which full-length 16S rRNA sequence could be found using the rRNA gene finder RNAmmer [19]. The 32 genome sequences included are shown in Table 1.

The gene annotations as provided in GenBank were used, except for those genomes marked "Easygene" in Table 1 where protein annotation was not available in the RefSeq file at the time of analysis, and we used EasyGene [20] to identify the genes. As a control, an available GenBank annotation was compared to a generated Easygene annotation to confirm that the number of identified genes was comparable.

Ribosomal RNA Analysis

RNAmmer [19] was used to identify 16S rRNA sequences within the 32 genomes. Sequences were considered reliable if they were between 1,400 and 1,700 nucleotides long and had an RNAmmer score above 1,800. In cases where the program found multiple and variable 16S sequences within a genome, one of these (with satisfactory RNAmmer scores) was arbitrarily chosen. The sequences were aligned using PRANK [23, 24], and the program MEGA4 was used to elucidate a phylogenetic tree [45]. Within MEGA4, the tree was created using the Neighbor-Joining method with the uniform rate Jukes–Cantor distance measure and the complete-delete option. Five hundred resamplings were done to find the bootstrap values.

Pan-Genome Family Clustering

Clustering based on shared gene families from the *Vibrio* pan-genome was constructed, based on BLASTP similarity using default settings. A BLASTP hit was considered significant if the alignment produced at least 50% identity for at least 50% of the length of the longest gene (either query or subject). Using this criterion, each pair of genes producing a significant reciprocal best hit was scored as belonging to the same gene family. A genome matrix was constructed, containing one row for each genome and one

Table 1 *Vibrionaceae* genomes used in this analysis

GPID	Organism	Contigs	Accession/GenBank	Status	No. of genes	Ref.
36	*V. cholerae* N16961[a]	2	AE003852.1	Fully sequenced	3,828	[15]
15667	*V. cholerae* O395 TIGR[a]	2	CP000626.1	Fully sequenced	3,875	[11]
32853	*V. cholerae* O395 TEDA[a]	2	CP001235.1	Fully sequenced	3,934	[49]
33555	*V. cholerae* MJ-1236[a]	2	CP001485.1	Fully sequenced	3,774	[31]
15666	*V. cholerae* MO10[a]	153	NZ_AAKF00000000	Unfinished (Easygene)	3,421	[5]
15670	*V. cholerae* V52[a]	268	NZ_AAKJ00000000	Unfinished (NCBI)	3,815	[16]
33559	*V. cholerae* BX330286[a]	8	NZ_ACIA00000000	Unfinished (NCBI)	3,632	[31]
33557	*V. cholerae* B33[a]	17	NZ_ACHZ00000000	Unfinished (NCBI)	3,748	[31]
33553	*V. cholerae* RC9[a]	11	NZ_ACHX00000000	Unfinished (NCBI)	3,811	[31]
32851	*V. cholerae* M66-2	2	CP001233.1	Fully sequenced	3,693	[49]
18495	*V. cholerae* MZO-2	162	NZ_AAWF00000000	Unfinished (NCBI)	3,425	[16]
18265	*V. cholerae* 1587	254	NZ_AAUR00000000	Unfinished (NCBI)	3,758	[16]
18253	*V. cholerae* 2740-80	257	NZ_AAUT00000000	Unfinished (NCBI)	3,771	[16]
17723	*V. cholerae* AM-19226	154	NZ_AATY00000000	Unfinished (Easygene)	3,407	[33]
33561	*V. cholerae* 12129	12	NZ_ACFQ00000000	Unfinished (NCBI)	3,574	[31]
33549	*V. cholerae* VL426	5	NZ_ACHV00000000	Unfinished (NCBI)	3,461	[31]
33579	*V. cholerae* TM 11079-80	35	NZ_ACHW00000000	Unfinished (NCBI)	3,621	[31]
33551	*V. cholerae* TMA 21	20	NZ_ACHY00000000	Unfinished (NCBI)	3,600	[31]
13564	*V. campbellii* AND4	143	NZ_ABGR00000000	Unfinished (NCBI)	3,935	[13]
19857	*V. harveyi* BAA-1116	3	CP000789.1	Fully sequenced	6,064	[1]
349	*V. vulnificus* CMCP6	2	AE016795.2	Fully sequenced	4,538	[38]
1430	*V. vulnificus* YJ016	3	BA000037.2	Fully sequenced	5,028	[3]
19397	*V. shilonii* AK1	158	NZ_ABCH00000000	Unfinished (NCBI)	5,360	[41]
15693	*Vibrio* sp. Ex25	222	NZ_AAKK00000000	Unfinished (Easygene)	4,004	[16]
13616	*Vibrio sp.* MED222	99	NZ_AAND00000000	Unfinished (NCBI)	4,590	[36]
32815	*V. splendidus* LGP32	2	FM954973.1	Fully sequenced	4,434	[27]
19395	*V. parahaemolyticus* 16	78	NZ_ACCV00000000	Unfinished (Easygene)	3,780	[9]
360	*V. parahaemolyticus* 2210633	2	BA000031.2	Fully sequenced	4,832	[25]
12986	*A. fischeri* ES114	3	CP000020.1	Fully sequenced	3,823	[42]
19393	*A. fischeri* MJ11	3	CP001133.1	Fully sequenced	4,039	[26]
30703	*A. salmonicida* LFI1238	6	FM178379.1	Fully sequenced	4,284	[17]
13128	*P. profundum* SS9	3	CR354531.1	Fully sequenced	5,480	[48]

GPID genome project identifier at NCBI. *Contigs* the number of contiguous sequences, which for a completely sequenced genome is at least two (for two chromosomes) and can be up to six when plasmids are present. Unfinished sequences are represented by multiple contigs per chromosome

[a] Strains containing the genes encoding the cholera enterotoxin subunits are indicated

column for each gene family. Cell (i, j) in this matrix is 1 if genome i has a member in gene family j, 0 otherwise. A hierarchical clustering, with average linkage based on the Manhattan distance between genomes was then performed. Two trees were made, one with more weight given to gene families present in most (90%, or between 27 and 30) *Vibrio* genomes ("stabilome"), and the other with more weight given to gene families present in only a few (two, three, or four) genomes ("mobilome"). Thus, the original Boolean matrix is now scaled differently, depending on the number of genomes in each gene family [44]. For both trees, singletons (families which are only found in one genome) have been excluded.

Pan- and Core Genome Analysis

The results of the BLAST analysis were also used to construct a pan- and core genome plot as follows. Based on clusterings from the pan-genome family tree, an ordered set of genomes was constructed with *V. cholerae* genomes at the start. For the first chosen genome, all BLAST hits found in the second genome were recorded and the accumulative

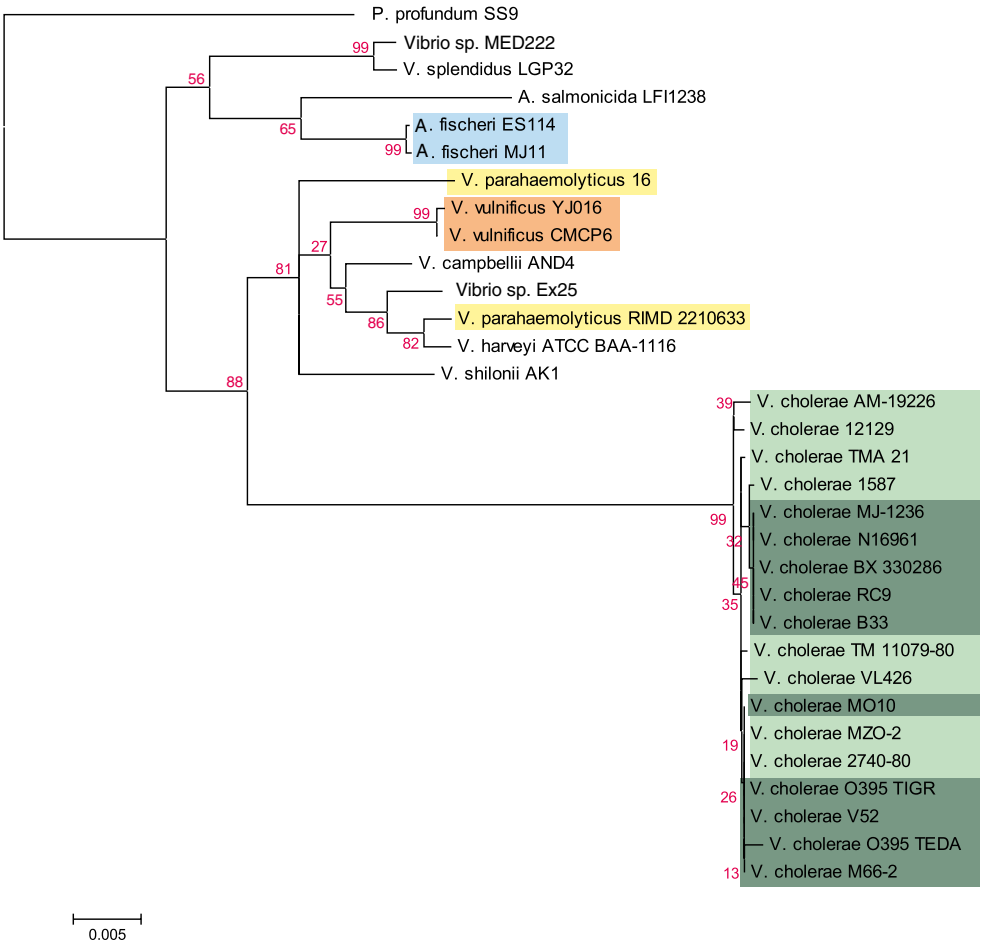


Figure 1 Phylogenetic tree of the 16S rRNA gene extracted from 32 sequenced *Vibrio* genomes listed in Table 1. Environmental *V. cholerae* lacking the cholera enterotoxin genes are highlighted in *bright green*, whilst pathogenic *V. cholerae* genomes are in *dark green*. Further colouring was used for species for which two genomes are represented

number of gene families (as defined above) now recognised in total was plotted for the pan-genome. The number of gene families with at least one representative gene in both genomes was plotted for the core genome. A running total is plotted for the pan-genome which increases as more genomes are added, whilst the core genome representing conserved gene families slowly decreases with the addition of more genomes.

Whole-Genome BLAST Analysis and Construction of a BLAST Matrix

The predicted genes of every genome (annotated or found by Easygene) were translated and every gene was compared, by BLASTP against every other genome and its own genome. In the latter case, the hit to self was ignored. The 50/50 rule for BLAST hits as described above was used. If these requirements were met, genes were combined in a gene family. The BLAST results were visualised in a BLAST matrix [2], which summarises the results of genomic pairwise comparisons and reports, both as percentage and as absolute numbers, the number of reciprocal BLAST hits as a fraction of the total number of gene families found in the two genomes. For easier visual inspection, the cells in the matrix are coloured darker as the fraction of similarity increases. Hits identified within a genome are differently coloured.

BLAST Atlas

BLAST results were also visualised in a BLAST atlas, this time visualising, for all genes in the reference genome *V cholerae* N16961, their best hit in all other genomes, again with a threshold of 50% identity over at least 50% of the length of the query protein. The atlas displays the hits as they are located in the reference strain [14]. The BLAST scores obtained for each queried gene is plotted, so that conserved and variable regions are located with respect to the reference genome. Note that genes absent in the reference genome are not shown in the lanes of the query genomes.

Results

Ribosomal RNA Analysis

A phylogenetic tree based on the 16S rRNA gene extracted from the 32 analysed *Vibrionaceae* genomes is shown in Fig. 1. The 18 *V. cholerae* genomes build a tight subcluster,

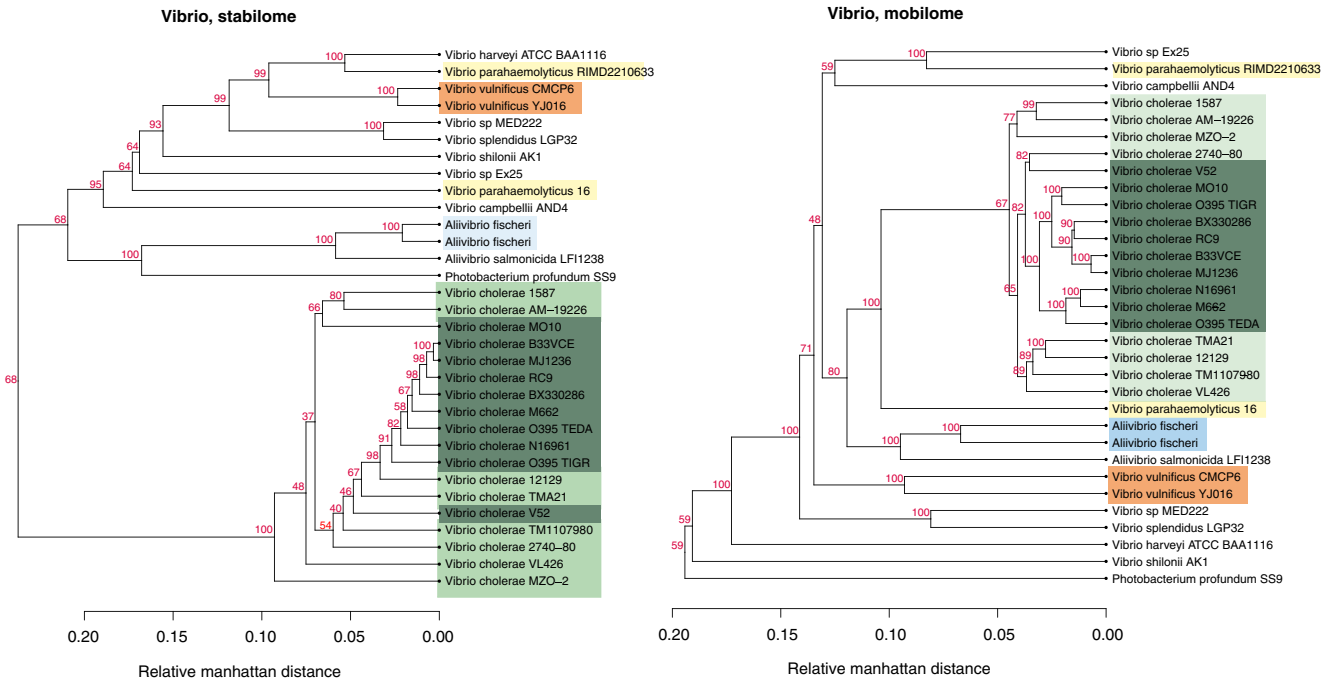


Figure 2 Pan-genome family clustering of the 32 *Vibrio* genome sequences. The two plots represent weighted values for genes present in at least 90% of the genomes (*stabilome*) or genes found in only a few (two to four) genomes (*mobilome*). The colours highlighting the species are the same as in Fig. 1

quite distanced from the other species. Above this in the figure, another subcluster comprising eight genomes representing at least six species is recognised, and within this cluster the two *V. parahaemolyticus* genes are not found on the same branch. A third cluster, a bit further removed, includes *Aliivibrio fischeri* and *A. almonidica* as well as *V. splendidus* and *Vibrio* species MED 222; the gene of *Photobacterium profundum* is the most distant.

Pan-Genome Family Trees

Starting with a database containing the total set of all *Vibrio* gene families, a profile of matching gene families was constructed for each individual genome. This was stored as a matrix, containing a column for each gene families, and a row for each genome. The rows contain a 0 or 1 representing the presence or absence of the gene family. This matrix was weighted to emphasise either the genes found in most genomes (the "stabilome") or in only a few genomes (the "mobilome"); from these weighted matrices, clustering of gene families yielded the resulting trees shown in Fig. 2. Shorter distances represent genomes with many gene families in common, and larger distances reflect genomes with fewer gene families in common. As expected, in both trees, genomes from the same species cluster together, whereby the depth of resolution within a species is considerably better than can be seen in the 16S rRNA tree in Fig. 1. Similarity between the unspeciated *Vibrio* isolate MED222 and *V. splendidus* is suggested by their close clustering; this is a connection also suggested by others [21]. Note that the unspeciated *Vibrio* isolate Ex25 and *V. parahaemolyticus* 2210633 cluster together in the mobilome tree, but are more distant in the stabilome. This implies that the genes shared between these two genomes are less common genes within the *Vibrio* genomes examined here. As already indicated by the 16S rRNA tree, the two *V. parahaemolyticus* isolates are quite dissimilar, and appear on separate branches. The *Aliivibrio* cluster is placed within *Vibrio* genomes in both the stabilome and the mobilome, as was the case for their 16S rRNA gene. *P. profundum* is not such an outlier as in the 16S rRNA tree, and in the stabilome. It is even positioned close to the *Aliivibrio* genomes. Zooming in at the genomes of *V. cholerae*, a division into two subclusters can be seen; these clusters correspond to environmental vs. clinical isolates (with the exception of V52 in the stabilome).

Pan- and Core Genome Plot

BLAST results were analysed to construct a pan-genome, which is a hypothetical collection of all the gene families that are found in the investigated genomes [28]. The core genome was constructed from all gene families that were represented at least once in every genome. Thus, the gene families conserved in all genomes represent their core genome; adding the remaining gene families produces the

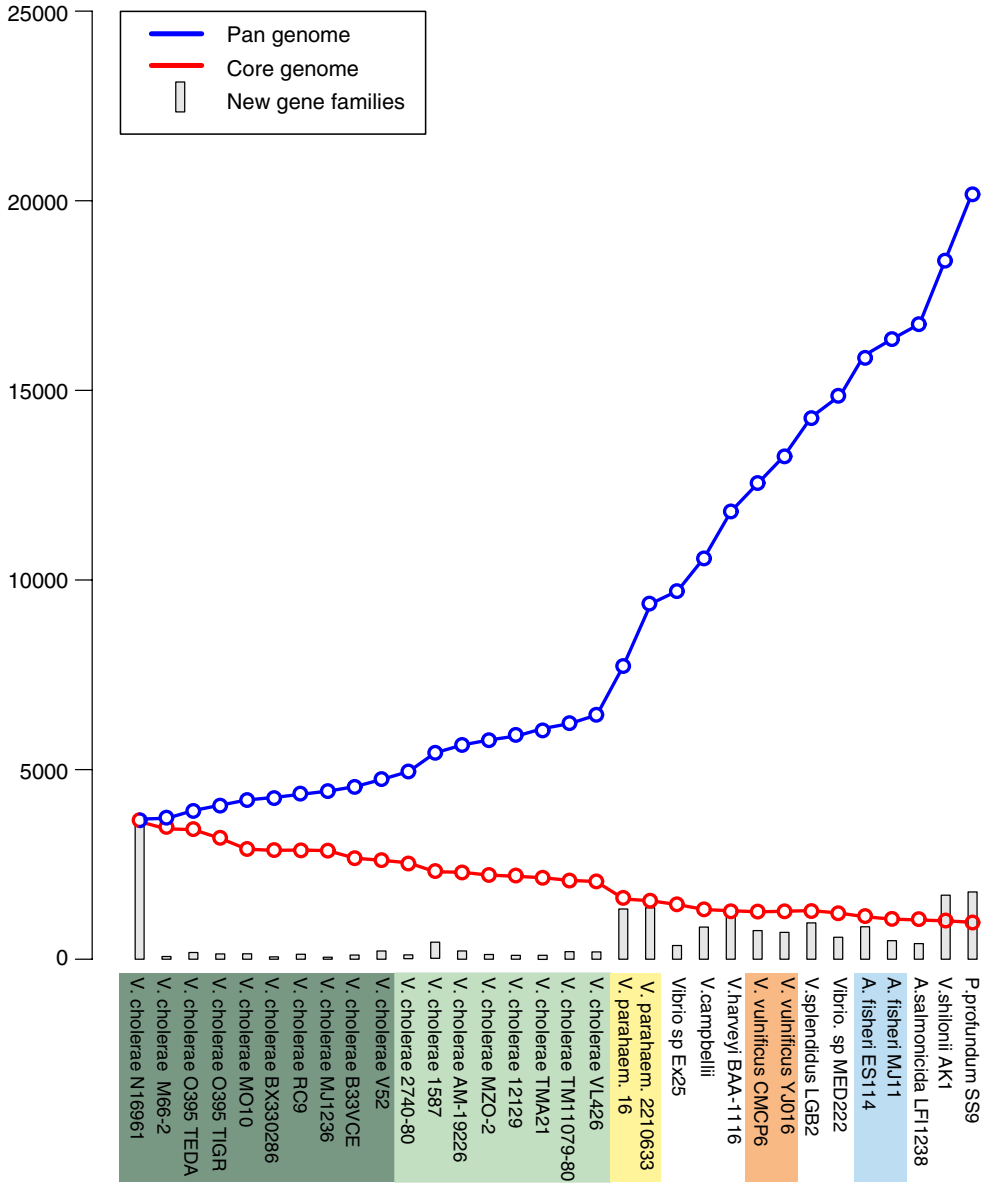


Figure 3 Pan- and core genome plot of the 32 *Vibrionaceae* genomes. The colours highlighting species are the same as in Fig. 1

pan-genome. The resulting pan- and core genome plot is shown in Fig. 3. The genomes start with the documented clinical isolates of *V. cholerae* and then follow the order suggested by the pan-genome family clustering (Fig. 2), although genomes from the same species were kept together (the two *V. parahaemolyticus* genomes were split in the trees). As more genomes are added in the plot, the number of gene families in the pan-genome (blue line) increases, and the number of conserved gene families (red line) in the core genome decreases, albeit at a lower rate. This is because every genome can add many novel (and frequently different) genes to the pan-genome but only decreases the core genome with a few genes that are absent in that particular strain but that were conserved in the previously analysed genomes. The pan-genome curve increases with a relative steep slope when a novel species is added, as is obvious when a *V. parahaemolyticus* genome is added after the last *V. cholerae*. A stable plateau can be seen for the pan-genome of *V. cholerae* around 6,500 genes. Nevertheless, a small increase occurs when adding *V. cholerae* 11587; this is caused by the difference between the two subclusters of *V. cholerae* seen in Fig. 2. *V. cholerae* strain 2740-80 behaves atypical in all the figures shown; although documented as an environmental isolate, it appears closer to the clinical isolates, in terms of overall genomic properties.

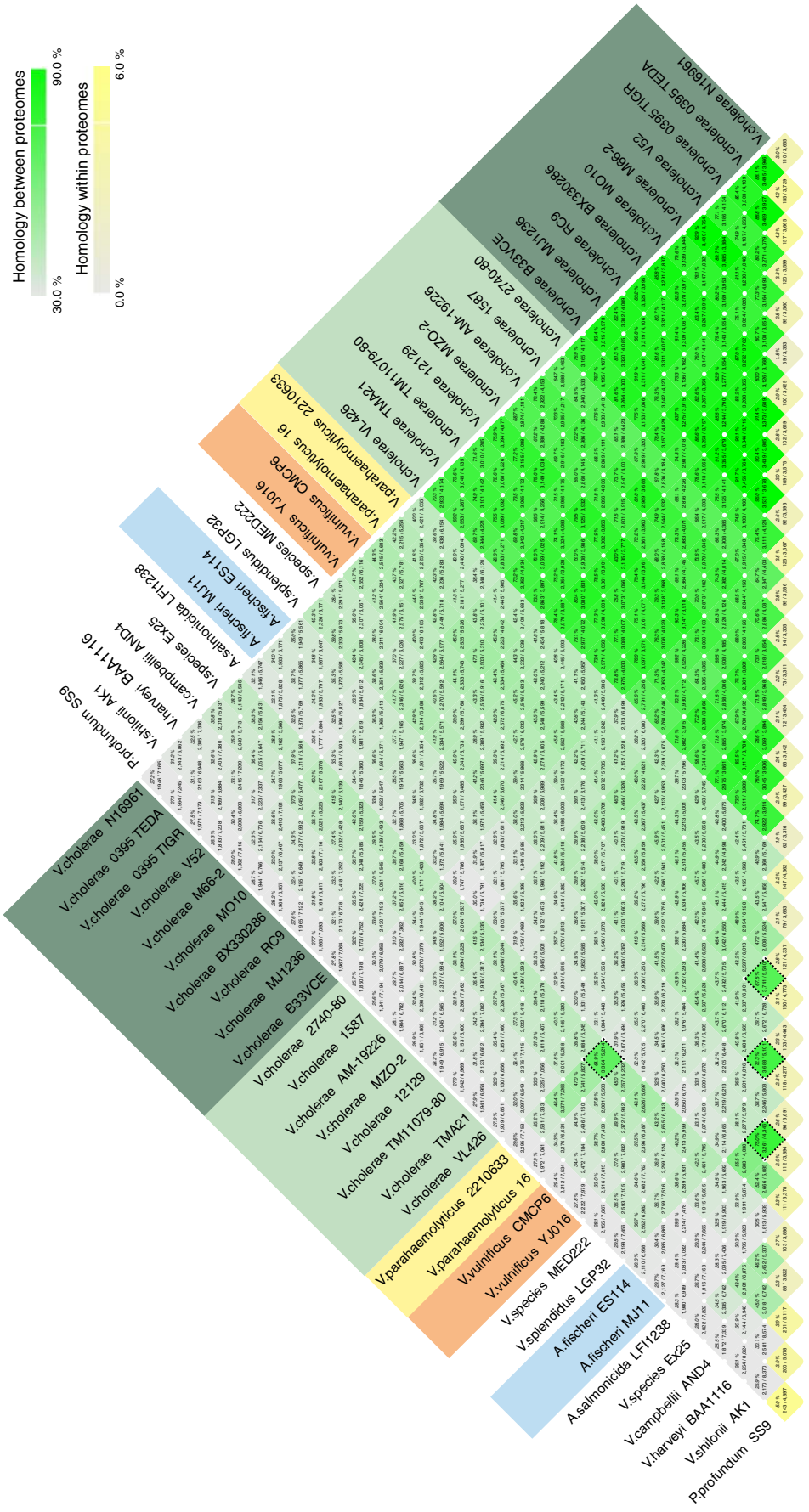
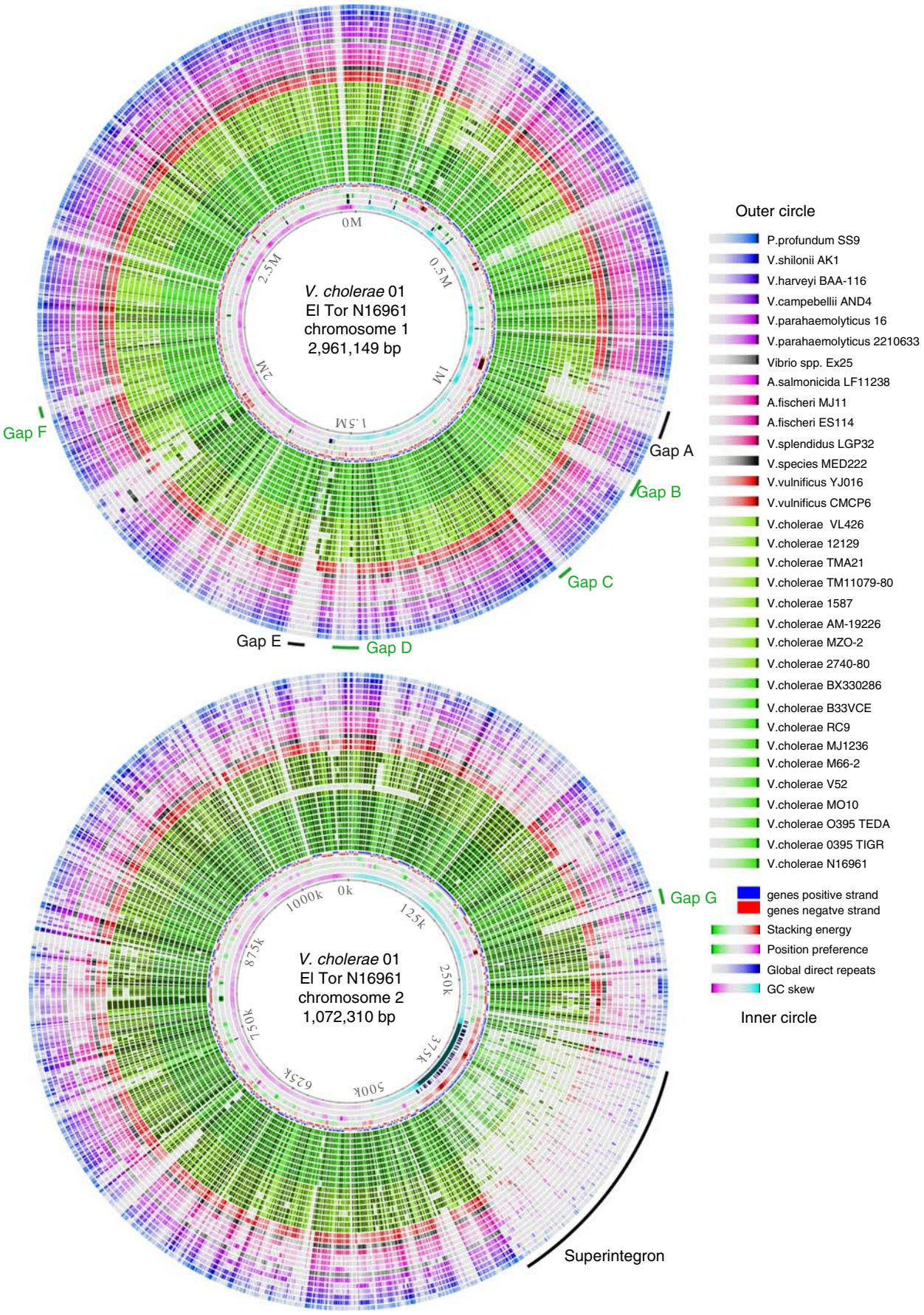


Figure 4 BLAST matrix of the 32 *Vibrionaceae* genomes. The colours highlighting the species are the same as in Fig. 1. Since the reciprocal similarity (reported as percent) is not readable at this resolution, every matrix cell is coloured using the scales as indicated. The *bottom row* identifies hits (other than hits-to-self) found within a genome. Four matrix cells reporting high pairwise similarities are *outlined*; their numbers are specified in the text


0M
0.5M
1M
1.5M
2M
2.5M
V. cholerae 01
El Tor N16961
chromosome 1
2,961,149 bp
Gap A
Gap B
Gap C
Gap D
Gap E
Gap F
0k
125k
250k
375k
500k
625k
750k
875k
1000k
V. cholerae 01
El Tor N16961
chromosome 2
1,072,310 bp
Gap G
Superintegron
Outer circle
P.profundum SS9
V.shilonii AK1
V.harveyi BAA-116
V.campebellii AND4
V.parahaemolyticus 16
V.parahaemolyticus 2210633
Vibrio spp. Ex25
A.salmonicida LF11238
A.fischeri MJ11
A.fischeri ES114
V.splendidus LGP32
V.species MED222
V.vulnificus YJ016
V.vulnificus CMCP6
V.cholerae VL426
V.cholerae 12129
V.cholerae TMA21
V.cholerae TM11079-80
V.cholerae 1587
V.cholerae AM-19226
V.cholerae MZO-2
V.cholerae 2740-80
V.cholerae BX330286
V.cholerae B33VCE
V.cholerae RC9
V.cholerae MJ1236
V.cholerae M66-2
V.cholerae V52
V.cholerae MO10
V.cholerae O395 TEDA
V.cholerae 0395 TIGR
V.cholerae N16961
genes positive strand
genes negatve strand
Stacking energy
Position preference
Global direct repeats
GC skew
Inner circle

When the first genome of *A. fischeri* is added, which is not a member of the *Vibrio* genus, it does not add significantly more novel genes to the pan-genome than *Vibrio* genomes did. This contrasts with *P. profundum* which produces a sharp increase in the pan-genome, as does, interestingly, *V. shilonii*. Note that there are approximately 20,200 total gene families within the 32 sequenced *Vibrionaceae* genomes, whereas the core genome decreases to approximately 1,000 gene families.

BLAST Comparison Visualised in a BLAST Matrix

A BLAST matrix provides a visual overview of reciprocal pairwise whole-genome comparisons, as shown in Fig. 4. The stronger a matrix cell is coloured, the more similarity was detected between the gene content of two genomes. As can be seen in the lower right triangle, all *V. cholerae* genomes are highly similar, with similarity ranging between 64% and 93% for any given pair of genomes. No statistical difference was observed when comparing clinical isolates to environmental isolates. The two *A. fischeri* and the two *V. vulnificus* genomes also share a high degree of identity within their species (75% and 67%, respectively), visible at the bottom of the matrix. In contrast, the two *V. parahaemolyticus* genomes only share 35% identity, which is not higher than the similarity detected between genomes of different species. With 72% similarity, isolate MED222 most closely matches *V. splendidus* and with 65% isolate EX25 again shares most similarity with *V. parahaemolyticus* 2210633.

BLAST Atlas

A BLAST atlas was constructed using *V. cholerae* N16961 (O1, El Tor) as the reference genome, shown in Fig. 5. The best blast hits identified in the query genomes are plotted in the lanes around the reference genome, with different colours for different species. In general, chromosome 1 is more strongly conserved than chromosome 2. A large part of chromosome 2 of N16961 displays very little conservation in the other genomes; this area represents a super integron [40] that contains the *V. cholerae*-specific repeat (VCR) sequences, as well as a high number of gene cassettes. The repeat sequences are visible as black boxes in the repeat lane of the reference genome (second inner lane). Although all *V. cholerae* genomes contain a superintegron, its genes are very diverse between isolates [34] which explains the lack of blast hits in this region.

Several regions of the atlas have been highlighted. Gaps B, C, D and F on chromosome 1 (indicated in green) contain genes that are conserved in the represented genomes of *V. cholerae* but not in the other *Vibrionaceae*. The gaps marked A, E and G indicate regions that are specific to the toxigenic, clinical isolates only. Annotated, *V. cholerae*-specific genes present in all these regions are listed in Table 2 (hypothetical genes are excluded). Genes specific for toxinogenic *V. cholerae* identified in gap A include, amongst others, biosynthesis genes for the toxin co-regulated pilus (which is required for transmission of the prophage CTXΦ carrying the enterotoxin genes), as well as genes encoding citrate lyase. Note that the genes in gap A are also found in the environmental isolate *V. cholerae* 2740-80.

Gap B contains a number of outer membrane protein genes involved in sugar modification that are found in all *V. cholerae* genomes. Genes from gap C encoding a histidine kinase two-component signal transduction regulatory system are also conserved within the species, as genes in gaps D and F, involved in chemotaxis and possible multidrug resistance.

Gap E, containing genes conserved in toxigenic strains only, holds the prophage CTXΦ that contains the genes encoding cholera enterotoxin subunits A and B; this enterotoxin is responsible for the excessive, watery diarrhoea typical for cholera. Upon binding to target cell GM1 gangliosides, enterotoxin enters the cell and stimulates adenylate cyclase by ADP ribosylation. The resultant increased cyclic AMP levels induce excessive electrolyte movement and sodium plus water secretion [43]. Strain M66-2 is believed to be a precursor of the seventh pandemic *V. cholerae* that lacks the prophage CTXΦ and the enterotoxin genes [11]. Gap E bears the RTX toxin operon, which encodes a pore-forming cytotoxin [22]. An RTX toxin is also present in environmental isolate 2740-80 and in *V. vulnificus*.

Gap G on chromosome 2 consists of a set of five genes, all in the same orientation, in a putative operon, flanked by genes on the complimentary strand. This appears to be a remnant of a mobile element, as these genes are flanked by a transposase gene on the 3′ end, and there is a small global repeat on the 5′ end. Only the first two of the five genes have an assigned function, with the first gene being a GMP reductase, and the second a putative DNA methyltransferase. The remaining three genes are hypothetical, but their strikingly strong conservation in all pathogenic strains and

◀ **Figure 5** BLAST atlas with *V. cholerae* strain N16961 as a reference strain, showing chromosomes 1 (*top*) and 2 (*bottom*). The best BLAST hits identified with genes from N16961 in the other *V. cholerae* genomes are represented in *dark red*, for the location as it appears in N16961. Blast hits in the other genomes are shown in various colours as indicated to the *right*. Major areas conserved in *V. cholerae* but not in other *Vibrionaceae* are identified as gap B, gap C, gap D and gap F in *green*; areas that are found in toxigenic *V. cholerae* only are marked *black* as gap A, gap E and gap G. The superintegron on chromosome 2 of *V. cholerae* is also indicated

Table 2 A selection of genes located in the gaps marked in Fig. 5

Gap A (850000–913000)	
852903–851557	Citrate/sodium symporter
853165–854235	Citrate (pro-3S)-lyase ligase
854287–854583	Citrate lyase subunit gamma
854565–855455	Citrate lyase, beta subunit
855391–856995	Citrate lyase, alpha subunit
856992–857528	citX protein
857506–858447	citG protein
869812–866873	Helicase-related protein
870391–869813	Tellurite resistance protein-related
871298–870819	Transcriptional regulator, putative
873242–874225	Transposase, putative
876974–880015	ToxR-activated gene A protein
881390–884728	Inner membrane protein, putative
885773–886267	tagD protein
888405–886543	Toxin co-regulated pilus biosynthesis
888846–889511	Toxin co-regulated pilus biosynthesis
889496–889906	Toxin co-regulated pilus biosynthesis
890449–891123	Toxin co-regulated pilin
891203–892495	Toxin co-regulated pilus biosynthesis
892495–892947	Toxin co-regulated pilus biosynthesis
892950–894419	Toxin co-regulated pilus biosynthesis
894412–894867	Toxin co-regulated pilus biosynthesis
894855–895691	Toxin co-regulated pilus biosynthesis
895707–896165	Toxin co-regulated pilus biosynthesis
896155–897666	Toxin co-regulated pilus biosynthesis
897641–898663	Toxin co-regulated pilus biosynthesis
898673–899689	Toxin co-regulated pilus biosynthesis
899896–900726	TCP pilus virulence regulatory protein
900726–901487	Leader peptidase TcpJ
901494–903374	Accessory colonization factor AcfB
903380–904150	Accessory colonization factor AcfC
904648–905556	tagE protein
906206–905559	Accessory colonization factor AcfA
914124–912856	Phage family integrase
Gap B (975000–1010000)	
978644–979144	Phosphotyrosine protein phosphatase
981833–982387	Serine acetyltransferase-related protein
982384–983532	Exopolysacch. biosynth protein EpsF
983529–984938	Polysacch. export protein, putative (gfcE)
986166–986597	Serine acetyltransferase-related protein
986597–987937	capK protein, putative
987913–989010	Polysaccharide biosynthesis protein, putative
1001910–1002437	Polysaccharide export-related protein (gfcE)
1002462–1004675	Putative exopolysacch. biosynth protein
Gap C (1130000–1160000)	
1139646–1142912	Chitinase, putative
1147856–1148998	Response regulator
1149033–1149398	Response regulator
1149990–1151309	Sensory box sensor histidine kinase
1151321–1152625	Sensor histidine kinase
1152625–1154235	Response regulator
1154252–1155595	Response regulator
1157228–1155624	Sensor histidine kinase
1158044–1157232	Periplasmic binding protein-related
Gap D (1478000–1520000)	
2086826–2087584	CDP-diacylglycerol-glyc.-3-phosph-3-phosphatidyltransferase
2087587–2088519	Phosphatidate cytidylyltransferase
2094741–2095604	PvcB protein
2098112–2097183	LysR family transcriptional regulator
2098432–2100258	pvcA protein
2117923–2119977	Methyl-accepting chemotaxis protein
2120575–2120030	Transcriptional regulator
2120663–2121826	Benzoate transport protein
Gap E (1537000–1587500)	
1541452–1543170	Sensor histidine kinase/response regulator
1545396–1543231	Toxin secretion transporter, putative
1546802–1545399	RTX toxin transporter
1548919–1546757	RTX toxin transporter
1549662–1550123	RTX toxin activating protein
1550108–1563784	RTX toxin RtxA
1564376–1564152	RstC protein
1564844–1564470	RstB1 protein
1565901–1564822	RstA1 protein
1566027–1566365	Transcriptional repressor RstR
1567341–1566967	Cholera enterotoxin, B subunit
1568114–1567338	Cholera enterotoxin, A subunit
1569412–1568213	Zona occludens toxin
1569702–1569409	Accessory cholera enterotoxin
1571241–1570993	Colonization factor
1571760–1571377	RstB2 protein
1572817–1571738	RstA1 protein
1572943–1573281	Transcriptional repressor RstR
1577272–1575704	Phage replication protein Cri
1582123–1580555	Phage replication protein Cri
1583160–1583513	Transposase OrfAB, subunit A
1583510–1584382	Transposase OrfAB, subunit B
Gap F (1896000–1956000)	
1896092–1897327	Phage family integrase
1900831–1898009	Helicase, putative
1903632–1902898	Chemotaxis protein MotB-related
1908858–1905790	Type I restriction enzyme HsdR
1916009–1913628	DNA methylase HsdM, putative
1933231–1935654	Neuraminidase
1936007–1935801	Transcriptional regulator
1936121–1936597	DNA repair protein RadC, putative
1938391–1937519	Transposase OrfAB, subunit B
1938732–1938388	Transposase OrfAB, subunit A
1941671–1941351	Transcriptional regulator, putative

Table 2 (continued)

1942032–1941658	Middle operon regulator-related
1944457–1943306	eha protein
Gap G (chromosome II, 21300–223000)	
213207–214250	GMP reductase
214574–215725	DNA methyltransferase
220262–219825	IS1004 transposase

All gene annotations are taken from the reference genome *V. cholerae* strain N16961. Hypothetical proteins were excluded. Gaps A, E and G are conserved in pathogenic strains, whereas gaps B, C, D and F are conserved in all *V. cholerae* genomes analysed (Figure 1)

complete absence of homologues in the other *Vibrio* genomes strongly point towards a potential biological significance.

Discussion

The recent availability of many *Vibrionaceae* genomes, including a substantial number of *V. cholerae* genomes, allows the possibility to take a closer look at the similarities and differences of species within the genus *Vibrio*. This can examine, on a genome scale, what distinguishes *V. cholerae* from the other *Vibrio* species. Since not all *V. cholerae* isolates are pathogenic, the presence of the prophage-bearing cholera enterotoxin, the main virulence factor for cholera, is not a suitable marker for this species. We attempted to identify a set of *V. cholerae*-specific genes, and also explored the internal diversity within the *V. cholerae* genomes that have been sequenced to date.

On a phylogenetic tree based on the 16S ribosomal RNA gene, those isolates that do not belong to the genus *Vibrio* were positioned as outliers, as expected. This tree further indicated the closest resembling 16S rRNA sequence for the two sequenced *Vibrio* strains that are currently not assigned to a species. It was observed that the two sequenced *V. parahaemolyticus* strains were not placed together. The complete gene content of each genome was next compared by BLAST and the results were pooled into gene families which were subjected to cluster analysis. This provided evidence that the 18 *V. cholerae* genomes fall into two subclusters, one mainly containing clinical isolates and the other environmental isolates.

The gene family clustering, subsequent pan-genome analysis and the pairwise BLAST results, as summarised in the BLAST matrix, all supported the relatedness of *Vibrio* species Ex25 to *V. parahaemolyticus* 2210633 but not to *V. parahaemolyticus* 16. This latter genome was quite different from *V. parahaemolyticus* 2210633 in all analyses. Although it is possible that the species *V. parahaemolyticus* is far more genetically diverse than *V. cholerae*, *A. fischeri* or *V. vulnificus*, an alternative explanation is that one of the sequenced isolates is perhaps incorrectly named as *V. parahaemolyticus*. The similarity between *Vibrio* species MED222 and *V. splendidus* based on gene families is in agreement with their related 16S rRNA genes and published data [21]. However, in contrast to what the ribosomal gene suggests, our whole-genome comparison indicates that the three *Aliivibrio* genomes (*A. salmonicida* and two *A. fischeri*) are not so different from *Vibrio* after all. Their recent placement in the genus *Aliivibrio*, a decision based on five genes (the 16S rRNA gene and four housekeeping genes) and phenotypical characteristics [47], appears not to be reflective of the whole genome picture presented here.

The BLAST results were graphically summarised in a BLAST atlas, which visualised *V. cholerae*-specific gene clusters. These coded for polysaccharide biosynthesis enzymes, response regulators and chemotaxis proteins, amongst others. In addition, a *V. cholerae*-specific, histidine kinase two-component signal transduction regulatory system was identified. The two-component signal transduction pathway is a powerful regulating system for bacteria to adapt to a particular ecological niche. There is a precedent for this claim, as the introduction of a single regulatory protein in *Vibrio fischeri* strain MJ11 has been shown to specifically enable colonization of the squid *Euprymna scolopes* [26].

As expected, the main differences observed between *V. cholerae* clinical isolates and the environmental strains are due to genes related to virulence. Two exceptions are the presence of a number of virulence genes in the environmental strain *V. cholerae* 2740-80 and the absence of enterotoxin genes in clinical isolate M66-2. It has already been suggested that M66-2 might be a predecessor of pandemic, enterotoxic *V. cholerae* [11]. From sequence comparison of four housekeeping genes, it was concluded that *V. cholerae* 2740-80 is intermediary between toxigenic and non-toxigenic isolates [30]. This view is confirmed by the data presented here, although we propose to consider the possibility that the isolate arose from a pandemic clone that has lost the CTXΦ prophage, rather than being a precursor of a pathogen.

In conclusion, several different methods of genome comparisons have yielded a picture of *V. cholerae* genomes as forming a distinct cluster, compared to related species, and a relatively small number of genes might be responsible for environmental niche adaptation and hence for generation of this distinct species. Likely candidates include multiple two-component signal transduction regulatory proteins as well as chemotaxis proteins.

Acknowledgements We would like to thank Tim Binnewies for early work on this project, and also to the Danish Research Councils and the DTU Globalization funds for financial support.

References

1. Bassler B et al. (2007) CP000789.1: Direct submission to GenBank
2. Binnewies TT, Hallin PF, Staerfeldt HH, Ussery DW (2005) Genome update: proteome comparisons. Microbiol 151:1–4
3. Chen CY, Wu KM, Chang YC, Chang CH, Tsai HC, Liao TL, Liu YM, Chen HJ, Shen AB, Li JC, Su TL, Shao CP, Lee CT, Hor LI, Tsai SF (2003) Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Genome Res 13:2577–2587
4. Clayton RA, Sutton G, Hinkle PS, Bult C, Fields C (1995) Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int J Syst Bacteriol 45:595–599
5. Colwell R, Grim CJ, Young S, Jaffe D, Gnerre S, Berlin A, Heiman D, Hepburn T, Shea T, Sykes S, Alvarado L, Kodira C, Heidelberg J, Lander E, Galagan J, Nusbaum C, Birren B (2008) NZ_AAKF00000000: Direct submission to GenBank
6. Doolittle WF (1995) Phylogenetic classification and the universal tree. Science 284:2124–2129
7. Doolittle WF, Papke RT (2006) Genomics and the bacterial species problem. Genome Biol 7:116
8. Doolittle WF, Zhaxybayeva O (2009) On the origin of prokaryotic species. Genome Res 19:744–756
9. Edwards R, Ferriera S, Johnson J, Kravitz S, Beeson K, Sutton G, Rogers Y-H, Friedman R, Frazier M, Venter JC (2008) NZ_ACCV00000000: Direct submission to GenBank
10. Farmer JJ, Janda JM (2005) Vibrionaceae. In: Bergey's manual of systematic bacteriology, 2nd edn, vol 2 part B. Springer, New York, pp 491–546
11. Feng L, Reeves PR, Lan R, Ren Y, Gao C, Zhou Z, Ren Y, Cheng J, Wang W, Wang J, Qian W, Li D, Wang L (2008) A recalibrated molecular clock and independent origins for the cholera pandemic clones. PLoS ONE 3:e4053
12. Gevers D, Cohan FM, Lawrence JG, Sprat BG, Coeyne T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739
13. Hagstrom A, Ferriera S, Johnson J, Kravitz S, Beeson K, Sutton G, Rogers Y-H, Friedman R, Frazier M, Venter JC (2007) NZ_ABGR00000000: Direct submission to GenBank
14. Hallin PF, Binnewies TT, Ussery DW (2008) The genome BLASTatlas—a GeneWiz extension for visualization of whole-genome homology. Mol Biosyst 4:363–371
15. Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406:477–483
16. Heidelberg J, Sebastian Y. NZ_AAKJ00000000, NZ_AAUT00000000, NZ_AAKK00000000, NZ_AAUR00000000, NZ_AAWF00000000: Direct submission to GenBank
17. Hjerde E, Lorentzen MS, Holden MT, Seeger K, Paulsen S, Bason N, Churcher C, Harris D, Norbertczak H, Quail MA, Sanders S, Thurston S, Parkhill J, Willassen NP, Thomson NR (2008) The genome sequence of the fish pathogen *Aliivibrio salmonicida* strain LFI1238 shows extensive evidence of gene decay. BMC Genomics 9:616
18. Konstantinidis T, Ramette A, Tiedje JA (2006) The bacterial species definition in the genomic era. Phil Trans R Soc B 361:1929–1940
19. Lagesen K, Hallin P, Rødland EA, Staerfeldt HH, Rognes T, Ussery DW (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35:3100–3108
20. Larsen TS, Krogh A (2003) EasyGene—a prokaryotic gene finder that ranks ORFs by statistical significance. BMC Bioinformatics 4:29
21. Le Roux F, Zouine M, Chakroun N, Binesse J, Saulnier D, Bouchier C, Zidane N, Ma L, Rusniok C, Lajus A, Buchrieser C, Médigue C, Polz MF, Mazel D (2009) Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. Environ Microbiol 11:1959–1970
22. Lin W, Fullner KJ, Clayton R, Sexton JA, Rogers MB, Calia KE, Calderwood SB, Fraser C, Mekalanos JJ (1999) Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. Proc Natl Acad Sci U S A 96:1071–1076
23. Loytynoja A, Goldman N (2005) An algorithm for progressive multiple alignment of sequences with insertions. Proc Natl Acad Sci U S A 102:10557–10562
24. Loytynoja A, Goldman N (2008) Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. Science 320:1632–1635
25. Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K, Iijima Y, Najima M, Nakano M, Yamashita A, Kubota Y, Kimura S, Yasunaga T, Honda T, Shinagawa H, Hattori M, Iida T (2003) Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. Lancet 361:743–749
26. Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG (2009) A single regulatory gene is sufficient to alter bacterial host range. Nature 458:215–218
27. Mazel D, Le Roux F (2008) FM954973.1: Direct submission to GenBank
28. Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R (2005) The microbial pan-genome. Curr Opin Genet Dev 15:589–594
29. Medrano-Soto A, Moreno-Hagelsieb G, Vinuesa P, Christen JA, Collado-Vides J (2001) Succesful lateral transfer requires codon usage compatibility between foreign genes and recipient genomes. Mol Biol Evol 21:1884–1894
30. Mohapatra SS, Ramachandran D, Mantri CK, Colwell RR, Singh DV (2009) Determination of relationships among non-toxigenic *Vibrio cholerae* O1 biotype El Tor strains from housekeeping gene sequences and ribotype patterns. Res Microbiol 160: 57–62
31. Munk A, Tapia R, Green L, Rogers Y, Detter JC, Bruce D, Brettin TS, Colwell R, Grim C, Vonstein V, Bartels D. CP001485.1, NZ_ACHV00000000, NZ_ACHY00000000, NZ_ACHW00000000, NZ_ACHX00000000, NZ_ACHZ00000000, NZ_ACIA00000000, NZ_ACFQ00000000: Direct submission to GenBank
32. Murray RG, Stackebrandt E (1995) Taxonomic note: implementation of the provisional status Candidatus for incompletely described procaryotes. Int J Syst Bacteriol 45:186–187
33. Nierman WC (2006) NZ_AATY00000000: Direct submission to GenBank
34. Pang B, Yan M, Cui Z, Ye X, Diao B, Ren Y, Gao S, Zhang L, Kan B (2007) Genetic diversity of toxigenic and nontoxigenic *Vibrio cholerae* serogroups O1 and O139 revealed by array-based comparative genomic hybridization. J Bacteriol 189:4837–4879
35. Philippe H, Douady CJ (2003) Horizontal gene transfer and phylogenetics. Curr Opin Microbiol 6:498–505

36. Pinhassi J, Pedros-Alio C, Ferriera S, Johnson J, Kravitz S, Halpern A, Remington K, Beeson K, Tran B, Rogers Y-H, Friedman R, Venter JC (2006) NZ_AAND00000000: Direct submission to GenBank
37. Pupo GM, Lan R, Reeves PR (2000) Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc Natl Acad Sci U S A 97:10567–10572
38. Rhee JH, Kim SY, Chung SS, Lee SE, Choy HE (2002) AE016795.2: Direct submission to GenBank
39. Riley MA, Lizotte-Waniewski M (2009) Population genomics and the bacterial species concept. Methods Mol Biol 532:367–377
40. Rowe-Magnus DA, Guérout AM, Mazel D (1999) Super-integrons. Res Microbiol 150:641–651
41. Rosenberg E, Ferriera S, Johnson J, Kravitz S, Beeson K, Sutton G, Rogers Y-H, Friedman R, Frazier M. Venter JC (2006) NZ_ABCH00000000: Direct submission to GenBank
42. 3Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, Lupp C, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP (2005) Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci U S A 102:3004–3009
43. Sánchez J, Holmgren J (2005) Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhoea. Curr Opin Immunol 17:388–398
44. Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kämpfer P, Maiden MC, Nesme X, Rosselló-Mora R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1047
45. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
46. Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68:403–431
47. Urbanczyk H, Ast JC, Higgins MJ, Carson J, Dunlap PV (2007) Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. Int J Syst Evol Microbiol 57:2823–2829
48. Vezzi A, Campanaro S, D'Angelo M, Simonato F, Vitulo N, Lauro FM, Cestaro A, Malacrida G, Simionati B, Cannata N, Romualdi C, Bartlett DH, Valle G (2005) Life at depth: *Photobacterium profundum* genome sequence and expression analysis. Science 30:1459–1461
49. Wang L, Feng L, Reeves P, Lan R, Ren Y, Gao C, Zhou Z, Ren Y, Wang W (2008) CP001233.1. CP001235.1: Direct submission to GenBank
50. Woese CR (1987) Bacterial evolution. Microbial Rev 51:221–271