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Conservation of the Chitin Utilization Pathway in the *Vibrionaceae*▽†

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Vibrionaceae **are regarded as important marine chitin degraders, and attachment to chitin regulates important biological functions; yet, the degree of chitin pathway conservation in *Vibrionaceae* is unknown. Here, a core chitin degradation pathway is proposed based on comparison of 19 *Vibrio* and *Photobacterium* genomes with a detailed metabolic map assembled for *V. cholerae* from published biochemical, genomic, and transcriptomic results. Further, to assess whether chitin degradation is a conserved property of *Vibrionaceae*, a set of 54 strains from 32 taxa were tested for the ability to grow on various forms of chitin. All strains grew on *N*-acetylglucosamine (GlcNAc), the monomer of chitin. The majority of isolates grew on α (crab shell) and β (squid pen) chitin and contained chitinase A (*chiA*) genes. *chiA* sequencing and phylogenetic analysis suggest that this gene is a good indicator of chitin metabolism but appears subject to horizontal gene transfer and duplication. Overall, chitin metabolism appears to be a core function of *Vibrionaceae*, but individual pathway components exhibit dynamic evolutionary histories.**

Chitin is the second most abundant biopolymer after cellulose and, particularly in the marine environment, may comprise an important source of organic carbon and nitrogen (2, 25). Chitin is composed of chains of *N*-acetylglucosamine (GlcNAc) residues arranged in antiparallel (α) or parallel (β) configurations. Both forms are found in the marine environment: β-chitin is produced by diatoms and is a major component of squid pens, while the more recalcitrant α form makes up crustacean shells. While the ability to grow on the chitin monomer GlcNAc is thought to be widespread among bacteria (37), likely because it is a component of peptidoglycan, chitinoclastic ability is limited to a number of bacterial groups within *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (8).

We focus here on bacteria of the family *Vibrionaceae*, which includes *Vibrio* and *Photobacterium* as its primary genera, since they have been studied extensively with respect to growth on chitin. Vibrios are ubiquitous and easily cultivatable members of the coastal marine bacterioplankton community; as obligate heterotrophs, they can utilize a wide range of carbon sources for energy (42). Moreover, there has been growing interest in the effect of chitin on pathogenicity and regulation of gene expression in the vibrios (27). Attachment of pathogenic *V. cholerae* to chitinous zooplankton may not only provide a nutrient-rich habitat (12) but could play a role in enhancing human disease transmission (7, 13). Chitin has been shown to change the physiology of the vibrios by inducing competence (26), upregulating attachment/colonization proteins involved in pathogenesis (20, 36), and increasing survival during temperature stress and exposure to stomach acid (3, 29). Thus, chitin has a strong influence on the growth and physiology of vibrios. It is estimated that chitin can support up to 10% of marine bacterial production (19), and it has been speculated that the ubiquity of the vibrios can be explained by their ability to degrade chitin (37).

Chitin degradation is achieved by a complex pathway including multiple chitinases (39); however, most studies of chitinase diversity in the aquatic environment focus on the distribution of the extracellular endochitinase chitinase A (*chiA*), since this gene is thought to be conserved in both *Proteobacteria* and *Firmicutes* (8, 22). Additionally, for organisms with multiple chitinases, *chiA* appears to have the highest expression and activity in response to crab shell chitin (31, 39), suggesting that it may be most active in the environment and thus is a potentially useful indicator of chitinoclastic ability.

In this study, we propose a chitin degradation pathway for *V. cholerae* by incorporating bioinformatic predictions, biochemical studies, and expression data. We then ask how this pathway maps onto sequenced *Vibrio* and *Photobacterium* genomes to determine whether there is a conserved chitin degradation core. Second, we evaluate how widespread chitin metabolism is among *Vibrionaceae* isolates, which cover the coexisting diversity in temperate coastal waters (41, 43), by assaying growth on different forms of chitin (α and β). Third, we explore conservation of the chitin degradation pathway in strains by using the *chiA* gene, and we evaluate its evolutionary dynamics in *Vibrionaceae*.

MATERIALS AND METHODS

In silico analysis of the chitin pathway. The annotated protein and DNA sequences from *Vibrionaceae* genome sequences (complete and unfinished) were obtained from the National Center for Biotechnology Information (NCBI) website on 10 May 2007; *Shewanella oneidensis* MR-1 was included as an outgroup. A list of genomes and their accession numbers is contained in Table S1 in the supplemental material. OrthoMCL (23) was used to identify orthologous groups (families) in the sequenced genomes. This program takes all-against-all BLASTp as input and defines putative pairs of orthologs or recent paralogs based on the reciprocal best BLAST hit. Recent paralogs are identified as genes within the same genome that are more similar to each other than any sequence from another genome. OrthoMCL then converts the reciprocal BLASTp values to a normalized similarity matrix that is analyzed by a Markov Cluster algorithm

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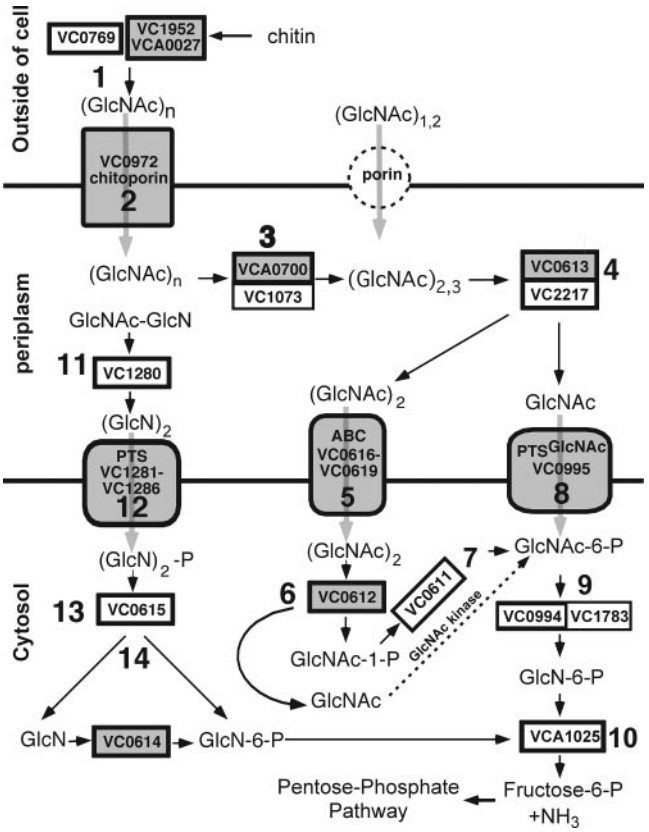


FIG. 1. Schematic of the chitin catabolic cascade in *V. cholerae*. Enzymes and transporters are given gene identifiers from *V. cholerae* N16961 when possible. The boxes around gene identifiers denote how functions were predicted: gray shading, biochemical evidence in the vibrios; thick outline, microarray expression data (27); thin lines, bioinformatic prediction only; and dashed lines, predicted functions based on experimental evidence. (Expanded from reference 34 with permission of the publisher.)

(MCL). In return, the MCL yields a set of clusters, with each cluster containing a set of orthologs and/or recent paralogs. OrthoMCL was run with a BLAST E-value cutoff of 1e−6 and an inflation parameter of 1.5. Families related to chitin metabolism were obtained from the chitin pathway defined for *Vibrio cholerae* (Fig. 1) and by using a keyword search for "chitin" in the annotated genomes. For each of the chitin-related families, the orthologous genes were identified for all *Vibrionaceae* genomes with OrthoMCL, and a presence/absence profile was constructed. A complete list of locus tags and gene locations is provided in Table S2 in the supplemental material.

***Vibrionaceae* genome phylogeny.** A "whole-genome phylogeny" was generated for the annotated genomes by taking 100 randomly selected, single-copy genes present in all genomes. These were aligned in MUSCLE (9), and poorly aligned regions were removed; this concatenated alignment was used to estimate a maximum-likelihood phylogenetic tree by using the PhyML program with 100 bootstrap replicates (10), with options "0 i 1 100 GTR e e 4 e BIONJ y y."

Growth assays. *Vibrionaceae* strains were tested for growth on GlcNAc, α, and β chitin as the nitrogen and carbon nutrient sources (Table 1). Cultures were grown overnight in 0.25× 2216 medium (Difco) and diluted 1:100 in minimal medium containing chitin substrates. The minimal medium was derived from that used by Meibom et al. (26), i.e., 234 mmol/liter (brackish) or 428 mmol/liter (marine) NaCl, 27.5 mmol/liter $MgSO_4$, 4.95 mmol/liter $CaCl_2$, 5.15 mmol/liter KCl, 0.07 mmol/liter $Na_2B_4O_7$, 0.187 mmol/liter K_2HPO_4, 1× "K" trace metals (14), and 50 mmol/liter HEPES, pH 7.4, and supplemented with a filter-sterilized vitamin mixture (30). β-Chitin was isolated from squid pen (*Loligo pealei*) by treatment with 1 mol/liter NaOH for 5 h to remove protein, followed by extensive washing to remove residual base (6). Tubes containing media (15 ml) were supplemented with 25 mmol/liter GlcNAc or 0.05 g of either crab shell α-chitin (Sigma) or β-chitin. Strains were grown at room temperature (~22°C) with shaking at 150 rpm, and growth was assessed every 2 days. A starting optical density at 600 nm of less than or equal to 0.01 that increased to a value of at least 0.1 by day 30 was scored as positive.

PCR amplification and phylogeny of *chiA*. PCR primers designed to target all known proteobacterial *chiA* genes were used to amplify and sequence this gene in vibrio isolates: chiAf (GGN GGN TGG CAN YTN WSN GAY CCN TT) (8) and chiAr (ATR TCN CCR TTR TCN GCR TC) (22). DNA was obtained using a DNA extraction kit (Gentra) or Lyse'N Go (Pierce). The PCR mixture contained 1 μmol/liter final concentration of chiAf and chiAr, 0.75 U Jumpstart *Taq* (Sigma), 200 μmol/liter deoxynucleoside triphosphates, and 1× buffer. The PCRs were thermocycled as follows: 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final 6-min extension at 72°C. Alternate primers targeting *Photobacterium profundum chiA* family sequences were designed based on the sequences of strains SS9 and 3TCK and contain all codon degeneracies. Primers Pprof_chiAf (AAR CAY TTY CCN GAR ATG GCN GC) and Pprof_chiAr (TCR TTR TCN ACD ATR TAY TGN GC) were amplified as described above.

An alignment, including *chiA* gene sequences from diverse isolates, previously analyzed taxa (22), and whole genomes, was prepared using Clustal and refined manually. Ambiguously aligned regions were excluded, yielding an alignment of 603 nucleotide positions. The maximum-likelihood tree was constructed using PHYML under the GTR model with estimation of all parameters and generation of 100 bootstraps (10).

Additional gene sequencing. The partial 16S rRNA gene was amplified as described previously (41) and identified based on similarity to database sequences (1). For a limited subset of isolates, adenylate kinase (*adk*) and malate dehydrogenase (*mdh*) sequences were amplified as described previously (38).

Nucleotide sequence accession numbers. Sequences were submitted to GenBank with accession numbers EU177043 to EU177094.

RESULTS AND DISCUSSION

Chitinolytic pathway in *V. cholerae*. The chitinolytic system in the vibrios channels chitin monomers into the central metabolism as fructose-6-phosphate (fructose-6-P), acetate, and ammonium (18). We refine a previous representation of the chitinolytic pathway (34) by incorporating literature data on biochemical experiments, microarray expression data, and bioinformatic predictions to fill gaps in the pathway related to chitobiose metabolism and identify a core set of genes which are responsible for chitin degradation in vibrios.

Figure 1 depicts the proposed chitin catabolic cascade in *V. cholerae*, beginning with the breakdown of chitin polymer into oligomers by extracellular chitinases (Fig. 1, label 1). These genes are assumed to have differential activity or regulation and act collectively to degrade chitin into $(GlcNAc)_{n>2}$ oligosaccharides (31, 39), which are transported into the periplasmic space via a specific porin (Fig. 1, label 2) (15). The monomer GlcNAc and dimer *N*,*N*′-diacetylchitobiose are thought to enter the periplasm by nonspecific porins. Once in the periplasm, chitin oligosaccharides are degraded by periplamic chitinodextrinases (Fig. 1, label 3) (17) and β-*N*-acetylglucosaminidases (Fig. 1, label 4) (16) to $(GlcNAc)_{1,2}$. $(GlcNAc)_2$ is transported across the inner membrane by an ABC-type transporter (Fig. 1, label 5) (24), whereas GlcNAc can be transported into the cytosol and phosphorylated via a PTS transporter (Fig. 1, label 8) (5). In the cytosol, $(GlcNAc)_2$ is converted into 2(GlcNAc-6-P) by an *N*,*N*′-diacetylchitobiose phosphorylase (Fig. 1, label 6) (32), a GlcNAc-1P-mutase (Fig. 1, label 7) (24), and a predicted GlcNAc-specific ATP-dependent kinase (gene not identified) (4). The GlcNAc-6-P generated either during uptake by the PTS or by the *N*,*N*′-deacetylchitobiose phosphorylase pathway is converted into fructose-6-P via the action of an *N*-acetylglucosamine-6-phosphate deacetylase

TABLE 1. Growth of vibrio isolates on different forms of chitin and GlcNAc

Organism (BLAST identity [%])[a]	Strain	*chiA* PCR result[b]	Growth assay result[c]			Reference or source[d]
			GlcNAc	α-Chitin	β-Chitin	
P. damselae (99)	12H05	−	+	+	+	37
P. kishitanii (95)	14H04	−	+	+	+	37
P. phosphoreum (96)	14E11	−	+	+	+	37
P. profundum (96)	14G04	−	+	+	+	37
P. profundum (99)	7A02	−	+	−	−	37
V. aestuarianus (99)	12C03	+	+	+	+	37
V. alginolyticus	PWH3a	+	+	−	+	
V. alginolyticus (99)	14C03	+	+	+	+	37
V. alginolyticus (99)	12G01	+	+	+	+	37
V. anguillarum	ATCC 14181	+	+	+	+	KB
V. calviensis (99)	FALF182	+	+	+	+	IBYC
V. cholerae	0395	+	+	+	+	JM
V. cholerae	VO-146	+	+	−	+	JM
V. cholerae (99)	OP3D	+	+	+	+	OP
V. cholerae (99)	OP7F	+	+	+	+	OP
V. cholerae 569B	ATCC 25870	+	+	+	+	JM
V. cholerae E7946	ATCC 55056	+	+	+	+	JM
V. fischeri (98)	14A09	+	+	+	+	37
V. fischeri (98)	14A08	+	+	−	−	37
V. fischeri (99)	14C05	+	+	+	+	37
V. fischeri (99)	7H01	−	+	−	−	37
V. fortis (99)	12F11	+	+	+	+	37
V. furnissii (99)	12F04	+	+	+	+	37
V. halioticoli (97)	7A03	−	+	−	−	37
V. halioticoli (97)	7H03	−	+	−	−	37
V. halioticoli (98)	1C10	−	+	−	−	37
V. halioticoli (99)	1A06	−	+	−	−	37
V. halioticoli (99)	1A07	−	+	−	−	JM
V. harveyi	B392	+	+	+	+	KB
V. hispanicus (98)	FALF230	−	+	−	−	IBYC
V. ichthyoenteri (94)	FALF124	+	+	+	+	IBYC
V. lentus (98)	12B10	+	+	−	+	37
V. logei	ATCC 35077	+	+	−	+	KB
V. logei (99)	7A08	−	+	−	+	37
V. metschnikovii (99)	OP5F	+	+	−	+	OP
V. mytili (98)	1B04	+	+	+	+	37
V. natriegens	ATCC 14048	+	+	−	+	KB
V. neptunius (98)	FALF109	+	+	+	+	IBYC
V. ordalii	ATCC 33509	+	+	+	+	KB
V. ordalii (100)	14C08	+	+	+	+	37
V. orientalis	ATCC 33434	+	+	+	+	KB
V. parahaemolyticus	ATCC 17802	+	+	+	+	ATCC
V. parahaemolyticus (97)	1A02	−	+	−	−	37
V. ponticus (97)	12D02	−	+	+	+	37
V. rumoiensis (95)	1C01	+	+	−	−	37
V. shilonii (99)	12F08	+	+	+	+	37
Vibrio sp.	MED222	+	+	+	+	JP
V. splendidus (97)	14F04	+	+	−	+	37
V. splendidus (99)	12B01	+	+	+	+	37
V. splendidus biovar 2 (99)	1C05	+	+	−	+	37
V. tasmaniensis (98)	13B08	+	+	−	+	37
V. tubiashii	ATCC 19105	+	+	+	+	KB
V. vulnificus	"kathy"	+	+	+	+	KB
V. vulnificus	ATCC 27562	+	+	+	+	ATCC

[a] Environmental isolates were named using the best BLAST (1) hit for the partial 16S rRNA gene.
[b] +, a PCR band of the correct size was amplified; −, two or more PCRs failed to amplify a band of the correct size.
[c] +, an optical density at 600 nm of >0.1 was reached by day 30; −, no observable growth on the chitin substrate.
[d] Type strains used to assay chitin growth and amplify chitinase A sequences were obtained from ATCC (American Type Culture Collection), JM (laboratory of John Mekalanos, Harvard Medical School), JP (laboratory of Jarone Pinhassi, Kalmar University), or KB (laboratory of Kathy Boetcher, University of Maine). Environmental isolates were obtained from a previous study (41), and new strains were isolated from the IBYC (Ipswich Bay Yacht Club, Ipswich, MA) and OP (Oyster Pond, Falmouth, MA) as described previously (41).

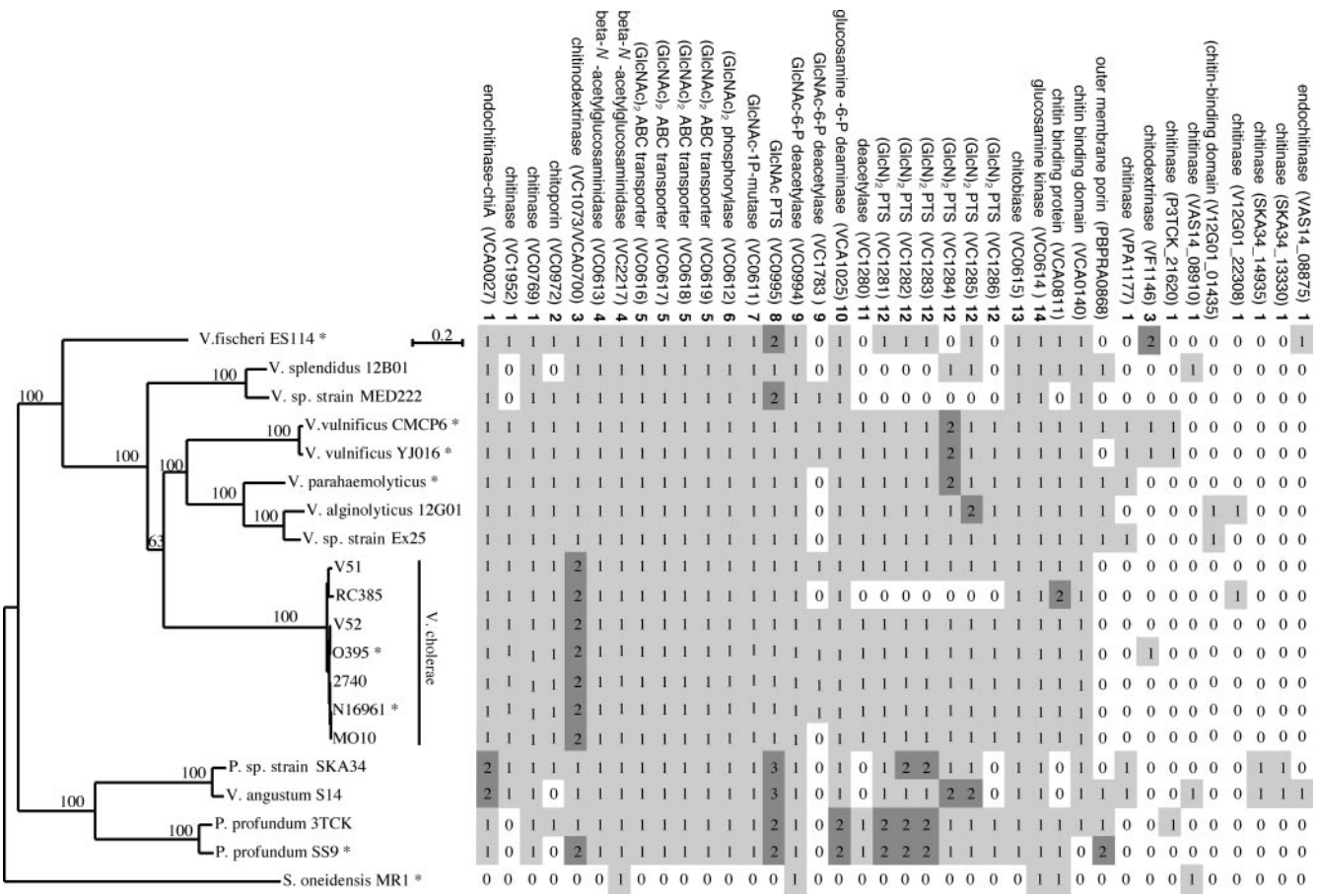
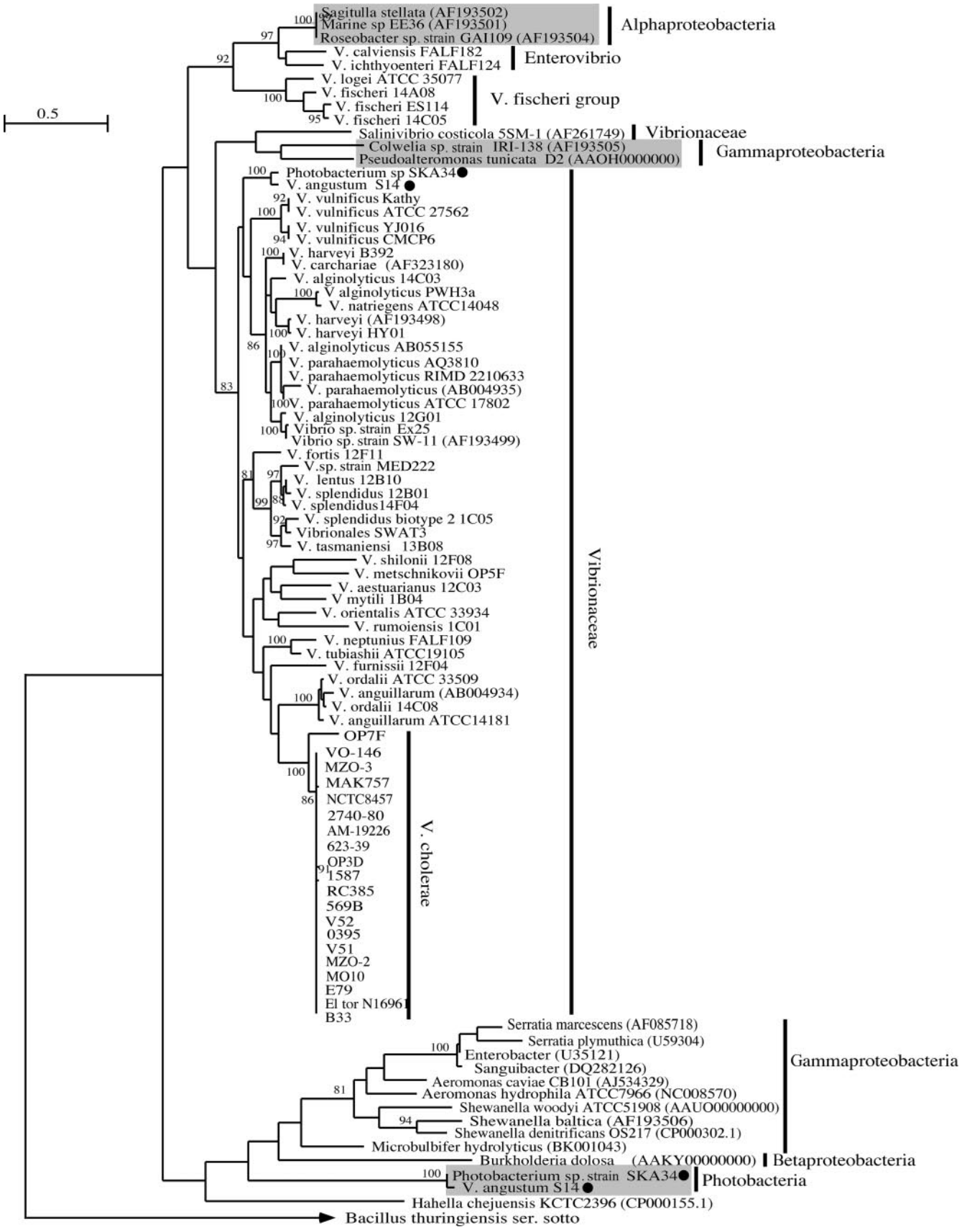


FIG. 2. Distribution of predicted chitin pathway genes among *Vibrionaceae* genomes. The phylogenetic relationship is based on maximum-likelihood analysis of a concatenation of 100 shared genes. Numbers at nodes represent values based on 100 bootstrap replicates. Each of the columns corresponds to a chitin metabolism-related gene family, with the family name indicating the predicted function and the number indicating the reaction or transport mechanism identifier from Fig. 1, with a representative gene designation in parentheses. The number within the box indicates the number of copies of that gene family in the corresponding genome, which is further indicated by light-gray shading for one gene copy or dark-gray shading for two or more gene copies. An asterisk indicates a complete genome sequence.

(Fig. 1, label 9) and a glucosamine-6-phosphate deaminase (Fig. 1, label 10) (11).

Complete degradation of chitin must also take into account the assimilation of deacetylated residues (GlcN), which can comprise up to a sixth of the residues in natural forms of chitin (28). Here, we propose a mechanism by which GlcN could be incorporated into the chitin catabolic cascade. Recently, a set of genes annotated as a cellobiose PTS transporter (Fig. 1, label 12) (VC1281 to VC1286) was demonstrated to transport $(GlcN)_2$ into the cytosol (27). An adjacent gene (VC1280) was also upregulated upon addition of $(GlcN)_2$ and has a predicted deacetylase function (Fig. 1, label 11), suggesting it converts GlcN-GlcNAc to $(GlcN)_2$. Once in the cytoplasm, the β1-4 linkage between the glucosamine residues could be broken by an enzyme (Fig. 1, label 13) currently characterized as a cellobiase (33). We reannotate this gene as a chitobiase, as *V. cholerae* does not grow on cellobiose and both substrates consist of β1-4-linked glucose. Further, this gene is upregulated by growth on chitin (27) and is adjacent to components of the chitin metabolic pathway. The cytoplasmic GlcN can be phosphorylated by an ATP-dependent glucosamine kinase (Fig. 1, label 14) (34) and converted to fructose-6-P (Fig. 1, label 10). The proposed chitin utilization scheme described above identifies a predicted chitin degradation core; the question is how well conserved this pathway is in the vibrios.

Distribution of chitin pathway genes in *Vibrionaceae* genomes. The conservation of the chitin degradation pathway in the sequenced *Vibrionaceae* genomes suggests that chitin metabolism is an ancestral feature of the vibrios (Fig. 2). In Fig. 2, the left panel depicts the phylogenetic relationships of the sequenced genomes, which demonstrate that gene presence/absence in the right panel has a phylogenetic context (e.g., the second copy of chitinodextrinase is shared among all *V. cholerae* genomes but not other isolates). The chitin degradation genes identified in *V. cholerae* (Fig. 1) appear to be almost universally conserved, with homologs identified for 91% of core gene matrix positions in sequenced genomes (Fig. 2). The genes in *V. cholerae* which are not well conserved in other genomes include the second copy of a GlcNAc-6-P deacetylase, an alternative chitinase (VC1952), and the $(GlcN)_2$ PTS transporter. We note that the *V. angustum* S14 whole-genome phylogeny and 16S rRNA gene sequence place this strain


0.5
Sagitulla stellata (AF193502)
Marine sp EE36 (AF193501)
Roseobacter sp. strain GAI109 (AF193504)
Alphaproteobacteria
V. calviensis FALF182
V. ichthyoenteri FALF124
Enterovibrio
V. logei ATCC 35077
V. fischeri 14A08
V. fischeri ES114
V. fischeri 14C05
V. fischeri group
Salinivibrio costicola 5SM-1 (AF261749)
Vibrionaceae
Colwelia sp. strain IRI-138 (AF193505)
Pseudoalteromonas tunicata D2 (AAOH0000000)
Gammaproteobacteria
Photobacterium sp SKA34
V. angustum S14
V. vulnificus Kathy
V. vulnificus ATCC 27562
V. vulnificus YJ016
V. vulnificus CMCP6
V. harveyi B392
V. carchariae (AF323180)
V. alginolyticus 14C03
V alginolyticus PWH3a
V. natriegens ATCC14048
V. harveyi (AF193498)
V. harveyi HY01
V. alginolyticus AB055155
V. parahaemolyticus AQ3810
V. parahaemolyticus RIMD 2210633
V. parahaemolyticus (AB004935)
V. parahaemolyticus ATCC 17802
V. alginolyticus 12G01
Vibrio sp. strain Ex25
Vibrio sp. strain SW-11 (AF193499)
V. fortis 12F11
V.sp. strain MED222
V. lentus 12B10
V. splendidus 12B01
V. splendidus14F04
V. splendidus biotype 2 1C05
Vibrionales SWAT3
V. tasmaniensi 13B08
V. shilonii 12F08
V. metschnikovii OP5F
V. aestuarianus 12C03
V mytili 1B04
V. orientalis ATCC 33934
V. rumoiensis 1C01
V. neptunius FALF109
V. tubiashii ATCC19105
V. furnissii 12F04
V. ordalii ATCC 33509
V. anguillarum (AB004934)
V. ordalii 14C08
V. anguillarum ATCC14181
OP7F
VO-146
MZO-3
MAK757
NCTC8457
2740-80
AM-19226
623-39
OP3D
1587
RC385
569B
V52
0395
V51
MZO-2
MO10
E79
El tor N16961
B33
V. cholerae
Vibrionaceae
Serratia marcescens (AF085718)
Serratia plymuthica (U59304)
Enterobacter (U35121)
Sanguibacter (DQ282126)
Aeromonas caviae CB101 (AJ534329)
Aeromonas hydrophila ATCC7966 (NC008570)
Shewanella woodyi ATCC51908 (AAUO00000000)
Shewanella baltica (AF193506)
Shewanella denitrificans OS217 (CP000302.1)
Microbulbifer hydrolyticus (BK001043)
Gammaproteobacteria
Burkholderia dolosa (AAKY00000000)
Betaproteobacteria
Photobacterium sp. strain SKA34
V. angustum S14
Photobacteria
Hahella chejuensis KCTC2396 (CP000155.1)
Bacillus thuringiensis ser. sotto

within the genus *Photobacterium*, and it is included in this group for subsequent analyses. Gene families annotated with chitin-related functions and present in at least two genomes are also shown in Fig. 2; these genes have a spotty distribution in *Vibrionaceae* genomes, suggesting that outside of the chitin degradation core, there is tremendous gene content flexibility.

There is also evidence for several gene duplications. For example, the chitodextrinases (Fig. 1, label 3) contain two orthologous copies in all *V. cholerae* genomes and in *P. profundum* SS9, although one of the copies (VC1073) is not upregulated in the presence of chitin (27), suggesting that this gene may no longer be active in chitin degradation. Additionally, the *chiA* gene family has two copies in *Photobacterium* sp. strain SKA34 and *V. angustum*, one of which clusters with the vibrios while the second is more closely related to other proteobacteria (Fig. 3). However, multiple copies of PTS genes (Fig. 1, labels 8 and 12) may reflect similarities between transporters for different substrates rather than multiple copies of the same gene.

Growth of *Vibrionaceae* environmental isolates on chitin substrates. Although the genome analysis suggests that chitin utilization is a universal characteristic among *Vibrionaceae*, a previous study indicated that growth on chitin was distributed spottily among *Vibrionaceae* isolates (35). Therefore, a more diverse set of isolates was tested for growth on α and β chitin as well as GlcNAc, the monomer of chitin. Growth on GlcNAc is common among marine bacteria, even among those not capable of metabolizing chitin (44). Indeed, all 54 *Vibrionaceae* strains assayed grew on GlcNAc as the sole nitrogen and carbon nutrient, including the few strains which did not grow on chitin and appeared to lack *chiA* genes (e.g., *V. halioticoli* and *V. hispanicus*) (Table 1). This suggests that growth on GlcNAc is not a good indicator of chitin metabolism and is consistent with the previously suggested GlcNAc uptake by the PTS system, which is independent of chitin degradation (Fig. 1).

The majority of isolates also grew on both α and β chitin, although 10 strains grew only on the more enzymatically accessible β form. Overall, the broad distribution of chitin metabolism suggests that chitin degradation is indeed an ancestral capability of the vibrios. However, several isolates were incapable of chitin degradation (Table 1), corroborating that it is not a universally conserved characteristic within the vibrios and that strains within a family may have alternate lifestyles. However, the fraction of isolates which displayed growth on chitin was much higher than that reported in the previous study (35), and we attribute this to more complete media containing trace metals and vitamins. Several isolates, including both *V. ordalii* strains, *V. ichthyoenteri*, and *V. calviensis*, produced a yellow pigment when attached to chitin but not when grown on rich media, glucose, or GlcNAc, indicating that chitin or perhaps biofilm growth regulates pigment production.

Diversity of chitinase A among *Vibrionaceae*. The *chiA* gene fragments amplified from strains listed in Table 1 were sequenced and found to be highly divergent, with a maximum nucleotide divergence of 55% within the genus *Vibrio*, compared to 22% for *recA* (40) and ~10% for the 16S rRNA gene within *Vibrionaceae* (21). The photobacterial *chiA* sequences are even more diverse, with the second copy of the strain S14 and SKA34 *chiA* genes grouping with nonvibrio proteobacteria (Fig. 3), while the *P. profundum chiA* family genes share only ~30% amino acid identity with other vibrio sequences. The majority of *Vibrionaceae* strains form a large clade albeit without strong bootstrap support (Fig. 3), although the *Enterovibrio* sp. and *V. fischeri* sequences are distinct from this large cluster.

A positive *chiA* PCR assay was a good predictor of chitin metabolism; however, several *Photobacterium* and *Vibrio* strains gave negative PCR results but still grew on chitin. Indeed, the *P. profundum* genomes harbor highly divergent sequences (see Table S2 in the supplemental material), which are distinct from the other vibrio *chiA* sequences but contain the conserved catalytic site motif, suggesting chitinase activity (22). Because the "universal" proteobacterial *chiA* primers do not match these *P. profundum chiA* sequences, new primers were designed for the divergent *chiA* genes (see Table S2 in the supplemental material). However, these new primers did not capture additional *chiA* sequences in strains positive for growth on chitin, suggesting that *chiA* is either not necessary for chitin degradation or more diverse than previously anticipated. The second possibility is supported by phylogenetic analysis using additional genes (*hsp60*, *mdh*, and *adk*) for five *Vibrionaceae* isolates, which grew on chitin but had negative PCR results for *chiA*. Four of the strains, with 16S rRNA gene sequences most similar to those of *P. damselae* and *P. phosphoreum*, formed two deep clades within the photobacteria distinct from the sequenced genomes (see Fig. S1 in the supplemental material). Given that the sequenced photobacterial genomes contain divergent *chiA* sequences, these additional clades (see Fig. S1 in the supplemental material) may harbor highly differentiated *chiA* genes. This is an indication that even core chitin degradation genes are apparently subject to duplication and transfer.

The use of *chiA* to identify chitin degraders (8, 22, 35) is problematic, as even within a single bacterial family, the *chiA* gene family is too divergent to capture with PCR primers. Additionally, there is evidence for lateral gene transfer (LGT) or duplication of this gene, which will make developing relationships with the organismal phylogeny difficult (8). The phylogeny of the *chiA* gene suggests several other instances of LGT (Fig. 3); the most obvious is the placement of alphaproteobacterial sequences in a node within the *Enterovibrio* group that has a well-supported bootstrap value. While some *Alphaproteobacteria* contain the pathways to assimilate GlcNAc, chitinase-like sequences have not been observed thus far in sequenced genomes (44). Moreover, Cottrell et al. (8) found that the *chiA*-containing alphaproteobacterial isolates did not grow on chitin, suggesting a nonfunctional chitinoclastic path-

FIG. 3. Phylogenetic relationships of partial *chiA* gene sequences from *Vibrionaceae* and related organisms based on maximum-likelihood analysis. Numbers shown at nodes represent values based on 100 bootstrap replicates; only nodes with values of >80 are shown. Branch length to the outgroup is truncated, as indicated by the arrow. GenBank accession numbers are given for previously sequenced genes. Gray boxes indicate potential LGT events. Round circles indicate the two copies of *chiA* family genes in *Photobacteria.*

way, potentially a hallmark of LGT into a strain without a complete metabolic pathway. Perhaps the chitinase gene in these strains has taken on another role, such as serving as a chitin attachment protein. Less well-supported evidence of gene transfer includes the presence of a second *chiA* family gene in *Photobacterium* SKA34 and *V. angustum* S14 more closely related to nonvibrio proteobacteria, as well as several gammaproteobacterial sequences that cluster within the vibrios (Fig. 2 and 3). Although *chiA* appears subject to lateral transfer and/or duplication, there is no other gene that serves as a good indicator of growth on chitin; alternate exochitinases are either not present in all sequenced genomes (Fig. 2) or not upregulated in the presence of chitin (Fig. 1). Additional genome sequencing of photobacteria may reveal alternate genes/pathways of chitin metabolism.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Aluwihare, L. I., D. J. Repeta, S. Pantoja, and C. G. Johnson.** 2005. Two chemically distinct pools of organic nitrogen accumulate in the ocean. Science **308:**1007–1010.
3. **Amako, K., S. Shimodori, T. Imoto, S. Miake, and A. Umeda.** 1987. Effects of chitin and its soluble derivatives on survival of *Vibrio cholerae* O1 at low temperature. Appl. Environ. Microbiol. **53:**603–605.
4. **Bassler, B. L., C. Yu, Y. C. Lee, and S. Roseman.** 1991. Chitin utilization by marine bacteria—degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. J. Biol. Chem. **266:**24276–24286.
5. **Bouma, C. L., and S. Roseman.** 1996. Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii*—molecular cloning and analysis of the glucose and N-acetylglucosamine permeases. J. Biol. Chem. **271:**33457–33467.
6. **Chaussard, G., and A. Domard.** 2004. New aspects of the extraction of chitin from squid pens. Biomacromolecules **5:**559–564.
7. **Colwell, R. R.** 1996. Global climate and infectious disease: the cholera paradigm. Science **274:**2025–2031.
8. **Cottrell, M. T., D. N. Wood, L. Y. Yu, and D. L. Kirchman.** 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the α- and γ-subclasses of the proteobacteria. Appl. Environ. Microbiol. **66:**1195–1201.
9. **Edgar, R. C.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.
10. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
11. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Y. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
12. **Heidelberg, J. F., K. B. Heidelberg, and R. R. Colwell.** 2002. Bacteria of the γ-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. Appl. Environ. Microbiol. **68:**5498–5507.
13. **Huq, A., R. B. Sack, A. Nizam, I. M. Longini, G. B. Nair, A. Ali, J. G. Morris, M. N. H. Khan, A. K. Siddique, M. Yunus, M. J. Albert, D. A. Sack, and R. R. Colwell.** 2005. Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. Appl. Environ. Microbiol. **71:**4645–4654.
14. **Keller, M. D., R. C. Selvin, W. Claus, and R. R. L. Guillard.** 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. **23:**633–638.
15. **Keyhani, N. O., X. B. Li, and S. Roseman.** 2000. Chitin catabolism in the marine bacterium *Vibrio furnissii*—identification and molecular cloning of a chitoporin. J. Biol. Chem. **275:**33068–33076.
16. **Keyhani, N. O., and S. Roseman.** 1996. The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*—molecular cloning, isolation, and characterization of a periplasmic beta-N-acetylglucosaminidase. J. Biol. Chem. **271:**33425–33432.
17. **Keyhani, N. O., and S. Roseman.** 1996. The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*—molecular cloning, isolation, and characterization of a periplasmic chitodextrinase. J. Biol. Chem. **271:**33414–33424.
18. **Keyhani, N. O., and S. Roseman.** 1999. Physiological aspects of chitin catabolism in marine bacteria. Biochim. Biophys. Acta **1473:**108–122.
19. **Kirchman, D. L., and J. White.** 1999. Hydrolysis and mineralization of chitin in the Delaware Estuary. Aquat. Microb. Ecol. **18:**187–196.
20. **Kirn, T. J., B. A. Jude, and R. K. Taylor.** 2005. A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature **438:**863–866.
21. **Kita-Tsukamoto, K., H. Oyaizu, K. Nanba, and U. Simidu.** 1993. Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. Int. J. Syst. Bacteriol. **43:**8–19.
22. **LeCleir, G. R., A. Buchan, and J. T. Hollibaugh.** 2004. Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment-specific distributions. Appl. Environ. Microbiol. **70:**6977–6983.
23. **Li, L., C. J. Stoeckert, and D. S. Roos.** 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. **13:**2178–2189.
24. **Li, X. B., and S. Roseman.** 2004. The chitinolytic cascade in vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. Proc. Natl. Acad. Sci. USA **101:**627–631.
25. **McCarthy, M., T. Pratum, J. Hedges, and R. Benner.** 1997. Chemical composition of dissolved organic nitrogen in the ocean. Nature **390:**150–154.
26. **Meibom, K. L., M. Blokesch, N. A. Dolganov, C. Y. Wu, and G. K. Schoolnik.** 2005. Chitin induces natural competence in *Vibrio cholerae*. Science **310:**1824–1827.
27. **Meibom, K. L., X. B. B. Li, A. T. Nielsen, C. Y. Wu, S. Roseman, and G. K. Schoolnik.** 2004. The *Vibrio cholerae* chitin utilization program. Proc. Natl. Acad. Sci. USA **101:**2524–2529.
28. **Muzzarelli, R. A. A.** 1973. Natural chelating polymers; alginic acid, chitin and chitosan. Pergamon Press, Oxford, United Kingdom.
29. **Nalin, D. R., V. Daya, A. Reid, M. M. Levine, and L. Cisneros.** 1979. Adsorption and growth of *Vibrio cholerae* on chitin. Infect. Immun. **25:**768–770.
30. **Newman, D. K., E. K. Kennedy, J. D. Coates, D. Ahmann, D. J. Ellis, D. R. Lovley, and F. M. M. Morel.** 1997. Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Arch. Microbiol. **168:**380–388.
31. **Orikoshi, H., S. Nakayama, K. Miyamoto, C. Hanato, M. Yasuda, Y. Inamori, and H. Tsujibo.** 2005. Roles of four chitinases (ChiA, ChiB, ChiC, and ChiD) in the chitin degradation system of marine bacterium *Alteromonas* sp. strain O-7. Appl. Environ. Microbiol. **71:**1811–1815.
32. **Park, J. K., N. O. Keyhani, and S. Roseman.** 2000. Chitin catabolism in the marine bacterium *Vibrio furnissii*—identification, molecular cloning, and characterization of a N-N′-diacetylchitobiose phosphorylase. J. Biol. Chem. **275:**33077–33083.
33. **Park, J. K., L. X. Wang, H. V. Patel, and S. Roseman.** 2002. Molecular cloning and characterization of a unique beta-glucosidase from *Vibrio cholerae*. J. Biol. Chem. **277:**29555–29560.
34. **Park, J. K., L. X. Wang, and S. Roseman.** 2002. Isolation of a glucosamine-specific kinase, a unique enzyme of *Vibrio cholerae*. J. Biol. Chem. **277:**15573–15578.
35. **Ramaiah, N., R. T. Hill, J. Chun, J. Ravel, M. H. Matte, W. L. Straube, and R. R. Colwell.** 2000. Use of a *chiA* probe for detection of chitinase genes in bacteria from the Chesapeake Bay. FEMS Microbiol. Ecol. **34:**63–71.
36. **Reguera, G., and R. Kolter.** 2005. Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. J. Bacteriol. **187:**3551–3555.
37. **Riemann, L., and F. Azam.** 2002. Widespread *N*-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. Appl. Environ. Microbiol. **68:**5554–5562.
38. **Santos, S. R., and H. Ochman.** 2004. Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. Environ. Microbiol. **6:**754–759.
39. **Svitil, A. L., S. M. N. Chadhain, J. A. Moore, and D. L. Kirchman.** 1997. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Appl. Environ. Microbiol. **63:**408–413.
40. **Thompson, F. L., D. Gevers, C. C. Thompson, P. Dawyndt, S. Naser, B. Hoste, C. B. Munn, and J. Swings.** 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl. Environ. Microbiol. **71:**5107–5115.
41. **Thompson, J. R., S. Pacocha, C. Pharino, V. Klepac-Ceraj, D. E. Hunt, J. Benoit, R. Sarma-Rupavtarm, D. L. Distel, and M. F. Polz.** 2005. Genotypic

diversity within a natural coastal bacterioplankton population. Science **307:** 1311–1313.

42. **Thompson, J. R., and M. F. Polz.** 2006. Dynamics of vibrio populations and their role in environmental nutrient cycling, p. 190–203. *In* F. L. Thompson, B. Austin, and J. Swings (ed.), The biology of vibrios. ASM Press, Washington, DC.
43. **Thompson, J. R., M. A. Randa, L. Marcelino, A. Tomita-Mitchell, E. Lim, and M. F. Polz.** 2004. Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Appl. Environ. Microbiol. **70:**4103–4110.
44. **Yang, C., D. A. Rodionov, X. Q. Li, O. N. Laikova, M. S. Gelfand, O. P. Zagnitko, M. F. Romine, A. Y. Obraztsova, K. H. Nealson, and A. L. Osterman.** 2006. Comparative genomics and experimental characterization of N-acetylglucosamine utilization pathway of *Shewanella oneidensis*. J. Biol. Chem. **281:**29872–29885.