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Identification of a Cellobiose Utilization Gene Cluster with Cryptic β-Galactosidase Activity in *Vibrio fischeri*▽

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Cellobiose utilization is a variable trait that is often used to differentiate members of the family *Vibrionaceae*. We investigated how *Vibrio fischeri* ES114 utilizes cellobiose and found a cluster of genes required for growth on this β-1,4-linked glucose disaccharide. This cluster includes genes annotated as a phosphotransferase system II (*celA*, *celB*, and *celC*), a glucokinase (*celK*), and a glucosidase (*celG*). Directly downstream of *celCBGKA* is *celI*, which encodes a LacI family regulator that represses *cel* transcription in the absence of cellobiose. When the *celCBGKAI* gene cluster was transferred to cellobiose-negative strains of *Vibrio* and *Photobacterium*, the cluster conferred the ability to utilize cellobiose. Genomic analyses of naturally cellobiose-positive *Vibrio* species revealed that *V. salmonicida* has a homolog of the *celCBGKAI* cluster, but *V. vulnificus* does not. Moreover, bioinformatic analyses revealed that CelG and CelK share the greatest homology with glucosidases and glucokinases in the phylum *Firmicutes*. These observations suggest that distinct genes for cellobiose utilization have been acquired by different lineages within the family *Vibrionaceae*. In addition, the loss of the *celI* regulator, but not the structural genes, attenuated the ability of *V. fischeri* to compete for colonization of its natural host, *Euprymna scolopes*, suggesting that repression of the *cel* gene cluster is important in this symbiosis. Finally, we show that the *V. fischeri* cellobioase (CelG) preferentially cleaves β-D-glucose linkages but also cleaves β-D-galactose-linked substrates such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), a finding that has important implications for the use of *lacZ* as a marker or reporter gene in *V. fischeri*.

The family *Vibrionaceae* encompasses a diverse collection of marine bacteria that are studied both for their direct significance to humankind and as models for a variety of basic biological phenomena (54). For example, our research group and others study *Vibrio fischeri* as a model of bacterial bioluminescence and for its symbiotic host-bacteria interactions and pheromone-mediated gene regulation (4, 38, 53, 58, 68). Other *Vibrio* species are investigated because of their pathogenicity for humans or marine animals, their contributions to aquatic ecosystems and processes, or their genomic plasticity and remarkable adaptability (15, 18, 61, 63). Although there are exceptions, a hallmark of this family is the ability to grow rapidly in culture and to use a variety of nutrient sources. There is considerable interspecies variability in metabolic patterns, and such differences may reflect ecologically important traits for individual species. Nutrient utilization profiles have also been important in practice both for developing taxonomic schemes to identify *Vibrio* species and for growing and manipulating these bacteria in the laboratory.

Traditionally, carbon source utilization patterns have been among the criteria used to distinguish different species within the family *Vibrionaceae* (6, 18), and this information has also guided the development of semiselective media to enrich for specific species (14). For example, cellobiose utilization is among the variable traits used to describe and distinguish species within the family *Vibrionaceae* (6), and cellobiose-based media have been used to enrich for certain species, particularly *Vibrio vulnificus* (11, 33). Molecular markers and DNA sequence analyses are becoming more widespread taxonomic tools with great value, but carbon source utilization phenotypes are still useful discriminators and have been tested through decades of research (6, 18). In the future, once the genetic basis for the utilization of particular carbon sources is better understood, it should be possible to generate molecular DNA-based techniques that draw directly on the wealth of phenotypic information available for identifying *Vibrio* species.

Understanding the genetic basis for various metabolism patterns among different *Vibrio* species will also help elucidate the evolutionary history of the *Vibrionaceae*. Despite interest in both the metabolic variability of this family and the mechanisms underlying its evolution, much remains to be learned about these subjects. For example, it is not known whether cellobiose utilization was an ancestral trait lost by some members of this family or if it was a trait acquired by certain lineages more recently. Bioinformatic analyses of genome sequences in the family *Vibrionaceae* (12, 21, 32, 46, 52, 64) promise to help answer such questions, but gene and pathway annotations can be ambiguous or incorrect. Therefore, continued experimental determination of metabolic pathways will be necessary to connect genomic and phenotypic variability.

In this study, we describe a gene cluster that is both necessary for cellobiose utilization by *V. fischeri* and sufficient to confer cellobiose utilization on other *Vibrio* species. Based on our results and bioinformatic analyses, we propose a model for cellobiose utilization arising from the acquisi-

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TABLE 1. Bacterial strains, select plasmids, and oligonucleotides

Bacteria, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference
E. coli		
CC118-λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	22
DH5α	F$^-$ Φ80d*lacZ*Δ(*lacZYA-argF*) *U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	20
DH5α-λ*pir*	DH5α lysogenized with λ*pir*	16
TOP10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA1 ara*Δ139 Δ(*ara-leu*)7697 *galU galK rpsL* (St^r) *endA1 nupG*	Invitrogen
Vibrionaceae		
ATCC 33653	*Vibrio mimicus*	E. Lipp
ATCC 33564	*Vibrio hollisae*	E. Lipp
ATCC 17803	*Vibrio parahaemolyticus*	E. Lipp
DMA401	ES114; *celI*::mini-Tn*5*-Em and 2-bp insertion in *celG*	This study
DMA420	ES114; *celI*::mini-Tn*5*-Em (insertion at bp 914)	This study
DMA421	ES114; *celI*::mini-Tn*5*-Em (insertion 53 bp before the predicted start of *celI*)	This study
DMA422	ES114; *celK*::mini-Tn*5*-Em (insertion at bp 548)	This study
DMA423	ES114; *celA*::mini-Tn*5*-Em (insertion at bp 20)	This study
DMA424	ES114; *ptsI*::mini-Tn*5*-Em (insertion at bp 247)	This study
DMA425	ES114; *celG*::mini-Tn*5*-Em (insertion at bp 502)	This study
DMA426	ES114; *celC*::mini-Tn*5*-Em (insertion at bp 1166)	This study
DMA427	ES114; *celB*::mini-Tn*5*-Em (insertion at bp 39)	This study
DMA428	ES114; VF2408::mini-Tn*5*-Em (insertion at bp 614) and 11-bp deletion in *celI* (at bp 566)	This study
DMA429	ES114; VF0170::mini-Tn*5*-Em (insertion at bp 314) and 11-bp deletion at in *celI* (at bp 566)	This study
ES114	Wild-type isolate from *E. scolopes*	7
KNH6	*Photobacterium leiognathi*	57
KV1319	ES114; 2-bp insertion in *celG*	This study
KV2801	ES114; *ptsI*::pTMO151	66
VC4056	*Vibrio cholerae*	R. Colwell
VC4103	*Vibrio cholerae*	R. Colwell
Plasmids		
pDMA171	*celI* in pVSV107	This study
pDMA193	P_{cel}-*gfp* reporter in pVSV209	This study
pEVS79	$oriV_{ColE1}$ $oriT_{RP4}$ Cm^r	57
pEVS170	mini-Tn*5*-Em, $oriV_{R6K\gamma}$ $oriT_{RP4}$ Kn^r	N. Lyell
pKV151	*celCBGKAI* plus 824 bp upstream of putative start in pVO8	This study
pKV162	2-bp insertion in *celG* and flanking sequences in pEVS79; source of *celG* allele in KV1319 and DMA401	This study
pVO8	$oriV_{P15A}$ $oriT_{RP4}$ Cm^r Em^r *lacZ*α	67
pVSV107	$oriV_{R6K\gamma}$ $oriT_{RP4}$ $oriV_{pES213}$ Tp^r *lacZ*α	17
pVSV209	$oriV_{R6K\gamma}$ $oriT_{RP4}$ $oriV_{pES213}$ *rfp* Kn^r promoterless Cm^r-*gfp*	17
Oligonucleotides		
dma91	5′CGGCGCTAGCGGTGCACGCCCAAGATCATATTATGAC 3′	This study
dma92	5′CGCCGCTAGCCGCTGTAACAGCCAGAGCAACAGG 3′	This study
dma93	5′GCCGCTAGCTGTGACTTCCTATATTTCAGCTTT 3′	This study
dma94	5′GGCGCTAGCTGTTCACCCCTAATTAGAATTATAATTTA 3′	This study

[a] St^r, streptomycin resistance; Kn^r, kanamycin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance.

tion of distinct pathways by different lineages within the family *Vibrionaceae*. We also show that this cellobiose utilization cluster in *V. fischeri* is responsible for an unexpected cryptic β-galactosidase activity. This observation has immediate practical significance, because the β-galactosidase gene *lacZ* from *Escherichia coli* has been used as both a marker (17, 24) and a transcriptional reporter (30, 69, 72, 73) in *V. fischeri*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are described in Table 1. Plasmids were maintained in *E. coli* strain DH5α (20), except conjugative helper plasmid pEVS104 (57), which was maintained in CC118λ*pir* (22); a pCR Blunt-TOPO-derivative, which was maintained in TOP-10 (Invitrogen, Carlsbad, CA); and other plasmids containing the R6Kγ replication origin, which were maintained in DH5αλ*pir* (16). *E. coli* was incubated at 37°C in LB medium (34) or brain heart infusion (BHI) medium (Difco, Sparks, MD). For selection of *E. coli*, chloramphenicol, kanamycin, and trimethoprim were used at concentrations of 20, 40, and 10 μg ml^{-1}, respectively. For the selection of *E. coli* with erythromycin (Em), 150 μg ml^{-1} was added to BHI medium. *V. fischeri* and all other *Vibrionaceae* strains were grown at 28°C in LBS medium (55) or by using a specific carbon source, as indicated, added to a minimal salts medium (0.340 mM $NaPO_4$ [pH 7.5], 0.05 M Tris [pH 7.5], 0.3 M NaCl, 0.05 M $MgSO_4$-$7H_2O$, 0.01 M $CaCl_2$-$2H_2O$, 0.01 M NH_4Cl, 0.01 M KCl, 0.01 mM $FeSO_4$-$7H_2O$, plus a carbon source). When chloramphenicol, kanamycin, Em, and trimethoprim were added to LBS or minimal medium for selection of *V. fischeri* or other *Vibrionaceae* strains, they were used at concentrations of 2, 100, 5, and 10 μg ml^{-1}, respectively. D-Cellobiose (Acros Organics, Geel, Belgium) was added to solid and liquid media at concentrations of 5 mM and 10 mM, respectively. Glucose was added to media at a concentration of 20 mM. Bromocresol purple sodium salt (BCP; Eastman Kodak, Rochester, NY) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Research Products International, Prospect, IL) were added to LBS medium at concentrations of 0.02 and 100 μg ml^{-1}, respectively. Agar was added to a final concentration of 1.5% for solid media.

DNA and plasmid manipulations. Standard methods were used to generate plasmids and to clone DNA fragments. Plasmids were mobilized from *E. coli* into recipients by triparental mating, using pEVS104 as a conjugative helper, as described previously (57). Restriction enzymes, DNA ligase, and Klenow fragment were obtained from New England Biolabs (Ipswich, MA). Chromosomal DNA was purified using an Easy-DNA kit (Invitrogen, Carlsbad, CA). Plasmids were isolated using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) or a GenElute plasmid miniprep kit (Sigma, St. Louis, MO). The Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA) was used to clone PCR products into pCR-BluntII-TOPO. DNA fragments were purified using a DNA Clean-up & Concentrator-5 kit (Zymo Research, Orange, CA). PCR was performed using KOD HiFi DNA polymerase (Novagen, Madison, WI), following the manufacturer's recommendations for cycle programs based on predicted DNA product size. Oligonucleotides (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA). The annealing temperature for primer pair dma91 and dma92 was 55°C, and for dma93 and dma94, it was 47°C. PCR was performed by using an iCycler unit (Bio-Rad Laboratories, Hercules, CA). DNA sequencing was conducted on an ABI automated DNA sequencer at the University of Michigan DNA sequencing core, and sequences were analyzed using Sequencher version 4.6 software (Gene Codes, Ann Arbor, MI).

Construction of mutants and complementation plasmids. Descriptions of select plasmids and the primers used in their construction are provided in Table 1. Details of plasmid construction are as follows. To generate a 2-bp insertion in *celG*, we first screened an existing library of XbaI-digested ES114 DNA cloned into pBluescript (Stratagene, La Jolla, CA) to isolate pKV150, which contains the *cel* gene cluster. pKV150 was digested with BglII, and the *cel* gene cluster was subcloned into BamHI-digested pEVS79, yielding pKV153. pKV153 was digested with SpeI and self-ligated to roughly center *celG* within the insert, making pKV156. pKV156 was then digested with ClaI, the overhangs were filled in with Klenow fragment, and the plasmid was recircularized by self-ligation, yielding pKV162, which has a frame-shifting 2-bp insertion in *celG*. This mutation was placed on the chromosomes of ES114 and DMA420 by allelic exchange, yielding strains KV1319 and DMA401, respectively.

The *celI* complementation plasmid pDMA171 was generated by first amplifying *celI* with ~500 bp of upstream sequence, thereby incorporating NheI sites that were engineered near the 5′ end of each primer (dma91 and dma92). This PCR product was cleaned and digested with NheI before being cloned directly into AvrII-digested pVSV107. Plasmid pKV151 contains the active *cel* cluster and was isolated from a library of BglII-digested ES114 DNA cloned into pV08 (2).

To construct pDMA193, the P_{cel}-*gfp* reporter, the intergenic DNA upstream of *celC* was PCR amplified with NheI sites incorporated near the 5′ ends of the primers (dma93 and dma94) and cloned into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA), yielding pDMA181. pDMA181 was digested with SpeI and XhoI, and the promoter fragment was subcloned into pEVS79, digested with the same enzymes, making pDMA184. pDMA184 was digested with NheI, and the promoter was subcloned into AvrII-digested pVSV209, which contains a promoterless *gfp* gene, completing the construction of pDMA193.

Transposon mutagenesis was performed by conjugating the mini-Tn*5* delivery plasmid pEVS170 (N. Lyell and E. Stabb, unpublished results) into wild-type *V. fischeri* strain ES114. After conjugation proceeded for more than 8 h, the conjugation mixture was diluted and plated onto selective media. The mini-Tn*5* mutagenesis was performed in three independent experiments for each screen, encompassing ~10,000 colonies per screen. In one screen, mutant colonies were isolated based on their blue color on LBS-X-gal supplemented with Em. In the other screen, mutant colonies were examined for yellowish-white color on LBS medium supplemented with cellobiose, Em, and X-gal. The site of transposon insertion in each mutant strain was determined by cloning the transposon and flanking DNA and then sequencing across the transposon::chromosome junction, using the M13 Forward primer. Insertions were cloned by digesting chromosomal DNA with HhaI, self-ligating the fragments, and recovering the transposon and flanking DNA as a plasmid, taking advantage of the origin of replication and Em resistance gene contained within the transposon.

Carbon utilization assays. The ability to grow on glucose or on cellobiose as the sole carbon source was tested by adding these sugars to a minimal medium and then streaking single colonies of each strain onto plates, which were incubated at 28°C for ~48 h and assessed for growth. To test for acid production by strains in the presence of glucose or cellobiose, single colonies were used to inoculate test tubes containing LBS medium with BCP and either cellobiose, glucose, or no sugar added. Cultures were incubated at 28°C with shaking (200 rpm) for 24 h, and acidification was scored as a change in the BCP from purple to yellow.

***cel* induction measurements in culture.** Overnight cultures of *V. fischeri* carrying pDMA193(P_{cel}-*gfp*) were grown in LBS with appropriate antibiotics and diluted 1:500 into 30 ml of antibiotic-free LBS medium, with or without cellobiose or glucose, in 125-ml baffled flasks and were then incubated at 24°C with shaking (200 rpm). The reporter and control plasmids used are derived from a vector that is stable in *V. fischeri* and does not require selection for maintenance (17). Samples (500-μl) were removed at intervals and the culture optical density at 595 nm (OD_{595}) was determined by using a BioPhotometer unit (Brinkman Instruments, Westbury, NY). Fluorescence was measured using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA), using excitation and emission filters of 486 nm and >510 nm, respectively. The fluorescence reported is the average of measurements taken when the OD_{595} readings were approximately 2.5. The fluorescence of strains carrying the promoterless *gfp* construct in pVSV209 was subtracted as background.

To examine the ability of various carbon sources to induce the *cel* operon, 15 μl of 100 mM stocks of cellobiose, raffinose, sucrose, maltose, lactose, *N*-acetylglucosamine, fructose, mannose, ribose, galactose, xylose, arabinose, and glucose were spotted onto filter disks placed on LBS-X-gal plates and spread-plated with ES114. After 24 h of incubation, plates were examined for the induction of the *cel* operon, which was scored as rings of blue in the lawn surrounding the sugar-impregnated disk. Parallel plates with the *celG* mutant KV1319 served as negative controls and did not develop blue color.

Enzyme assays using *p*NP-conjugated substrates. The strains tested were grown to an OD_{595} of ~2.0, pelleted, and lysed by freezing at −80°C for 20 min, and the pellets were resuspended in the original volume of a 500 mM sodium phosphate buffer (pH 7.0). One hundred microliters of this lysate was added to 400 μl of a 10 mM *p*-nitrophenol (*p*NP)-conjugated substrate dissolved in 50 mM sodium phosphate buffer (pH 7.0). Parallel reaction mixtures were incubated at 28°C and 37°C until a yellow color was observed or for a maximum of 24 h. The assay was stopped by adding 2 ml of 1 M Na_2CO_3 (final concentration of 800 mM). A 1-ml sample from the reaction mixture was centrifuged for 5 min to pellet cell debris. The absorbance was read at 410 nm (A_{410}) to determine the amount of *p*NP generated from enzymatic cleavage, and at A_{550} to determine light scattering from residual cell debris. To calculate pmol of *p*NP generated min^{-1} ml^{-1}, the A_{410} reading from each sample minus the A_{550} reading for each sample was compared to a linear standard of *p*NP, and this was divided by the incubation time, and the 0.1 ml of lysate was added to the reaction mixture.

Squid colonization assays. *E. scolopes* host animals were maintained in Instant Ocean (Aquarium Systems, Mantor, OH) mixed to ~36 ppt. To determine whether a mutant strain had a competitive disadvantage in the symbiosis relative to the wild type, cultures for inoculation were grown as previously described (17), and juvenile squid were exposed to a ~1:1 mixture of the wild-type and mutant strains for 14 h and then moved to *V. fischeri*-free Instant Ocean. Squid were homogenized after 48 h to determine the ratio of wild-type to mutant strains. The relative competitive index (RCI) was determined by dividing the mutant-to-wild type ratio for each individual squid by the ratio for the inoculum. Log-transformed data were used to calculate the average RCI and to determine statistical significance.

Bioinformatic analyses. Protein sequence comparisons to GenBank entries were generated using BLASTp (3). *V. salmonicida* LFI1238 sequence was obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/V_salmonicida/) as a shotgun database, and homologs of specific *V. fischeri* genes were determined by using Artemis software (48). Genomes with similar regions surrounding the CelC open reading frame (ORF) were found by using the SEED pinned region search (40). The similarities reported between homologs were determined by MatGAT software using the default settings (10). Phylogenetic and molecular evolutionary analyses were conducted with MEGA software version 4.0, using the default settings (60). Using the MEGA program, consensus neighbor-joining phylogenetic trees were constructed by using the amino-Poisson correction. The unweighted-pair group method with arithmetic mean (UPGMA) and minimum evolution trees were also constructed with similar results (data not shown). Bootstrap values for the trees were obtained from a consensus tree based on 1,000 randomly generated trees, using MEGA 4.0 software (60).

RESULTS

Mutations in *celI* reveal cryptic β-galactosidase activity in *V. fischeri*. This study was initiated by the serendipitous observation that when *V. fischeri* ES114 was mutagenized with mini-Tn*5* and plated on selective medium with X-gal, approximately

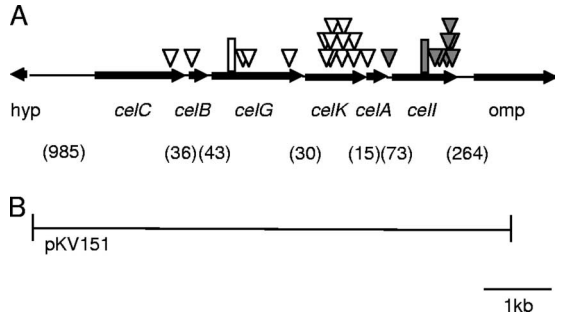

FIG. 1. Schematic representation of the genetic organization around the *cel* gene cluster in *V. fischeri* ES114. (A) Arrows represent ORFs and indicate the direction of gene transcription, as well as the gene size, which is presented relative to the scale bar. Shaded triangles represent transposon insertions that resulted in a blue colony phenotype on LBS-X-gal plates. Open triangles represent transposon insertions that resulted in a yellowish-white colony phenotype on LBS-X-gal cellobiose plates. A hypothetical gene is designated "hyp." The shaded rectangle denotes the 11-bp deletion detected in mutants DMA428 and DMA429. The open rectangle denotes the 2-bp insertion in strain KV1319. Numbers in parentheses represent the base pairs between ORFs. (B) The region cloned into plasmid pKV151.

1 in 1,000 colonies was dark blue, suggesting that some mutations could reveal a cryptic β-galactosidase activity in this strain. We isolated 10 such blue mutants and determined the location of the transposon insertion in these strains. Eight of the mutants had insertions in or directly upstream of VF0608, a gene that encodes a putative LacI family transcriptional regulator. We have now designated VF0608 as *celI*. The other two blue mutants, DMA428 and DMA429, had insertions in VF2408, a putative LuxR family transcriptional regulator, and VF0170, a putative O antigen flippase. However, these mutants, like the other eight, could be complemented to the parental yellowish-white colony color on LBS medium supplemented with X-gal (LBS-X-gal) by the reintroduction of *celI* on pDMA171 but not by the introduction of the parent vector pVSV107. Subsequent analyses showed that DMA428 and DMA429 each also had an identical 11-bp deletion in *celI*, which could account for their blue phenotype on LBS-X-gal plates (Fig. 1).

Identification of the cellobiose utilization gene cluster. Analysis of the genome near *celI* revealed that it was downstream of a gene cluster annotated as functioning in disaccharide uptake and catabolism, including a putative glucosidase (cellobioase) gene (Fig. 1). We hypothesized that the glucosidase gene encoded the enzyme responsible for the cleavage of X-gal and that *celI* repressed this operon in the absence of cellobiose. Consistent with this hypothesis, when wild-type ES114 was plated on LBS-X-gal and 5 mM cellobiose, all the colonies were blue. We then repeated the mini-Tn*5* mutagenesis but plated the mutants on LBS-X-gal and 5 mM cellobiose and screened for the loss of blue colony color, predicting that the inability to cleave X-gal would correlate with the loss of the ability to grow on cellobiose as the sole carbon source. After more than 10,000 colonies were screened, 16 transposon mutants were isolated based on their yellowish-white color on LBS-X-gal cellobiose plates. All but one insertion was localized to the predicted cellobiose utilization gene cluster upstream of *celI* (Fig. 1), and all of the mutants with insertions in this cluster grew poorly or did not grow with cellobiose as the sole carbon source. Moreover, in BCP assays, none of these mutants acidified the medium in the presence of cellobiose as the wild type did (Table 2). Each of the mutants with an insertion in the genes upstream of *celI* could be complemented to a wild-type-like phenotype upon reintroduction of this gene cluster on pKV151 (Fig. 1) but not by introduction of the parent vector pVO8. In light of these data, the genes in the cluster were named based on their predicted function: phosphotransferase system (PTS) IIA component, *celA* (VF0607); PTS IIB component, *celB* (VF0604); PTS IIC component, *celC* (VF0603); 6-phospho-β-glucosidase, *celG* (VF0605); glucokinase, *celK* (VF0606) and, as mentioned above, LacI-like family transcriptional regulator, *celI* (VF0608) (Fig. 1).

The single mutant with an insertion outside this gene cluster, DMA424, had an insertion in VF1896, which is annotated as a PTS system enzyme. Visick et al. (66) previously characterized a mutation in this gene and determined that the VF1895 and VF1896 ORFs were actually one gene, an ortholog to *E. coli ptsI*. Similarly to the *ptsI* mutant isolated by Visick et al. (66), we found that our *ptsI* mutant grew poorly in minimal medium with cellobiose or with glucose as the sole carbon source and had a slower growth rate than the wild type in LBS medium (data not shown). The *ptsI* gene encodes the E1 component of the PTS system, one of two proteins with essential roles as general components for all PTS systems. Because there is no other E1 encoded in *V. fischeri* ES114, the PTS II system comprised by CelA, CelB, and CelC should be severely attenuated or nonfunctional in the DMA424 mutant.

TABLE 2. Growth and acid production of *Vibrio* strains on glucose and cellobiose

Strain	Growth on minimal medium plus cellobiose		Acidification of LBS-BCP medium in the presence of:			
			Glucose		Cellobiose	
	pVO8	pKV151	pVO8	pKV151	pVO8	pKV151
ES114	+	+	+	+	+	+
KV1319	−	+	+	+	−	+
Photobacterium leiognathi	−	+	+	+	−	−
Vibrio cholerae VC4103	−	+	+	+	−	+
Vibrio cholerae VC4056	−	+	+	+	+	+
Vibrio parahaemolyticus	−	+	+	+	−	+
Vibrio hollisae	−	+	+	+	−	−
Vibrio mimicus	−	+	+	+	−	+

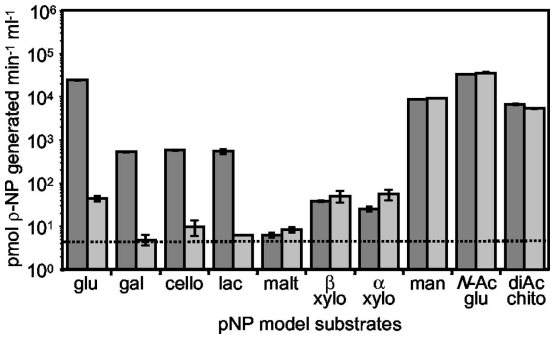

FIG. 2. *celG*-dependent enzymatic activity. Activities in lysates from DMA420 (*celI*::mini-Tn*5*-Em) are shown as dark gray bars, and activities from DMA401 lysates (*celG* frameshift, *celI*::mini-Tn*5*-Em) are shown as light gray bars. Activities represent the ability to cleave *p*NP from the substrate. Error bars, some too small to visualize, indicate standard error ($n = 3$). The dashed line represents the limit of detection. Results shown are representative of two experiments at 37°C and are also similar to data obtained from two experiments at 28°C. Substrate abbreviations: glu, *p*NP-β-D-glucopyranoside (model substrate for cellobiose); gal, *p*NP-β-D-galactopyranoside; cello, *p*NP-β-D-cellobioside; lac, *p*NP-β-D-lactopyranoside; malt, *p*NP-β-D-maltoside; β xylo, *p*NP-β-D-xylopyranoside; α xylo, *p*NP-α-D-xylopyranoside; man, *p*NP-β-D-mannopyranoside; *N*-Ac glu, *p*NP-*N*-acetyl-β-D-glucosaminide; and diAC chito, *p*NP-*N*,*N*′-diacetyl-β-D-chitobioside.

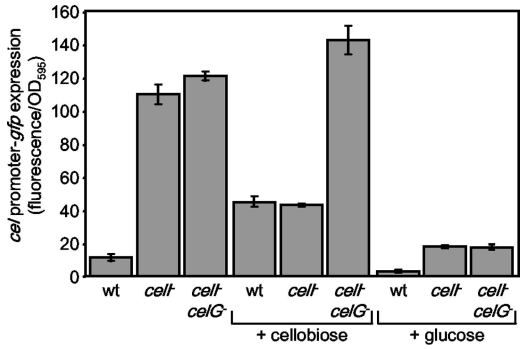

FIG. 3. Specific fluorescence generated from the P_{cel}-*gfp* reporter in ES114 (wild type [wt]), the mutant DMA420 (*celI* mutant), or DMA401 (*celI celG* double mutant). Cultures of strains carrying pDMA193 were grown in LBS with and without 10 mM cellobiose or 20 mM glucose. Data represent the average specific fluorescence with standard error ($n = 3$).

***celG* encodes a β-glucosidase with lower β-galactosidase activity.** To investigate whether the putative 6-phospho-β-glucosidase encoded by *celG* was responsible for the cleavage of cellobiose and X-gal, we generated mutant KV1319, which contains a 2-bp insertion in *celG*. KV1319 was unable to cleave X-gal and unable to utilize cellobiose as a sole carbon source and did not acidify cellobiose-containing media in BCP assays. This provides further evidence that CelG is responsible for the cleavage of both cellobiose and X-gal, a supposition that is also supported by the enzymatic assays described below. To test the prediction that the *celI* mutant colony's blue phenotype on LBS-X-gal was due to the loss of CelI-mediated repression of *celG*, we incorporated the 2-bp frameshifting mutation in *celG* into DMA420, a *celI* transposon mutant. As predicted, the resulting strain (DMA401 *celG*; mutant, *celI*::mini-Tn*5*-Em) frameshift was yellowish-white in contrast to the blue color of the *celI* mutant on LBS-X-gal plates (data not shown).

To further test the substrates targeted by CelG, we assayed enzymatic activity in cell lysates by using sugar substrates paralinked to a nitrophenol group. We examined lysates of the *celI* mutant DMA420, which should enhance CelG activity by allowing the derepression of *celG*. To determine whether the utilization of a particular substrate was specific to CelG and not to some other enzyme in the whole-cell lysate, we compared the activity of *celI* mutant lysates (Fig. 2, dark gray bars) to that of lysates of the *celI celG* double mutant, DMA401 (Fig. 2, light gray bars). Thus, Fig. 2 shows both CelG-dependent activity, which is the difference between the activities in strains DMA420 and DMA401, and CelG-independent activity, which is the activity in strain DMA401.

Figure 2 shows that the *celG* mutant loses the ability to cleave the model substrate for cellobiose cleavage, *p*NPβ-D-glucopyranoside (Fig. 2, glu). Lower but significant CelG-dependent activity levels were observed with *p*NP-β-D-galactopyranoside (Fig. 2, gal), *p*NPβ-D-cellobioside (Fig. 2, cello), and *p*NP-β-D-lactopyranoside (Fig. 2, lac). We also observed modest (0.25%) residual activity toward *p*NP-β-D-glucopyranoside (Fig. 2, glu) even in the *celG* mutant DMA401, which could indicate that the frameshift in *celG* does not completely eliminate CelG function or that other enzymes in the lysate catalyze a relatively minor amount of hydrolysis of the substrate. To differentiate between these possibilities, we tested the *celG*::mini-Tn*5*-Em mutant, DMA425, and found it had activity toward *p*NP-β-D-glucopyranoside (Fig. 2, glu) similar to that of the *celG* frameshift mutant, supporting the idea that the residual activity is due to an unidentified enzyme rather than partial activity from the frameshift allele (data not shown).

Although each of the other substrates cleaved by CelG contains a β-1,4 linkage, the promiscuity of the enzyme was evident in its activity as both a β-glucosidase and a β-galactosidase. The latter may reflect a coincidental and physiologically irrelevant activity. Although CelG is apparently able to cleave *p*NP-β-D-lactopyranoside (Fig. 2, lac), *V. fischeri* is unable to utilize lactose as a carbon source. Moreover, the data below indicate that CelG is induced by the presence of cellobiose but not by lactose (Fig. 3). Given the ability of CelG to direct X-gal cleavage, the β-galactosidase activity attributed to CelG in this assay was expected; however, it is worth noting that this activity was ~50-fold lower than the β-glucosidase activity (Fig. 2).

The assays with other *p*NP-linked substrates indicate that enzymes other than CelG in the lysates direct cleavage of *p*NP-β-D-mannopyranoside (Fig. 2, man), *p*NP-*N*-acetyl-β-D-glucosaminide (Fig. 2, *N*-Ac glu), *p*NP-*N*,*N*′-diacetyl-β-D-chitobioside (Fig. 2, diAC chito), *p*NP-β-D-xylopyranoside (Fig. 2, β xylo), and *p*NP-α-D-xylopyranoside (Fig. 2, α xylo). We saw no enzymatic activity toward *p*NP-β-D-maltoside (Fig. 2, malt).

***celI*-cellobiose- and glucose-mediated control of *cel* expression.** To determine when the *cel* genes are induced, we developed a transcriptional reporter plasmid, pDMA193, containing the region immediately upstream of the *cel* gene cluster, driving the expression of *gfp*. Without cellobiose, the reporter's fluorescence in ES114 was slightly elevated above that of the background, but with growth in cellobiose, there was an increase of almost 5-fold in fluorescence (Fig. 3). In the *celI*::mini-Tn*5*-Em mutant DMA420, the reporter was ex-

pressed with and without added cellobiose, further supporting our prediction that CelI represses the *cel* gene cluster when cellobiose is not present.

Interestingly, in the presence of cellobiose, fluorescence of the reporter in the *celI* mutant DMA420 decreases compared to when it is growing without cellobiose. We hypothesized that the generation of glucose due to the cleavage of cellobiose might cause catabolite repression of the gene cluster, resulting in this inhibitory effect of cellobiose on *cel* expression. To examine this, both the wild type ES114 and the *celI* mutant DMA420 containing the reporter plasmid were grown in the presence of glucose. In both strains, fluorescence from the reporter plasmid decreased substantially when cells were grown in LBS medium supplemented with glucose (Fig. 3). Furthermore, we moved the reporter plasmid into DMA401, the *celI celG* double mutant, to assess whether the loss of CelG, which should reduce the breakdown of cellobiose to glucose, would allow induction of the gene cluster in the presence of cellobiose. In media with glucose, reporter expression in DMA401 was reduced, as it was in the other strains (Fig. 3). However, supplementation with cellobiose did not affect reporter expression in DMA401 (Fig. 3). Thus, it appears that *cel* expression is repressed by glucose that is either added exogenously or generated by CelG-dependent cleavage of cellobiose.

Taking advantage of the promiscuity of CelG and its ability to degrade X-gal, we next determined whether carbon sources other than cellobiose could induce the expression of the *cel* gene cluster, using a disc assay with ES114 or the *celG* mutant KV1319 plated on LBS-X-gal plates. We tested glucose, galactose, lactose, chitin-hexamers, cellulose, *N*-acetyl-glucosamine, and cellobiose and found that only cellobiose was able to induce the expression of the *cel* gene cluster, resulting in CelG-dependent cleavage of X-gal and development of blue color in the growth of ES114 cells around the disc (data not shown). Thus, even if CelG is able to cleave substrates other than cellobiose (as described above), the substrates above do not induce *celG* and are therefore unlikely to be physiologically relevant targets for the *cel* gene cluster.

The *celCBGKAI* gene cluster on pKV151 confers cellobiose utilization on six cellobiose-negative *Vibrio* strains. To determine whether this cellobiose-utilizing gene cluster in *V. fischeri* was sufficient to confer cellobiose utilization to other *Vibrio* strains, pKV151 (Fig. 1) was moved into six different *Vibrio* or *Photobacterium* strains that are negative for cellobiose utilization (Table 2). Transconjugants were tested for growth on solid medium with cellobiose as the sole carbon source and were also tested for growth in the presence of glucose (the breakdown product of cellobiose) as a control. Both ES114 and the *celG* frameshift mutant were also included as positive and negative controls, respectively. All of the strains grew regardless of the plasmid when glucose was the sole carbon source (data not shown); however, of the strains carrying the insertless vector pVO8, only ES114 grew on cellobiose (Table 2). Thus, for each of the other *Vibrio* or *Photobacterium* strains, the *V. fischeri cel* gene cluster on pKV151 conferred the ability to grow on cellobiose (Table 2).

Substrate utilization is often tested indirectly based on the production of fermentation acids in the presence of a particular sugar, resulting in a pH shift that can be detected by the colorimetric change in the dye BCP. We therefore also tested strains carrying pKV151 in BCP assays. All strains with either the control vector or pKV151 acidified glucose-containing LBS medium (Table 2). For *Vibrio cholerae* VC4103, *V. mimicus*, and *V. parahaemolyticus*, pKV151 conferred not only the ability to grow on cellobiose but also the production of acid in LBS supplemented with cellobiose. For the other strains, the ability to grow on cellobiose did not correlate with acid production in the presence of cellobiose. *Photobacterium leiognathi* and *V. hollisae* were unable to acidify the cellobiose-containing medium regardless of whether they carried the control vector or pKV151, whereas *V. cholerae* VC4056 acidified the medium regardless of whether it contained pKV151 or the control vector. Thus, although acid production is often used as an indirect indicator of sugar catabolism by *Vibrio* species (6, 18), direct testing for growth on cellobiose was a more reliable measure of this metabolic capability.

Bioinformatic analyses of the *cel* gene cluster. Using a combination of bioinformatic programs and databases (see Materials and Methods), we sought to determine whether *celCBGKAI* was an ancestral locus present in all cellobiose-utilizing members of the family *Vibrionaceae* and, if this was not the case, to determine the likely origin(s) of these genes. Comparisons of nucleotide or encoded protein sequences yielded similar results, and for the most part, only the latter are reported here, with nucleotide sequences used for reporting gene arrangement. We found that *V. salmonicida* strain LFI1238, which utilizes cellobiose (N.-P. Willassen, personal communication), has a homologous *celCBGKAI* cluster. The *cel* gene order is conserved in *V. salmonicida*, the encoded proteins were >90% similar to the respective homologs in *V. fischeri*, and the *V. salmonicida cel* gene cluster included both the genes for cellobiose utilization (Fig. 4A) and the regulator *celI* (not shown).

Photobacterium profundum SS9 and *V. vulnificus*, which are cellobiose-positive bacteria (18, 37), also had gene clusters that included PTS II system genes, a glucosidase gene, and, in the latter bacterium, a glucokinase gene as well; however, neither gene had impressive similarity to the *V. fischeri cel* cluster. For example, the putative glucosidase and glucokinase encoded by this *V. vulnificus* cluster shared only 29% and 33% similarity to CelG and CelK, respectively (Fig. 4A). Although *V. vulnificus* is able to grow on cellobiose, we speculate that this might not be the gene cluster responsible for cellobiose catabolism. In support of this idea, *V. cholerae* and *V. parahaemolyticus* are both cellobiose-negative bacteria, yet both have a gene cluster that is highly homologous to that described above in *V. vulnificus* (Fig. 4B). Partial genomic sequences are available for other cellobiose-utilizing *Vibrio* species, but additional clusters similar to *celCBGKAI* were not found. Indeed, homologs of the individual *V. fischeri* CelG and CelK proteins were notably absent. Taken together, our analyses suggest that *celCBGKAI* underpins cellobiose utilization in the *V. fischeri*/*V. salmonicida* clade but that distinct pathways may direct cellobiose catabolism in other members of the family *Vibrionaceae*.

Despite further analyses, the ancestry of the *celCBGKAI* genes in *V. fischeri* and *V. salmonicida* remains uncertain, and these genes may have multiple origins. It seems likely that *celI*, which encodes the LacI family regulator, originated within the family *Vibrionaceae*, as it shares high similarity with many regulators in this bacterial family (data not shown). Interestingly, however, CelG and CelK clustered most closely with ORFs

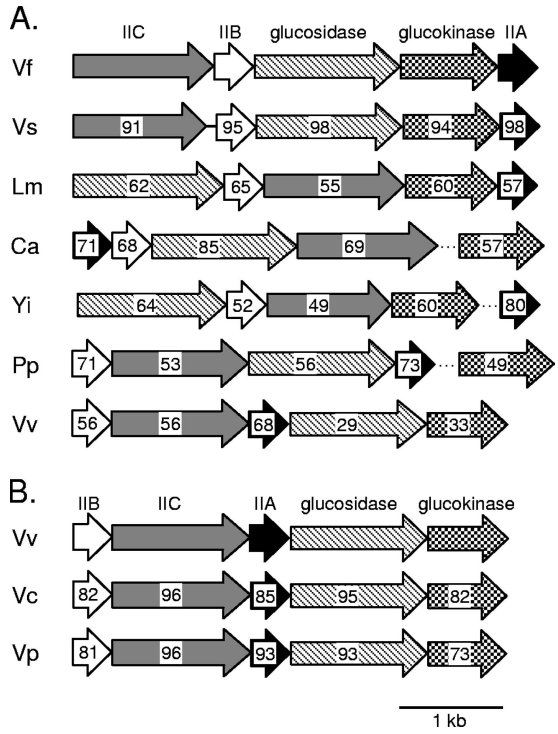

FIG. 4. Homologs of the *V. fischeri* cellobiose-utilizing genes. Gene arrangement and orientation for each particular strain are indicated with arrows. Arrows with the same shading or pattern are putative homologs. Genes encoding PTS components A, B, and C are shaded black, white, or gray, respectively. Genes encoding glucokinases or glucosidases are filled with checkerboard or crosshatch patterns, respectively. Dotted lines indicate that glucokinase or PTS IIA genes in these bacteria are not genetically linked to the other genes shown. Numbers within the arrows represent the overall similarity to the respective protein from *V. fischeri* (panel A) or *V. vulnificus* (panel B) as determined by MatGAT software (10). Species abbreviations are: Vf, *Vibrio fischeri*; Vs, *Vibrio salmonicida*; Lm, *Listeria monocytogenes*; Ca, *Clostridium acetobutylicum*; Yi, *Yersinia intermedia*; Pp, *Photobacterium profundum*; Vv, *Vibrio vulnificus*; Vc, *Vibrio cholerae*; Vp, *Vibrio parahaemolyticus*.

found in the phylum *Firmicutes*, particularly in the *Clostridium*, *Bacillus*, and *Listeria* species. This is illustrated both in the comparisons of similar gene clusters (Fig. 4) and in the neighbor-joining trees that compare the specific proteins encoded by these gene clusters (Fig. 5). Gene clusters in *Clostridium acetobutylicum* and *Listeria monocytogenes* were more similar to the *V. fischeri cel* cluster than were the gene clusters from other *Vibrio* species (Fig. 4A). The closest homolog of CelG was found in *C. acetobutylicum* (Fig. 4A and 5D), while close homologs to CelK were found in *L. monocytogenes* and *Yersinia intermedia* (Fig. 4A and 5C). Although *Y. intermedia* is a gammaproteobacterium, it appeared to be exceptional in this group in that the proteins encoded by the *Y. intermedia* cluster group more closely with homologs in *Listeria* than with proteins in other proteobacteria. The gene cluster in *L. monocytogenes* is also similar to the *V. fischeri cel* cluster, and the gene order is conserved with that in *V. fischeri*, except that the PTS IIC and glucosidase genes are switched (Fig. 4A). Moreover, codon usage by *Listeria* is so similar to that in *V. fischeri* that genes transferred between the two cannot be recognized as foreign by using this criterion (data not shown). The origin of the *celA*, *celB*, and *celC* genes is especially difficult to infer, as the PTS IIC component tends to group with homologs in the phylum *Firmicutes* (Fig. 5B), whereas the PTS IIB and IIA components tend to group more closely with homologs within the family *Vibrionaceae* (Fig. 5A and data not shown). The PTS IIB and IIA components must interface with other endogenous proteins, and the respective genes might be expected to face greater selective pressure to adapt to a new host and therefore appear less foreign. Overall, it seems plausible that at least some of the genes for cellobiose uptake and catabolism may have been transferred horizontally from a marine *Firmicutes* organism to an ancestor of the *V. fischeri*/*V. salmonicida* clade, although other scenarios remain possible.

Symbiotic colonization of *E. scolopes*. Our laboratories and others study the symbiotic interaction between *V. fischeri* ES114 and the Hawaiian bobtail squid *E. scolopes*, and we therefore investigated the symbiotic phenotype of the *cel* mutants. If cellobiose utilization contributed to colonization proficiency, this would provide insight into the symbiotic nutritional environment of the host light organ. On the other hand, if mutants had no symbiotic attenuation, then mutant *cel* alleles could be useful as neutral markers, with a readily scored phenotype on X-gal.

Mutants with insertions in each of the *cel* genes were checked for their ability to compete with the wild type for colonization in *E. scolopes*. Mutants with an insertion in *celI* or in the noncoding region upstream of *celI* were consistently outcompeted by ~2.2-fold as indicated by RCI values of ~0.45 (Table 3). In contrast, strains with mutations in genes responsible for the transport and degradation of cellobiose, *celA*, *celB*, *celC*, *celG*, and *celK*, had no significant competitive defect relative to that of the wild type (Table 3). We competed DMA401 (*celG* mutant, *celI*::mini-Tn*5*-Em) to see if the competitive defect of a mutation in *celI* was dependent on the overexpression of a functional *cel* gene cluster and cellobioase activity. This competition yielded an RCI that was essentially the same as the single *celI* mutant alone, indicating that the negative effect of knocking out *celI* on symbiotic colonization is independent of cellobioase activity. Instead, this attenuation of competitiveness may simply be from the overexpression of the Cel proteins. Not surprisingly, the *ptsI* mutant DMA424 was outcompeted by the wild type; however, this mutant's slower growth in culture indicates that its defect in colonizing the host cannot be considered symbiosis specific.

DISCUSSION

In this study, we describe a gene cluster that is required for the utilization of cellobiose by *V. fischeri* and is sufficient to confer this property onto cellobiose-negative *Vibrio* species. The genes in this cluster encode a PTS transport system (*celA*, *celB*, and *celC*), a glucokinase (*celK*), a glucosidase (*celG*), and a LacI-like transcriptional regulator (*celI*) that inhibits expression of the cluster when cellobiose is not present (Fig. 1 and 3). Mutational analyses and enzymatic assays show that the *cel* cluster is responsible for cleavage of both cellobiose, which is a β-1,4-linked glucose disaccharide, and X-gal, which is a β-1,4 linked galactoside. Although CelG is promiscuous with respect to the β-1,4-linked substrates it cleaves and, theoretically, it

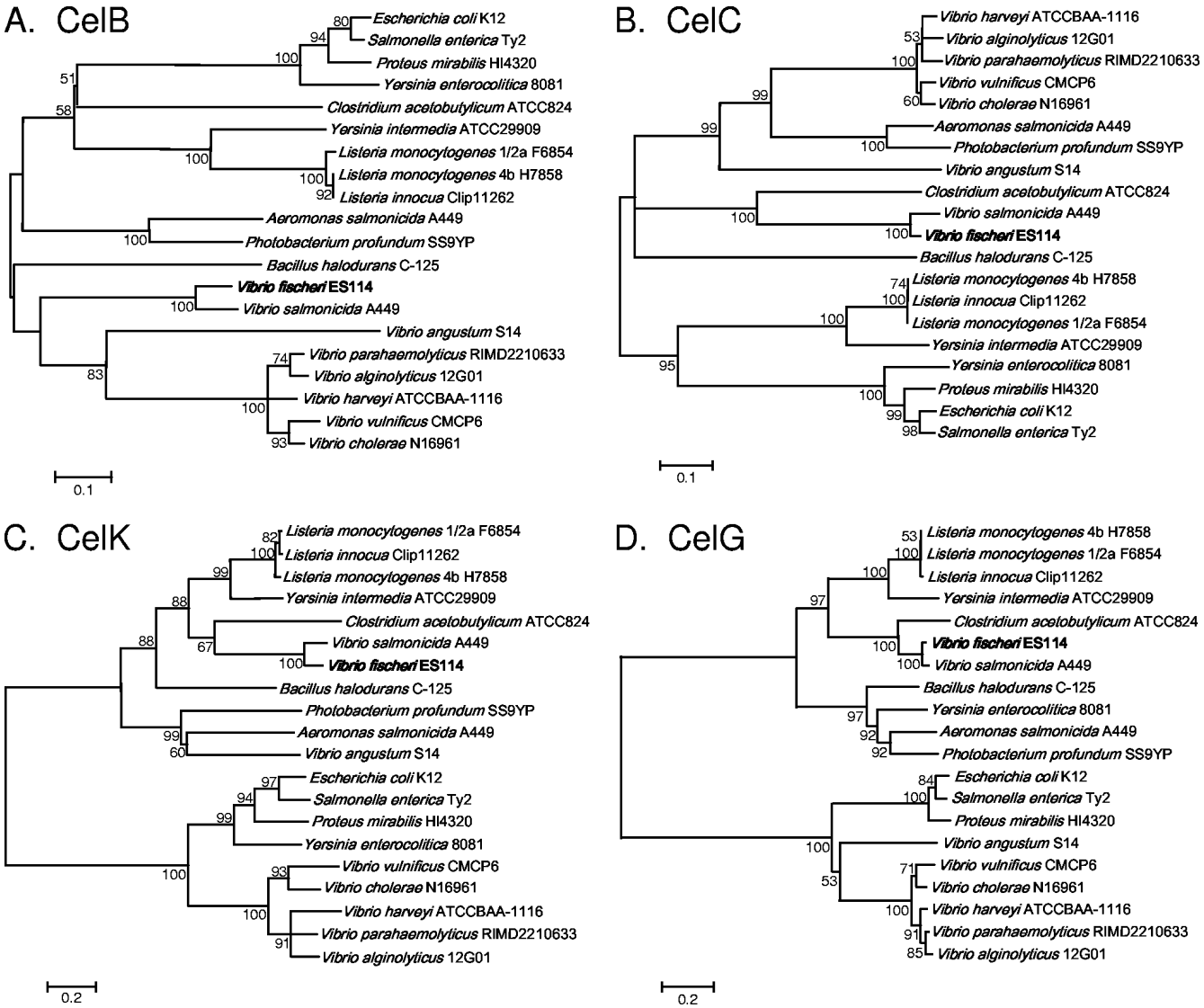

FIG. 5. Comparison of the *V. fischeri* CelB, CelC, CelK, and CelG proteins to proteins encoded by other bacteria with similar gene clusters. Consensus neighbor-joining trees constructed using MEGA 4.0 software (60) are shown. Trees constructed using both UPGMA and minimum evolution algorithms were similar to those shown in the figure (data not shown). Bootstrap values of >50% are indicated at the respective nodes. The scale bar represents a corrected sequence divergence of 0.1 or 0.2 as indicated. Trees in panels A, B, C, and D include *V. fischeri* proteins CelB, CelC, CelK, and CelG, respectively.

could direct catabolism of a β-1,4-galactoside such as lactose, we propose that its activity as a β-1,4-glucosidase is physiologically relevant, whereas its galactosidase activity is coincidental. In support of this, CelG appeared to have greater activity as a glucosidase, and it was required for growth on cellobiose, while lactose is not even utilized by *V. fischeri* as a carbon source. Moreover, the glucoside cellobiose (or a mutation in *celI*) induced the *cel* cluster, whereas several other sugars including lactose did not.

Cellobiose is a disaccharide-breakdown product of cellulose, but *V. fischeri* apparently lacks cellulose-degrading capacity, and the importance of its *cel* gene cluster is unclear. There is no evidence that cellobiose is released by *E. scolopes* to symbiotic *V. fischeri*, and it seems unlikely that this predatory invertebrate would produce or accumulate cellulose or cellobiose. Moreover, losing the ability to utilize cellobiose did not result in symbiotic attenuation (Table 3). *V. fischeri* has been isolated from the water column, even in areas where symbiotic hosts are not found (27, 45), and it has also been isolated from the bacterial consortium in the guts of herbivorous marine fishes, where it can survive and persist (42, 59). These observations suggest a niche where cellobioase activity may be important for *V. fischeri*. Many herbivorous fish partially digest cellulose by acid hydrolysis in their stomachs (74), and it is believed that bacterial cellulases contribute to the digestion of the cellulose in the gut, as with termites and ruminants (47, 70). Although *V. fischeri* does not contain a cellulase, it is possible that either the acidity of the fish's stomach or other bacteria in a fish's intestine could break down cellulose into cellobiose, allowing *V. fischeri* to consume it.

TABLE 3. Colonization competitiveness of *V. fischeri cel* mutants relative to that of ES114

Strain	Genotype	No. of animals[a]	RCI[b]
DMA420	*celI*::mini-Tn*5*-Em	42	0.40[c]
		41	0.48[c]
		44	0.40[c]
DMA421	*celI* upstream region::Tn*5*	44	0.44[c]
DMA401	*celI*::mini-Tn*5*-Em, *celG* mutant (frameshift)	32	0.39[c]
DMA425	*celG*::Tn*5*-Em	32	0.90
KV1319	*celG* mutant (frameshift)	32	1.21
DMA422	*celK*::mini-Tn*5*-Em	26	0.68
DMA423	*celA*::mini-Tn*5*-Em	29	0.95
DMA427	*celB*::mini-Tn*5*-Em	32	1.04
DMA426	*celC*::mini-Tn*5*-Em	32	0.91
DMA424	*ptsI*::mini-Tn*5*-Em	32	0.02[c]

[a] Number of animals used in an individual experiment.
[b] The RCI was derived by the ratio of the mutant strain to the wild type in the light organ at 48 h postinoculation divided by the ratio in the inoculum.
[c] The mutant was significantly outcompeted by ES114; P value of <0.01.

Interestingly, other cellobiose-utilizing *Vibrio* species, notably *V. vulnificus*, lack the *celCBGKAI* gene cluster, and we speculate that cellobiose catabolism may have arisen in members of the family *Vibrionaceae* by multiple distinct events. Based on our bioinformatic data, we speculate that a *Firmicutes* species horizontally transferred the cellobiose degradation glucokinase and glucosidase genes, and possibly the PTS genes, to an ancestor of the *V. fischeri* and *V. salmonicida* lineages. *Firmicutes* species have been found in the guts of marine fishes (23, 36) and in the marine environment (9, 44), so it seems plausible for the ancestor of *V. fischeri* to have acquired the gene cluster from a member of the phylum *Firmicutes* by horizontal gene transfer. Moreover, codon usage patterns of *V. fischeri* and *Listeria* species isolated from marine environments are not distinguishably different, suggesting that the expression of genes transferred between these species may be readily possible. As genome sequences become available for additional marine species, particularly those of *Firmicutes*, the origin of the *V. fischeri* gene cluster may become more apparent.

Our laboratory and those of others use the β-galactosidase gene *lacZ* as a transcriptional reporter (30, 69, 72, 73) in *V. fischeri*, and the discovery of a cryptic β-galactosidase activity in *V. fischeri* strikes a cautionary note for such applications. For example, when *lacZ* is used as a transcriptional reporter and transposon mutants are screened to find regulators of these *lacZ* fusions, knockouts of *celI* will also result in blue colonies on media containing X-gal. It may be useful in such situations to use the *celG* mutant allele in KV1319 in the reporter strain to prevent *celG* expression from confounding screens for *lacZ* activity. Alternatively, *celI* could be introduced on the pDMA171 plasmid into strains with apparent increases in *lacZ* activity to eliminate the possibility that a *celI* mutation and concomitant *celG* expression are responsible for β-galactosidase activity. Whatever the experimental setup, appropriate controls and careful interpretations are warranted whenever *lacZ* is used in a *celG*$^+$ background.

lacZ has also been used as a marker in *V. fischeri*, so that the ratio of two strains in a mixed inoculum or infection can be determined by blue/white screen plating on media with X-gal. Determining strain ratios underlies competition experiments, which enable researchers to detect even subtle differences in symbiotic fitness (8, 24, 26, 29, 31, 35, 39, 56, 65, 66, 71, 72). Recently, *V. fischeri* strains have been marked for competition assays with the introduction of a stable plasmid containing *lacZ* (1, 17, 24). However, the *lacZ*-carrying plasmids can be lost, albeit at a low rate, and their use is inconsistent with other plasmids (e.g., for complementation). Our data suggest a fresh approach that does not rely on a plasmid-borne *lacZ* gene but retains the convenience of blue/white scoring to determine strain ratios. In this approach, one strain could be marked with the *celG* mutant allele present in KV1319. We have shown that this mutation has no effect on colonization competitiveness (Table 3), yet it results in the loss of blue color when it is plated on medium containing cellobiose and X-gal.

The family *Vibrionaceae* is an important and diverse family of bacteria in which species are continually being discovered (5, 25, 28, 41, 43, 50, 62) and with an apparent capacity for rapid evolution, given the periodic emergence of new pathogenic biotypes (13, 19, 49, 51). Traditionally, phenotypic markers such as strains' catabolic capacities have been used to help define *Vibrio* species. However, as more genomes are sequenced for important *Vibrio* species, molecular probes and DNA-based techniques will likely play an ever-larger role in identifying and defining important species or emergent biotypes. Our bioinformatic and phenotypic analyses suggest, not surprisingly, that caution is warranted when automated genome annotations are viewed. For example, an automated annotation of *celA* indicated that it directed "diacetylchitobiose-specific" transport, which seems, clearly, not to be the case given its importance in cellobiose catabolism. Similarly, PTS gene clusters were annotated as cellobiose transport systems in *V. parahaemolyticus* and *V. cholerae*, two species that are cellobiose negative. Experimental studies linking genes with taxonomically useful phenotypes, such as our dissection of the *cel* gene cluster reported here, will be useful in the future to improve *Vibrio* genome annotations and to connect molecular and phenotypic identification techniques.

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