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Cloning and Sequence Analysis of a Novel Hemolysin Gene (*vllY*) from *Vibrio vulnificus*

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A gene (*vllY*) encoding a novel hemolysin of *Vibrio vulnificus* CKM-1 has been cloned and sequenced. When the *vllY* gene was expressed in minicells, a unique peptide of approximately 40 kDa was identified. Subcellular fractionation of *Escherichia coli* cells carrying the *vllY* gene indicated that the VllY protein was distributed in both the cytoplasmic and the periplasmic fractions, with the notable ability to appear in the latter compartment. Nucleotide sequence analysis predicted a single open reading frame of 1,071 bp encoding a 357-amino-acid polypeptide with an estimated pI of 5.02. The deduced amino acid sequence of VllY showed high similarity to the sequence of legiolysin, responsible for hemolysis, pigment production, and fluorescence in *Legionella pneumophila*. The enzyme also exhibited sequence homology to the MelA protein sequence of *Shewanella colwelliana* and the sequences of 4-hydroxyphenylpyruvate dioxygenase family proteins from various organisms. PCR screening and Southern blotting of *V. vulnificus* strains revealed that all of the 41 *V. vulnificus* clinical isolates contained *vllY*-like genes.

Vibrio vulnificus is a halophilic, gram-negative bacterium that has recently become notorious for causing both serious wound infections and fatal septicemia in humans, particularly in certain underlying diseases (27, 43). Primary septicemia may be acquired after consumption of seafoods containing this organism, with mortality exceeding 50%. Infections are associated with exposure of wounds to seawater, with a mortality rate of about 25% (2, 5, 17). Over the last decade, there has been a dramatic increase in the number of cases due to *V. vulnificus* infection in the southern part of Taiwan (6).

A variety of factors have been proposed as possible virulence determinants for *V. vulnificus* (13, 18, 25, 36, 37, 41). Among these factors, hemolysin is known to be cytotoxic to Chinese hamster ovary (CHO) cells and to disrupt the membranes of various mammalian erythrocytes (19). It also has the ability to enhance vascular permeability and is lethal to mice (13). However, studies comparing the amount of hemolysin production and the virulence of *V. vulnificus* have yielded contradictory results. Both Oliver et al. (28) and Morris et al. (26) observed a lack of correlation between virulence and hemolysin production; also, Massad et al. (23) reported that mutants deficient in the production of hemolysin were still virulent for mice.

A gene (*vvhA*) encoding hemolysin with potent cytolytic activity has been cloned and sequenced (51), and regions of this gene showed homology to the structural gene for the *Vibrio cholerae* El Tor hemolysin (29). Our laboratory has been interested in the virulence mechanisms responsible for the pathogenicity of *V. vulnificus* for humans. The cloning and characterization of the gene (*empV*) encoding the *V. vulnificus* CKM-1 extracellular metalloprotease has been described elsewhere (7). In a further study of the virulence factors of clinically isolated *V. vulnificus*, we also found that most strains, including strain CKM-1, exhibited a clear hemolytic zone after cultivation on blood agar plates containing rabbit erythrocytes but showed an ambiguous green-brown color after cultivation on blood agar plates containing human or sheep erythrocytes. In this study, we describe the cloning and expression of a DNA fragment from *V. vulnificus* which is able to induce hemolytic activity and color production in *Escherichia coli.*

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria used in this study include *V. vulnificus* CKM-1, 40 *V. vulnificus* strains, 8 *Aeromonas hydrophila* strains, 4 *Pseudomonas aeruginosa* strains, 5 *Staphylococcus aureus* strains, and 3 *Klebsiella pneumoniae* strains, all isolated from the blood or stool culture of patients at the National Cheng Kung University Hospital. *E. coli* JA221 (1), JM109 (52), XL1B (Stratagene), and the minicell-producing strain p678-54 were used as transformation hosts. The clinical bacterial isolates and *E. coli* were grown in Luria-Bertani (LB) medium (33) at 28 and 37°C, respectively. Recombinant *E. coli* cells were evaluated for hemolytic phenotypes by using tryptic soy agar containing 5% human or sheep erythrocytes. Ampicillin (50 μg ml^{-1}) was added when appropriate for selection. Color production (browning) in strains and clones was examined by assay after cultivation on LB agar plates or PIG agar plates, which are LB agar plates supplemented with 0.025% ferric PPi, 0.04% cysteine, and 4 mM tyrosine (47). When necessary, the medium was supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. Ampicillin, ferric PPi, cysteine, and tyrosine were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Plasmid pBR322 (3) was used in cloning experiments; plasmids pUC18 and pUC19 (44) were used in the subcloning experiments. A 1.3-kb *Acc*I-*Bst*XI fragment was subcloned into the *Acc*I and *Bst*XI site of the high-expression vector pBC (Stratagene) to produce plasmid pBCV. This placed the initiation codon of VllY at an appropriate distance from the T7 promoter to allow high-level expression of the VllY protein.

Construction and screening of the genomic library. All DNA manipulations were carried out in accordance with the manufacturers' instructions as described by Sambrook et al. (33). The preparation of the genomic library containing *V. vulnificus* CKM-1 genomic DNA in the vector pBR322 has been described previously (4). The gene bank was used to transform *E. coli* JA221, and transformants were screened on blood agar plates. After 48 h of incubation at 37°C, the colonies that had clear zones around them were isolated and further screened on the PIG agar plates. The colonies that exhibited brown color were collected and subjected to advanced analysis at the molecular level.

Minicell isolation and labelling of proteins. Cells of *E. coli* p678-54 (*F⁻ thr leu thi supE lacY fhuA gal mal xyl mtl*) were transformed with the plasmids. Minicells were prepared in sucrose density gradients, and proteins were labelled with L-[^{35}S]methionine (>1,200 Ci/mmol; Amersham) according to the method of Dougan and Kehoe (9). After being labelled, components in cell lysates of the minicells were separated by 0.1% sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) (20). The gels were dried and exposed for 2 days to X-ray film at −70°C and developed by using Kodak (Rochester, N.Y.) developer and fixer.

DNA sequencing and sequence analysis. All sequences were determined on denatured double-stranded DNA templates by the dideoxy chain-termination

method (34), using a Sequenase 2.0 DNA sequencing kit (U.S. Biochemicals) and sequencing grade [^{35}S]dATPαS (>12.5 mCi ml^{-1}, >463 Mbq ml^{-1}; specific activity, 1,000 to 1,500 Ci mmol^{-1}, 37 to 55.5 TBq mmol^{-1}) (New England Nuclear). Universal and reverse primers were used to obtain the initial sequences within the inserts, and then specific primers for the sequences within the inserts were generated. Conditions for DNA sequencing were as described in the Sequenase brochure (U.S. Biochemicals). Denaturing 6% PAGE at 60 W was used to separate the reaction products. The gels were exposed to X-ray film (Omat-AR; Kodak) overnight. DNA was sequenced in both directions. In some cases, dITP was used in place of dGTP for a more reliable reading through regions of high G+C content. Sequence analysis was carried out with the PC/GENE software package (Intelligenetics).

Cellular fractionation and N-terminal amino acid sequence analysis. To express the *vllY* gene in *E. coli*, a DNA fragment containing the open reading frame (ORF) of *vllY* was inserted into pUC and pBC vectors. The *E. coli* cells containing plasmid DNA were collected, and cellular fractionation was performed as described by Chang et al. (4). The periplasmic proteins remained in the supernatant when the cells were pelleted. The cell pellets were lysed by five cycles of freezing-thawing. After removal of unlysed cells, the total membrane (pellet) and cytoplasmic (supernatant) fractions were collected by centrifugation (15,000 × *g*, 30 min). The proteins present in the various fractions of spheroplasts were separated by SDS-PAGE (20). The proteins separated on the gels were electroblotted onto a polyvinylidene difluoride membrane. After being visualized with Coomassie brilliant blue R250 stain, the membrane was cut into pieces containing the 40-kDa proteins corresponding to VllY. The membrane pieces were directly applied to a protein sequencer (model 477A; Applied Biosystems, Foster City, Calif.) for amino-terminal amino acid sequence analysis. The deduced amino acid sequence of VllY was analyzed by using BLAST E-mail searches of the databases at the National Center for Biotechnology Information. Multiple alignments of the amino acid sequences among the homologous proteins were performed with the CLUSTAL multiple sequence alignment program (14).

Hemolytic activity assay. Ten-milliliter volumes of sheep erythrocytes were each washed twice in 0.015 M Tris-HCl (pH 8.0)–0.17M NaCl and resuspended to 2.25% in the buffer. A sample (0.5 ml) of this solution was added to 1 ml of enzyme preparation. The mixtures were incubated at 37°C for 45 min and then centrifuged at 2,500 rpm (MSE Microcentaur; Serlabo, Paris, France) for 2 min. The amount of hemoglobin released was estimated by measuring the absorbance of the supernatant at 540 nm. Each assay was run two or three times (42).

PCR. PCR was performed with a model PE 2400 automated thermocycler with MicroAm tubes (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction was carried out in a 50-μl volume containing 5 μl of 10× buffer (supplied with *Taq*), 20 pmol of each primer, 1 mM deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Boehringer), and 1 μl of bacterial lysate or 100 ng of genomic DNA. About 1 to 10 ng of plasmid DNA or insert DNA was used. PCR conditions were as follows: 30 cycles at 94°C for 1.0 min, 52°C for 1.0 min, and 72°C for 1.0 min. A final extension step of 10 min at 72°C was included. Chromosomal DNAs from several clinically isolated strains, including *V. vulnificus*, *A. hydrophila*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*, were used as templates. Two oligodeoxyribonucleotides, 5′-GCAGTCTAGAAGGAGGAATTCCTG and 5′-GCGCTG GTCTCGAGAAGTGAA, were designed for the operon of *vllY*. The other oligodeoxyribonucleotides, 5′-CCGCGGTACAGGTTGGCGC and 5′-CGCC ACCCACTTTCGGGCC, which have been used to amplify a specific 519-bp region of the hemolysin previously shown to be unique to *V. vulnificus* (15), were used as PCR primers.

Southern hybridization. Chromosomal DNA was digested with the restriction endonuclease *Sma*I, separated by agarose gel electrophoresis, and transferred to Zeta-probe GT membranes (Bio-Rad Laboratories, Richmond, Calif.). An *Acc*I-*Bst*XI DNA fragment cut from plasmid pVK5 served as the probe after being labelled with [α-^{32}P]dCTP (>3,000 Ci/mmol; Amersham) by using the Prime-a-Gene labeling system (Promega, Madison, Wis.). Membranes were hybridized at 60°C for 16 h, exposed to X-ray film at −70°C for 2 days, and developed with Kodak developer and fixer as recommended by the manufacturer.

Nucleotide sequence accession number. The nucleotide sequence of the hemolysin gene *vllY* cloned from *V. vulnificus* CKM-1 has been assigned GenBank accession no. U97357.

RESULTS

Cloning of the *vllY* gene. To clone the determinants responsible for the hemolytic phenotypes of *V. vulnificus*, a genomic library derived from *V. vulnificus* CKM-1 was used to transform *E. coli* JA221. Transformed *E. coli* cells were screened on blood agar plates to select hemolytic clones. Seven clones which displayed hemolytic phenotypes on blood agar plates were isolated from an initial screen of approximately 6,000 *E. coli* transformants. Four of these clones exhibited a clear zone of hemolysis around the colonies, while the remaining three clones exhibited a zone of hemolysis and were able to display green-brown color after incubation at 37°C for 2 days. A gene

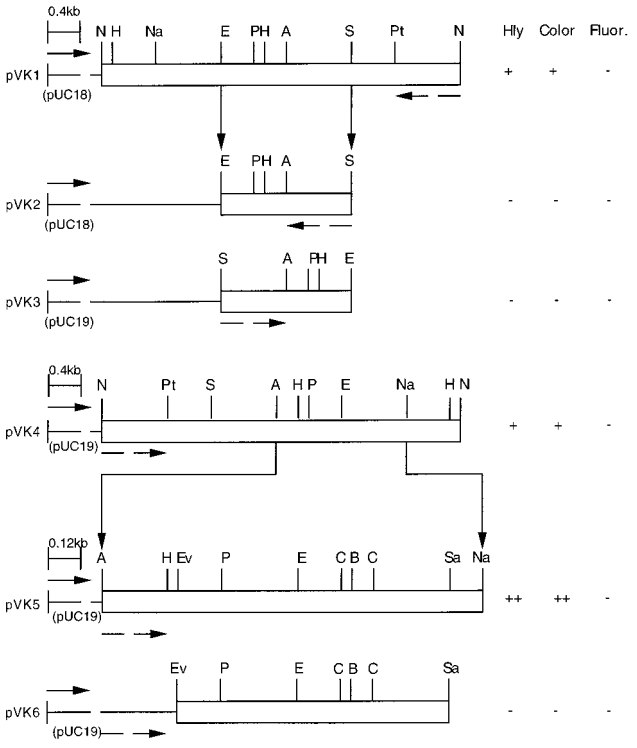

FIG. 1. Restriction endonuclease maps of plasmid clones harboring the hemolysin gene (*vllY*). A, *Acc*I; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; N, *Nar*I; Na, *Nla*IV; P, *Pvu*I; Pt, *Pst*I; S, *Sal*I; Sa, *Sca*I. Boxes indicate *V. vulnificus* DNA; thin lines indicate DNA from the plasmid vector pBR322, pUC18, or pUC19; solid horizontal arrows indicate the direction of transcription of the *lacZ* promoter; dashed arrows indicate the direction of the putative promoter of the cloned gene; vertical arrows indicate the regions that were subcloned into pUC18 or pUC19. Varying levels of hemolysis (Hly), color, and fluorescence (Fluor.) activity are represented by + and ++; − indicates no activity.

(*vvhA*) which codes for the *V. vulnificus* hemolysin that has cytolytic activity has been cloned and sequenced (48, 51). To test whether the *vvhA*-specific DNA sequence was present in these seven hemolytic phenotype clones, plasmid DNA was isolated from the clones and hybridized with synthetic DNA probe, which was designed according to the *vvhA* sequence of *V. vulnificus* EDL-174 (51). The results showed that four clones, which exhibited clear zones on blood agar plates, were positive and hybridized with the synthetic probe; the remaining three clones showed negative reactions (data not shown).

To further investigate the genes encoding proteins responsible for hemolysis and color production, plasmid DNA was isolated from the three negative clones and subjected to restriction endonuclease mapping. All three clones contained an identical 4.5-kb fragment. When this fragment was recloned into pUC18 and pUC19 (pVK1 and pVK4, respectively), both plasmids coded for the protein which confers hemolysis and color production on recombinant *E. coli* JM109. This indicated that the genes encoding the two phenotypes seem to be transcribed from a single promoter. To determine the approximate location of the gene responsible for the two phenotypes in the cloned DNA fragment, several subclones were generated from pVK4 and analyzed for the two properties. Subcloning revealed that a 1.3-kb *Acc*I-*Nla*IV fragment was sufficient to produce the two properties in *E. coli* JM109 (Fig. 1).

Detection of the VllY protein in minicells. To identify the gene product encoded by pVK5 (Fig. 1), the *E. coli* minicell-

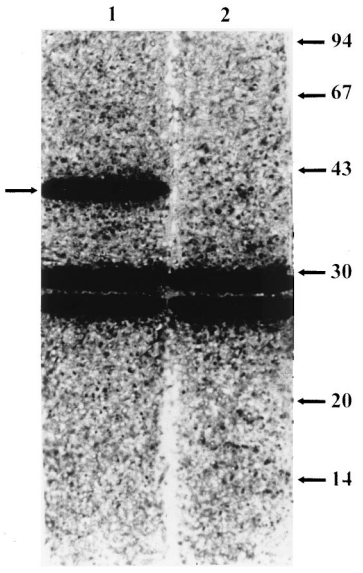

FIG. 2. Expression of pVK5 and pUC18 in minicells. Lane 1, the *vllY* gene product labelled with L-[^{35}S]methionine obtained from minicells carrying pVK5 is shown by an arrow; lane 2, the result obtained from minicells carrying pUC18. Numbers on the right of lane 2 represent size standards in kilodaltons.

producing strain (p678-54), which permits specific labelling of the plasmid-encoded proteins, was transformed with plasmids pVK5 and pUC18 and minicells were prepared and labelled with L-[^{35}S]methionine. A unique heavily labelled polypeptide of approximately 40 kDa was found in minicells carrying pVK5 (Fig. 2), and no such labelled polypeptide was observed in minicells carrying pUC18. Synthesis of all other identifiable proteins detected was conducted by the vector plasmid. The fact that only one polypeptide unique to pVK5 was observed by minicell analysis indicated that the *vllY* gene, encoding a protein of about 40 kDa, is responsible for hemolysis and color production in recombinant *E. coli* cells.

Nucleotide sequence of *vllY*. The nucleotide sequence of *vllY* and its flanking regions was determined on both strands (Fig. 3). The sequence revealed that an ORF of 1,071 bp encodes a polypeptide of 357 amino acids with a molecular mass of 40,223 Da, which is in agreement with the size of the protein expressed in the minicell labelling experiments. A possible ribosomal binding site (Shine-Dalgarno sequence) (40), AGGA, was found 7 bp upstream from the presumptive start codon, ATG. Although we have not yet determined the promoter sequences that function in *E. coli*, sequences homologous to *E. coli* −35 and −10 promoter consensus sequences (24) were observed 73 bp upstream from the start codon. Furthermore, an A+T-rich region, which had been shown to be involved in transcription regulation in *E. coli* (30), was found immediately upstream from the consensus sequences. At a point 84 bp downstream from the stop codon, there was a palindromic sequence between nucleotides 1,259 and 1,288. This structure would be expected to function as a transcriptional terminator. The G+C content of the coding region for VllY was 47.3%, which is nearly identical to those of β-*N*-acetylhexosaminidase (47% [38]) and cytolysin (48% [51]) and a little lower than that for the *fur* gene (50.3% [22]). The codon usage frequency in the *vllY* gene is similar to the pattern for the aforementioned genes of this species (data not shown).

Gene expression and localization of the VllY protein in *E. coli* transformants. *E. coli* JA221 cells containing plasmid pVK5 did not express the *vllY* gene efficiently; the *vllY* gene was placed directly under the control of the strong T7 promoter. The resulting plasmid was designated pBCV. Expression of the *vllY* gene upon induction with IPTG in *E. coli* XL1B cells containing plasmid pBCV is shown in Fig. 4. No major polypeptides could be detected in *E. coli* cells carrying only the pBC vector or in uninduced cultures. The overexpressed *vllY* gene product migrated as a 40-kDa polypeptide, which agreed well with the value calculated from minicell analysis and from the putative amino acid sequence. To determine the cellular location of the gene product, *E. coli* cells carrying pBCV in induced culture were grown to the exponential phase and fractionated. The hemolytic activity of VllY was determined in the cell-free supernatant and the periplasmic and cytoplasmic fractions. Marker enzymes, *E. coli* β-lactamase and β-galactosidase, were also assayed for each fraction to monitor cell lysis and the efficiency of cell fractionation. The results showed that 50% of the hemolytic activity and 85% of β-lactamase activity occurred in the periplasmic fraction and 45% of the hemolytic activity and 90% of β-galactosidase activity were found in the cytoplasmic fraction. No significant β-lactamase or β-galactosidase activity was detected in the culture supernatants (data not shown).

No significant cell lysis could be observed in the cytoplasmic fraction, where β-galactosidase activity was primarily found, while β-lactamase activity was located in the periplasm. Thus, the distribution of VllY in both the periplasmic and the cytoplasmic fractions should reflect the true destination of the enzyme. The distribution of VllY in both the periplasmic and the cytoplasmic fractions was also observed by SDS-PAGE (Fig. 4). From these measurements, it is apparent that VllY is capable of being processed in the periplasm in *E. coli*. Furthermore, the N-terminal sequence of the VllY protein obtained from the periplasmic fraction was determined to be Val-Asp-Ala-Ile-Asn-Pro-Leu-Gly-Thr-Asp-Gly, which was completely identical to the beginning of the amino acid sequence deduced from the DNA sequence, except that the N-terminal methionine residue was not present in the intact protein.

Alignment of VllY amino acid sequences with other proteins. Analysis of the deduced amino acid sequence of VllY for homology to other sequences in the GenBank database, using BLAST searches at the National Center for Biotechnology Information, revealed high degrees of similarity (76.1%) and identity (57.2%) to the Lly protein of *Legionella pneumophila* (47) without large gaps or insertions. The enzyme also showed high levels of similarity to the MelA protein of *Shewanella colwelliana* (63.7% similarity and 42.2% identity) (11) and those of HPPD (4-hydroxyphenylpyruvate dioxygenase) family proteins from various organisms (10, 12, 16, 21, 31, 35, 50). A multiple alignment of their amino acid sequences (Fig. 5) shows relatively poor N-terminal homology and a greater number of conserved regions in C-terminal parts of the proteins.

Conservation of the *vllY* gene among clinical *V. vulnificus* isolates. Because most clinically isolated *V. vulnificus* strains exhibited an ambiguous green-brown color after cultivation on blood agar plates containing human or sheep erythrocytes, we examined the possibility that the *vllY*-specific DNA sequence might be conserved. Two approaches were used to determine the presence of *vllY*-specific DNA sequences in the chromosomal DNAs from clinically isolated *V. vulnificus* strains. Southern blotting analysis of *Sma*I-digested chromosomal DNAs of several *V. vulnificus* strains (including CKM-1), probed with a 1.3-kb DNA fragment (which contains the entire 1,071-bp *vllY* operon), showed that similar *Sma*I DNA fragment patterns were conserved among the *V. vulnificus* strains

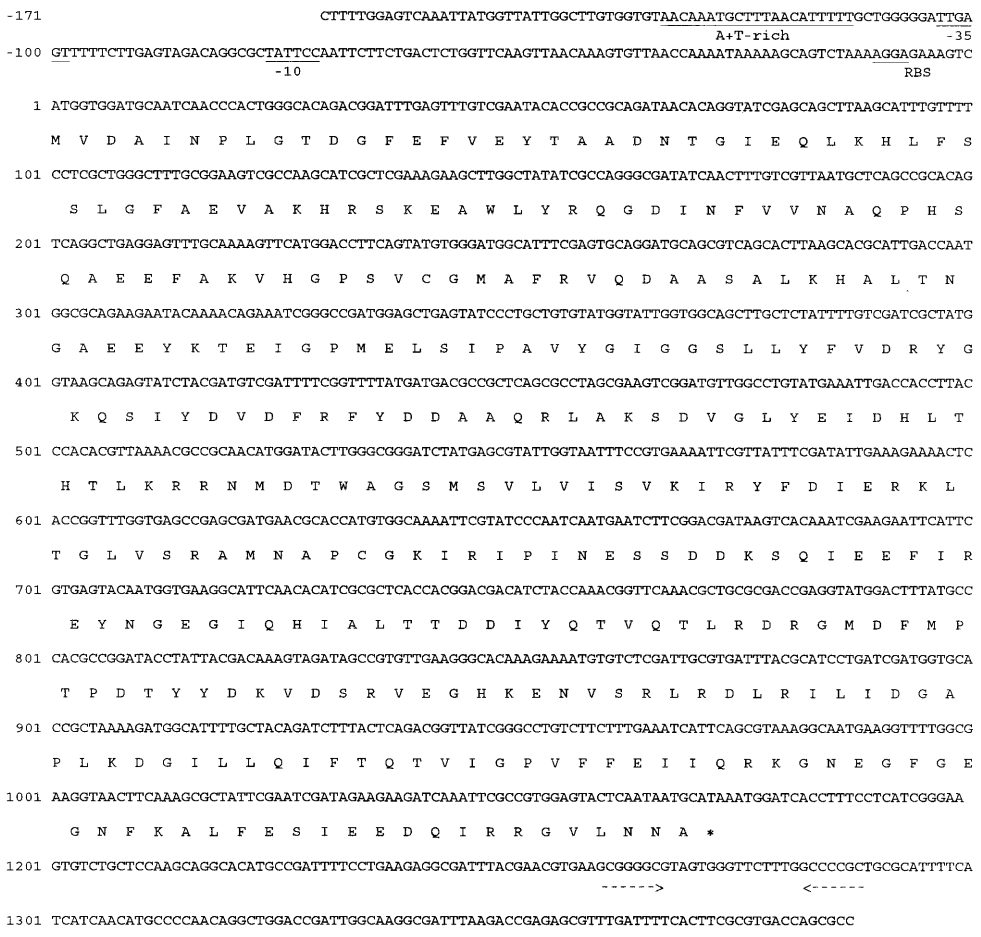

FIG. 3. Nucleotide sequence of *V. vulnificus* CKM-1 *vllY* DNA fragment in pVK5. The deduced amino acid sequence for the ORF of *vllY* is shown with each symbol below the first nucleotide of the corresponding codon. The nucleotides are numbered with the first letter of the initiator Met codon as +1, and the amino acid residues are numbered as +1 from the initiator Met. The A+T-rich region, *E. coli* −35 and −10 promoter consensus sequences, and a putative Shine-Dalgarno sequence for a ribosomal binding site are underlined and labelled. Stop codons are indicated by asterisks. A palindromic sequence is indicated by convergent arrows.

tested (Fig. 6), except for one strain, CKM-7. This difference was shown by the size of the *Sma*I restriction fragment, presumably as a result of restriction site polymorphisms in the flanking regions. No hybridization was observed with chromosomal DNAs of other bacterial strains, such as *A. hydrophila*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae* (data not shown). In addition, PCR analysis with primers that allowed amplification of the 1.3-kb DNA fragment which contains the operon of *vllY* produced the 1.3-kb DNA fragment from all *V. vulnificus* strains (Fig. 7). No significant DNA band was seen when chromosomal DNA of *A. hydrophila*, *P. aeruginosa*, *S. aureus*, or *K. pneumoniae* was used as the template (data not shown).

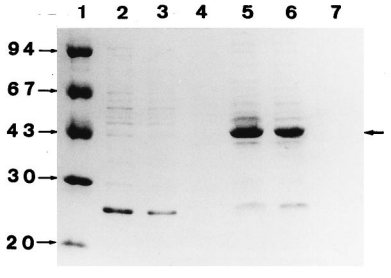

FIG. 4. Polypeptides from the various fractions of *E. coli* cells containing pBCV or pBC. Extracts were applied to the SDS-polyacrylamide gel as follows: lane 1, low-molecular-mass protein size standards (Pharmacia); lane 2, the cytoplasmic fraction of *E. coli* cells containing pBC; lane 3, the periplasmic fraction of *E. coli* cells containing pBC; lane 4, the total membrane fraction of *E. coli* cells containing pBC; lane 5, the cytoplasmic fraction of *E. coli* cells containing pBCV; lane 6, the periplasmic fraction of *E. coli* cells containing pBCV; lane 7, the total membrane fraction of *E. coli* cells containing pBCV. The position of the *vllY* gene product is shown by an arrow. Numbers on the left of lane 1 represent size standards in kilodaltons.

DISCUSSION

This paper describes the molecular cloning, sequencing, and identification of a *V. vulnificus* CKM-1 gene encoding a protein that induces hemolysis and color production in recombinant *E. coli* strains. The protein is encoded by an ORF of 1,071 bp coding for 357 amino acids with a predicted molecular mass of 40,223 Da and an estimated pI of 5.02. The amino acid sequence of the polypeptide showed strong similarity to that of legiolysin (Lly) of *L. pneumophila*, which causes hemolysis and pigment production in recombinant *E. coli* cells. We have therefore designated this gene *vllY*, for VllY protein of *V. vulnificus* CKM-1. No such protein has been described for the species *V. vulnificus*, and our results document for the first time the presence of a *vllY* gene in this species.

The extensive homology between *V. vulnificus* VllY and *L.*

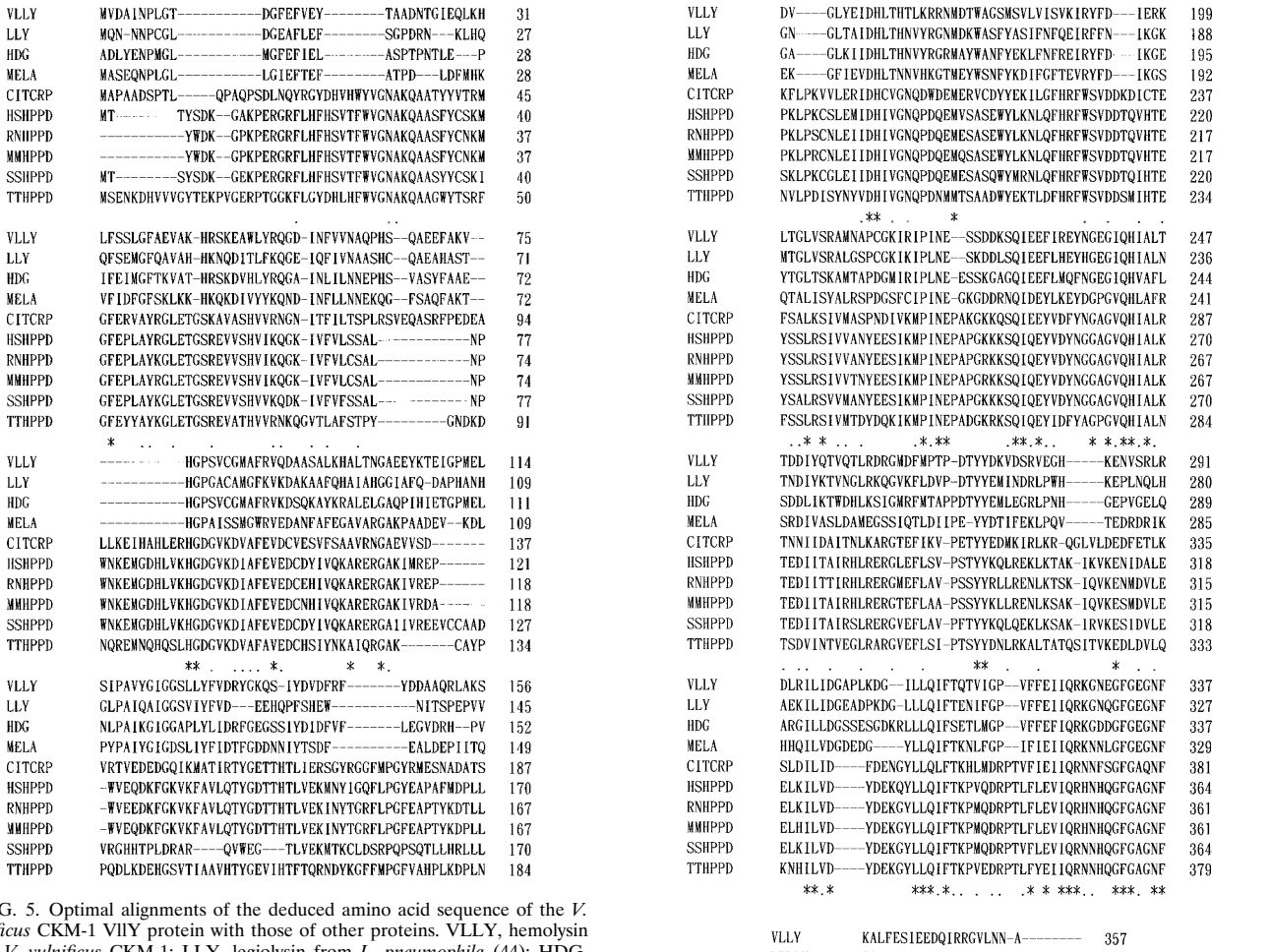

FIG. 5. Optimal alignments of the deduced amino acid sequence of the *V. vulnificus* CKM-1 VllY protein with those of other proteins. VLLY, hemolysin from *V. vulnificus* CKM-1; LLY, legiolysin from *L. pneumophila* (44); HDG, 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* spp. (28); MELA, a protein mediate-melanin synthesis from *S. colwelliana* (11); CITCRP, a T-cell-reactive protein from *Coccidioides immitis* (47); HSHPPD, human 4-hydroxyphenylpyruvate dioxygenase (29); RNHPPD, a liver-specific rat F antigen (12); MMHPPD, a mouse F protein (32); SSHPPD, 4-hydroxyphenylpyruvate dioxygenase from *Sus scrofa* (10); TTHPPD, an F antigen homolog from *Tetrahymena thermophila* (16). Asterisks indicate positions at which the amino acids are identical in all seven proteins. Dots indicate the location of homologous residues of the seven protein sequences. Dashes represent gaps introduced during the alignment process. Numbers refer to the amino acid located at the end of each line.

pneumophila Lly is an interesting finding. The Lly protein of *L. pneumophila* has been shown to confer the properties of hemolytic activity in human erythrocytes and pigment production when expressed by recombinant *E. coli* carrying an *lly* gene. Like the Lly protein, VllY also produces these two phenotypes of recombinant *E. coli*. It has been reported that the pigment produced by *Legionella* resembles melanin (46) and that the pigment production of various organisms is enhanced when the culture medium is supplemented with L-tyrosine. Likewise, the browning of the culture medium by recombinant *E. coli* harboring the *vllY* gene was observed after 2 days in culture at 37°C in both LB and PIG liquid media and was increased by the addition of tyrosine (data not shown). However, it has been reported that the Lly protein additionally confers the phenotypic characteristic of fluoresence, whereas this distinctive property was not observed in recombinant *E. coli* carrying a *vllY* gene when the cells were illuminated by a long-wave UV lamp in the dark. Some differences in the sequences of the two proteins may be responsible for the third phenotype produced by the Lly protein.

A BLAST search revealed that the VllY protein is also homologous to both prokaryotic and eukaryotic forms of the HPPD family proteins, including the related F antigen, as well as to *S. cowelliana* MelA, which has been identified as an HPPD-like protein. The C-terminal amino acid sequence is thought to be highly conserved in the HPPD family proteins. The multiple alignment of amino acid sequences shown in Fig. 5 revealed that the deduced structure of the *V. vulnificus* VllY presented here also contained these highly conserved sequence motifs of HPPD proteins, suggesting that VllY is an HPPD-like protein. The fact that pigment formation in *vllY*-positive *E. coli* is enhanced in the presence of tyrosine also suggests that VllY and 4-hydroxyphenylpyruvate dioxygenase have similar functions in catalyzing the formation of homogentisate from 4-hydroxypyruvate, a product which is formed following transamination of L-tyrosine (8). The high degree of homology

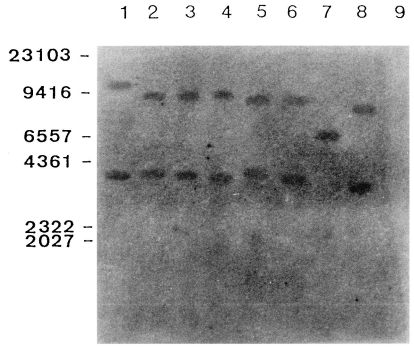

FIG. 6. Southern hybridization analysis of genomic DNA from several *V. vulnificus* strains and *E. coli* JM109. The genomic DNA of these strains was digested with *Sma*I and probed with a 1.3-kb *Acc*I-*Bst*XI DNA fragment containing the *V. vulnificus* CKM-1 hemolysin gene (*vllY*). Lane 1, *V. vulnificus* CKM-1; lane 2, *V. vulnificus* CKM-2; lane 3, *V. vulnificus* CKM-3; lane 4, *V. vulnificus* CKM-4; lane 5, *V. vulnificus* CKM-5; lane 6, *V. vulnificus* CKM-6; lane 7, *V. vulnificus* CKM-7; lane 8, *V. vulnificus* CKM-8; lane 9, *E. coli* JM109. Numbers on the left of lane 1 represent *Hin*dIII-digested lambda DNA size standards in base pairs.

in the C-terminal part of the HPPD family proteins suggests that this part of the protein is important to enzymatic function. Recently, it has been reported that several histidine and tyrosine residues are potential metal-binding sites in the *Pseudomonas* HPPD protein (31), and some of those residues seem to be highly conserved among the HPPD family proteins, as shown in Fig. 5.

Cellular fractionation studies revealed that VllY is present in both the periplasmic and cytoplasmic spaces of *E. coli*, indicating that VllY protein can traverse the inner membrane without the need for *V. vulnificus*-specific factors. However, an examination of the putative amino-terminal sequence of the VllY protein revealed no conventional signal sequence, such as those seen in a number of other secreted proteins characterized in bacteria (45). Furthermore, the N-terminal amino acid sequence obtained from the periplasmic fractions of *E. coli* cells carrying the *vllY* gene shows that only the initiating methionine is processed. Secretion of the VllY protein into the periplasmic space of *E. coli* is, therefore, independent of a signal sequence. It is speculated that VllY is secreted into the periplasmic space of *E. coli* through a novel secretion mechanism, which will be addressed in subsequent investigations.

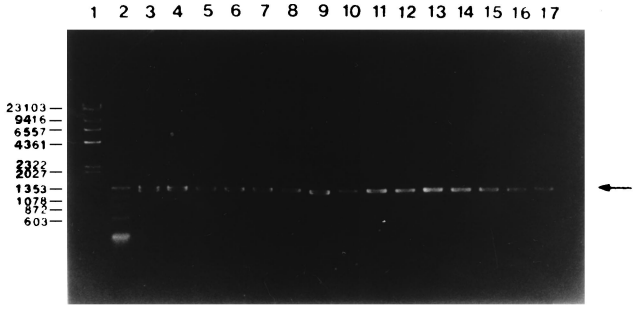

FIG. 7. Agarose gel electrophoresis of PCR products from different template DNAs. Lane 3, *V. vulnificus* CKM-1; lane 4, *V. vulnificus* CKM-2; lane 5, *V. vulnificus* CKM-3; lane 6, *V. vulnificus* CKM-4; lane 7, *V. vulnificus* CKM-5; lane 8, *V. vulnificus* CKM-6; lane 9, *V. vulnificus* CKM-8; lane 10, *V. vulnificus* CKM-9; lane 11, *V. vulnificus* CKM-10; lane 12, *V. vulnificus* CKM-11; lane 13, *V. vulnificus* CKM-12; lane 14, *V. vulnificus* CKM-13; lane 15, *V. vulnificus* CKM-14; lane 16, *V. vulnificus* CKM-15; lane 17, *V. vulnificus* CKM-16. Lanes 1 and 2 represent *Hin*dIII-digested lambda DNA and *Hae*III-digested ϕX174 DNA size standard markers, respectively. The unique 1.3-kb PCR product is shown by an arrow. Numbers on the left of lane 1 represent size standards in base pairs.

Under stringent hybridization conditions, the *vllY* gene hybridized with DNA from all of our clinical *V. vulnificus* isolates but not with DNA isolated from *A. hydrophila*, *P. aeruginosa*, *S. aureus*, or *K. pneumoniae*. It should be noted that the *V. vulnificus* strains that we tested were isolated from several patients over an extended time in the southern part of Taiwan. *V. vulnificus* elaborates cytotoxin-hemolysin, and DNA probes targeted to the relevant gene of *V. vulnificus* have been used for species identification (15). To elucidate whether the cytotoxin-hemolysin gene is unique to our clinical *V. vulnificus* isolates, a pair of PCR primers designed to amplify a 519-bp region within an ORF of a *Hin*cII fragment encoding the cytotoxin-hemolysin gene were constructed and PCR experiments were performed according to the method described by Hill et al. (15). The results revealed that the 519-bp DNA fragment could be obtained when DNA from any of our clinical *V. vulnificus* isolates was used as the template of the PCR but not when DNA isolated from other bacteria, including *A. hydrophila*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*, was used (data not shown). Furthermore, the Southern blotting results agreed with those of PCR (data not shown). From the results of both Southern analysis and PCR, we concluded that both the *vvhA* and *vllY* genes are unique to our clinical *V. vulnificus* isolates and all our clinical *V. vulnificus* isolates possess at least two different types of hemolytic proteins: cytotoxin-hemolysin protein and one that causes browning of the medium. However, data concerning the inactivation of the *V. vulnificus* EDL-174 hemolysin gene in the chromosomal DNA by transposon mutagenesis or marker exchange techniques, which results in a nonhemolytic phenotype (49), contrast with the results presented here. Two possible reasons for the discrepancy are that VllY is not detectable with the blood agar plates containing rabbit erythrocytes used by the authors and that *V. vulnificus* EDL-174 is a VllY-negative strain. At present, we are uncertain whether the *vllY* gene exists in all clinical and environmental isolates. It will be of interest to investigate whether the *vllY* gene can provide a useful general DNA probe for clinical or environmental *V. vulnificus* isolated from various geographic locations.

The precise role of the *vllY* gene in *V. vulnificus* is unclear. Its presence in all our clinical *V. vulnificus* isolates suggests that it may have a role in the pathogenicity of *V. vulnificus*. Recently, it has been reported that the cloned *lly* gene conferred increased resistance to light in recombinant *L. pneumophila* and *E. coli* K-12, suggesting that the Lly of *L. pneumophila* might be important for the survival of *L. pneumophila* stressed by light (39). We have cloned the *vllY* gene from *V. vulnificus* and have begun preliminary characterization of its protein product. Further study with genetic modifications for functional analyses and serological evaluation may help to elucidate whether the *vllY* gene is responsible for the survival of *V. vulnificus* and the possibility that VllY protein may also be a virulence determinant in the pathogenesis of *V. vulnificus.*

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